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CULTURE-DEPENDENT AND INDEPENDENT STUDIES OF SULFUR OXIDIZING BACTERIA FROM THE FRASASSI CAVES

A Thesis in

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by

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ABSTRACT

The Frasassi Caves (Italy) are developed in calcium carbonate rocks and contain sulfide from groundwater and oxygen from both the cave atmosphere and downward percolating meteoric water. The presence of sulfide and oxygen allows for both the abiotic and biotic formation of sulfuric acid, which subsequently reacts with calcium carbonate cave walls to enlarge the cave. While the contribution of abiotic processes on cave development has been studied, less research has focused on microbial contributions through the complete oxidation of reduced sulfur sources.

I used culture-dependent and culture-independent methods to study the metabolic properties of *Thiobacillus baregensis*, a dominant sulfur oxidizing bacterium in sulfidic streams within the cave system, and a novel strain of *Sulfuricurvum kujiense* (*Sulfuricurvum* sp. strain Frasassi), the first Epsilonproteobacterium successfully cultured from the Frasassi Caves. Since *T. baregensis* is abundant and capable of completely oxidizing multiple reduced sulfur sources, it is likely to contribute to cave development. While *Sulfuricurvum* sp. strain Frasassi exhibits growth on reduced sulfur sources, its ability to completely oxidize sulfur remains to be tested. Both *T. baregensis* and *Sulfuricurvum* sp. strain Frasassi show growth on nitrate, a chemical species perennially below detection limits in the Frasassi Caves. Future studies should focus on obtaining axenic cultures of both bacteria, using whole genome sequencing to elucidate their metabolic properties, and determining whether or not nitrate is present in Frasassi Cave streams.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

This thesis contains work contributed by multiple people. Metagenomics work was completed by Trinity Hamilton, Muammar Mansor, and Beatrice Aren Ajeng Laing. Phylogenetic trees in Chapters 2 and 3 were constructed by Trinity Hamilton. *Thiobacillus baregensis* inoculum and corresponding geochemical data was obtained by Jennifer Macalady. I personally completed fieldwork to obtain samples for *T. baregensis* area counts in 2013 with the help of Sandro Mariani, Alejandro Crocetti, Jennifer Macalady, Daniel Jones, and Katherine Dawson.

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CHAPTER 1: INTRODUCTION

1.1 Hydrogeological background of the Frasassi Caves

The Frasassi Caves are an extensive cave system in central Italy with over 25 km of passageways (Figure 1.1). Situated in the anticline transected by the Frasassi Gorge, the cave system is predominantly located in the Calcare Massiccio Fm. This formation contains over 99% calcium carbonate and is highly porous due to fractures within the formation and the origin of the limestone as a carbonate platform (Galdenzi and Maruoka, 2003). Above the Calcare Massiccio Fm. sits a thin layer of low-permeability limestone known as the Bugarone Fm. (Galdenzi *et al.*, 2008). Although the low-permeability of the Bugarone Fm. would impede meteoric water flow, fractures allow water to move from the overlying permeable Cretaceous limestone layers to the Calcare Massiccio Fm., as shown in Figure 1.2 (Galdenzi *et al.*, 2008). The geologic history of the Frasassi cave system allows for a unique combination of reduced and oxidized groundwater to co-exist in the caves.

Two chemically distinct types of groundwater, bicarbonate and sulfidic water, exist in the Frasassi caves (Sighinolfi, 1990; Tazioli *et al.*, 1990; Cocchioni *et al.*, 2003). The oxidized bicarbonate water originates from downward percolating meteoric water and is characterized by low salinity (200 to 400 mg L^{-1}), high dissolved oxygen, and low sulfate concentrations (Galdenzi *et al.*, 2008). This differs from the reduced sulfidic water, which flows upward, has higher salinity (up to 2 g L^{-1}) and is likely to have originated from older meteoric water that traveled through an underlying anhydrite formation (Tazioli *et al.*, 1990; Galdenzi *et al.*, 2008). Throughout the year, the bicarbonate water dilutes the sulfidic water between 30% and 60% based on seasonality (Galdenzi *et al.*, 2008). The redox disequilibria between the bicarbonate and sulfidic water allow for biological processes to contribute to cave development.

1.2 Sulfuric acid speleogenesis

Sulfuric acid speleogenesis (SAS) occurs when sulfuric acid produced from the complete oxidation of sulfide dissolves calcium carbonate cave walls (Principi, 1931; Egemeier, 1973). Originally thought to be abiotic, SAS was proposed to occur both above and below the water table (Davis, 1981; Galdenzi, 1990; Egemeier, 1981). Subaqueous dissolution occurs when sulfide in groundwater reacts with oxygen to produce sulfuric acid for calcium carbonate dissolution (equations 1 and 2). This differs from subaerial gypsum replacement, when sulfide volatilizes and reacts with atmospheric oxygen to form sulfuric acid, which subsequently reacts with the calcium carbonate walls to form gypsum crusts (equations 1 and 3). As the gypsum crusts develop and detach from the walls, the cave becomes larger. Although SAS is thought to take place in fewer than 10% of caves worldwide, it is responsible for the development of caves in Italy, the United States, Romania, and Mexico (Egemeier, 1973; Hill, 1990; Palmer, 1991; Galdenzi and Menichetti, 1995; Sarbu *et al.*, 1996; Hose *et al.*, 2000; Engel *et al.*, 2004b).

$$H_2S + 2\ O_2 \rightarrow H_2SO_4 \qquad (1)$$

$$H_2SO_4 + CaCO_3 \rightarrow Ca^{2+} + SO_4^{2-} + CO_2 + H_2O \qquad (2)$$

$$H_2SO_4 + CaCO_3 + H_2O \rightarrow CaSO_4{\cdot}2H_2O + CO_2 \qquad (3)$$

Although abiotic limestone dissolution was originally accepted as the primary mode of speleogenesis in caves affected by SAS, more recent evidence shows that sulfur oxidizing bacteria contribute to cave development (Hubbard *et al.*, 1990; Angert *et al.*, 1998; Hose *et al.*, 2000; Vlasceanu *et al.*, 2000; Engel *et al.*, 2001; Engel *et al.*, 2004b; Macalady *et al.*, 2007). In the process of energy production, sulfur oxidizing lithotrophs increase the amount of sulfuric acid in cave environments, causing faster rates of calcium carbonate dissolution (Porter *et al.*, 2009; Luther *et al.*, 2011). Furthermore, microbes are capable of concentrating sulfuric acid on

cave walls by colonizing surfaces for localized dissolution (Engel *et al.*, 2004b). The realization that microbes contribute to SAS created a new paradigm in the field.

In the Frasassi Caves, the rate of limestone dissolution from abiotic and biotic processes is comparable above and below the water table (Galdenzi *et al.*, 1997). Relevant abiotic processes include subaqueous calcium carbonate dissolution and subaerial gypsum replacement (Galdenzi, 1990; Galdenzi *et al.*, 1997; Galdenzi and Maruoka, 2003; Galdenzi *et al.*, 2008; Galdenzi, 2012). Relevant biotic processes include subaqueous and subaerial microbial sulfur oxidation (Macalady *et al.*, 2006; Macalady *et al.*, 2007; Jones *et al.*, 2008; Macalady *et al.*, 2008; Jones *et al.*, 2012). Although previous research has focused on the contributions of abiotic processes and cave wall biofilms to the development of the Frasassi Caves, less work has been completed on the contribution of subaqueous microbes to cave development. Since the streams host microbes capable of sulfur oxidation, and the cave system is dissolving at similar rates above and below the water table, it is likely that microbes in the cave streams contribute to SAS. It is therefore important to understand the metabolic contributions of dominant microbes within the cave streams.

1.3 Microbes in sulfidic caves

Sulfidic caves are aphotic environments that support a plethora of microbial life. Without carbon input from surface environments, such caves consist of trophic systems supported entirely by lithoautotrophy (Jones *et al.*, 2008; Porter *et al.*, 2009). Although microbial diversity in sulfidic caves varies geographically, there are recurring patterns in the taxonomic composition of cave walls and streams. Apart from microbial communities embedded in gypsum crusts, sulfidic caves generally have cave walls with irregular patterns of mud and clay known as biovermiculations, and biofilms that dangle from the cave wall known as snotties (Hose *et al.*,

2000; Vlasceanu et al., 2000; Engel *et al.*, 2001; Macalady *et al.*, 2007; Jones *et al.*, 2008). Cave streams typically contain conspicuous white biofilms dominated by sulfur oxidizing Epsilonproteobacteria and Gammaproteobacteria (Sarbu *et al.*, 1994; Angert *et al.*, 1998; Engel *et al.*, 2001; Engel *et al.*, 2003; Rohwerder *et al.*, 2003; Engel *et al.*, 2004a; Macalady *et al.*, 2006). Biovermiculations, snottites, and stream biofilms have been the subject of previous studies in the Frasassi Caves.

Although biovermiculations and snottites both exist on Frasassi Cave walls, they differ significantly in their physical microbial compositions. Biovermiculations were found to range between pH 5-10 and contained high microbial diversity, with 48 representative phylotypes from 15 bacterial lineages (Jones *et al.*, 2008). Conversely, snottites were consistently measured between pH 0-2 and were dominated by *Acidothiobacillus thiooxidans*, which accounted for greater than 70% of all cells (Macalady *et al.*, 2007; Jones *et al.*, 2011; Jones *et al.*, 2014). *A. thiooxidans* is a sulfur-oxidizing Gammaproteobacterium likely to be directly involved in SAS (Jones *et al.*, 2014).

Frasassi Cave streams are neutral in pH and contain white biofilms with microbes involved in sulfur cycling (Macalady *et al.*, 2006). Biofilms forming at the sediment-water interface over fine sediment are dominated by the filamentous sulfur-oxidizing bacterium *Beggiatoa*. Biofilms attached to rocks and coarse sediment in flowing water contain Deltaproteobacteria, Epsilonproteobacteria, and a large amount of the filamentous sulfur-oxidizing bacterium *Thiothrix* by biomass. Macalady *et al*. (2008) established a niche model with greater amounts of *Thiothrix* in areas with low sulfide to oxygen ratios, *Beggiatoa* prevailing in slow-flow regions regardless of sulfide to oxygen ratios, and filamentous Epsilonproteobacteria

dominating in environments with high sulfide to oxygen ratios (2008). Field photographs of typical stream biofilms studied in this project are shown in Figure 1.3.

Although preliminary work showed that *Thiobacillus baregensis* abundances do not correlate with sulfide and oxygen ratios, Macalady *et al.* determined that *T. baregensis* is both dominant and abundant in cave streams (2008). *T. baregensis* was originally isolated from sulfidic water in the Barèges region of France and has a SSU rDNA sequence deposited in the GenBank database. The culture was subsequently lost, and thus remains a candidate species (Hédoin *et al.*, 1996). Since *T. baregensis* is pervasive in the Frasassi Caves, its metabolic properties are likely to have a significant impact on the distribution and abundance of sulfur species, including sulfuric acid.

Determining the metabolic properties of Epsilonproteobacteria is of particular interest because Epsilonproteobacteria are the least studied class of Proteobacteria and have few cultured representatives. While the majority of Epsilonproteobacteria clones within the Frasassi Caves lie within *Arcobacter* spp. and the Sulfurovumales group, a few representatives are also found in the 1068 and Sulfuricurvales groups (Macalady *et al.*, 2008). Metagenomic analyses conducted in parallel with this project showed that *Arcobacter* spp. and *Sulfuricurvum* spp. may contribute to cave formation through the complete oxidation of sulfur sources, while bacteria within Sulfurovumales are likely to precipitate elemental sulfur through incomplete sulfide oxidation (Hamilton *et al.*, 2014). Isolating Epsilonproteobacteria from the aforementioned groups is of interest in order to increase the number of cultivated representatives. Culturing *T. baregensis* and Epsilonproteobacteria from the Frasassi Caves and studying their metabolic properties will allow for greater insight into the role of bacteria in sulfur cycling and cave development.

1.4 Figures

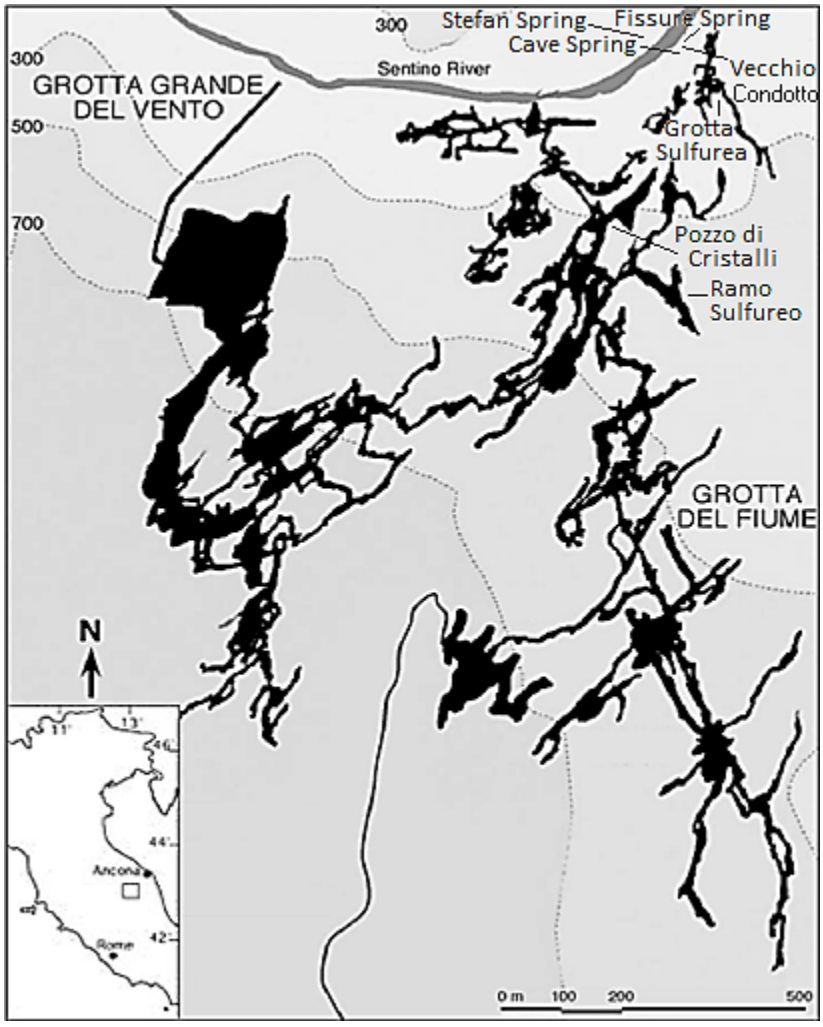

Figure 1.1 Map of the Frasassi Caves. Labels point to various sampling locations, thick black lines show major caves, and dashed lines reveal surface topography. The inset positions the caves within Italy. Base map courtesy of the Gruppo Speleologico CAI di Fabriano.

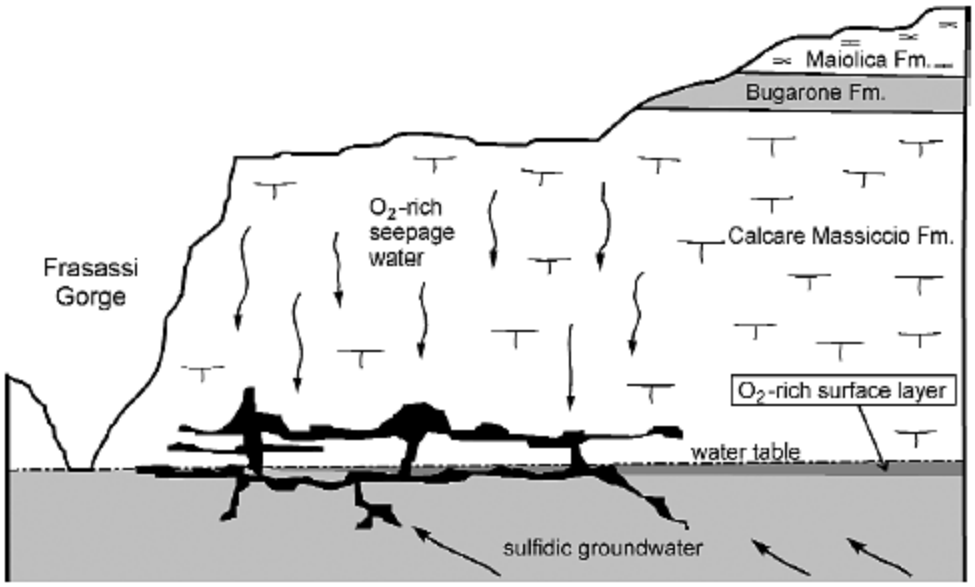

Figure 1.2 Hydrogeologic schematic of the Frasassi Caves. The white area filled with perpendicular black lines represents the Calcare Massaccio Fm., which consists of highly porous limestone. The gray area represents the Bugarone Fm., which consists of low-permeability limestone. The white area filled with parallel black lines represents porous limestone. Figure from Galdenzi and Maruoka, 2003.

Figure 1.3 Images of cave stream biofilms from Pozzo dei Cristalli with filled black arrows pointing to a biofilm with streamer morphology, and a black arrow outline pointing to *Beggiatoa* dominated biofilm at the sediment-water interface. (A) Close-up with carabiner for scale. (B) Pen for scale. Photo credits: J. Macalady.

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CHAPTER 2: *THIOBACILLUS BAREGENSIS*, A SUCCESSFUL BACTERIUM IN FRASASSI CAVE WATERS, IS METABOLICALLY VERSATILE

2.1 Abstract

Thiobacillus baregensis populations are both abundant and widespread in biofilms colonizing sulfidic streams in the Frasassi Caves. The original 1996 culture of *T. baregensis* (now lost) and close cultivated relatives *Thiofaba tepidiphila* and *Thiovirga sulfuroxydans* are known to oxidize sulfide with oxygen, similar to other ecologically successful populations in Frasassi stream biofilms. Previous work on the taxonomic composition of cave stream biofilms showed that the relative abundances of sulfur oxidizers in streamers can be predicted based on concentration ratios of dissolved total sulfide to oxygen. In apparent contrast to this prior result, fluorescent in-situ hybridization experiments showed that the area ratios of *Thiobacillus baregensis* relative to all bacteria do not correlate with sulfide concentrations (r-squared 0.11), oxygen concentrations (0.03), sulfide to oxygen ratios (r-squared 0.27), or oxygen to sulfide ratios (r-squared 0.04). The lack of correlation can be explained if *T. baregensis* uses alternative or additional electron donors and acceptors. Culture-dependent methods were used to test whether or not *T. baregensis* is capable of using sulfide, elemental sulfur, thiosulfate, and hydrogen as electron donors, and oxygen and nitrate as electron acceptors. *T. baregensis* grew on sulfide, elemental sulfur, and thiosulfate paired with nitrate prior to being outcompeted by competing populations. However, its growth in cultures containing hydrogen and cultures containing oxygen was difficult to establish due to quick overgrowth by other populations. The cleanest culture was obtained using thiosulfate and nitrate. These culturing results show that *T. baregensis* is capable of using multiple redox pairs to produce energy, and provides one possible explanation as to why its niche could not be adequately described based on sulfide and oxygen concentrations or ratios. My culturing results are supported by metagenomic evidence obtained in a parallel study. Together, the studies indicate that *T. baregensis* provides organic carbon to the cave system through carbon fixation (autotrophy), that it is likely to contribute to cave development through the formation of sulfuric acid, and that nitrate may be an important electron acceptor in Frasassi Cave stream waters even though nitrate concentrations are perenially below detection limits. Future work should focus on obtaining an axenic culture of *T. baregensis*, acquiring a whole-genome sequence, and determining whether or not *T. baregensis* directly contributes to cave development by measuring the amount of sulfuric acid produced by *T. baregensis* under various growth conditions, or by directly measuring the rate of calcium carbonate dissolution in cultures under various growth conditions.

2.2 Introduction

Candidatus Thiobacillus baregensis is a bacterium within class Betaproteobacteria (Hédoin *et al*., 1996). Originally described from sulfur-rich thermal waters in Barèges, France (Hédoin *et al*., 1996; Hédoin, 1997), closely related 16S rRNA sequences have since been retrieved from other sulfur-rich groundwaters and cave aquifers, including Frasassi Cave and

associated springs in Italy (Macalady *et al*., 2006; Macalady *et al.*, 2008), Parker Cave in the U.S.A. (Angert *et al*., 1998), Movile Cave in Romania (Chen *et al.*, 2009), and Acquasanta Terme in Italy (Jones *et al.*, 2010). Outside of groundwater environments, close relatives with sequence similarities greater than 90% have also been described from wastewater biofilms (Ito *et al.*, 2004; Ito *et al.*, 2005), column reactor biofilms (Ferrera *et al.*, 2004), a membrane bioreactor (Vannini *et al.*, 2008), and a Japanese hotspring (Mori and Suzuki, 2008). Although there are no publications describing the metabolic properties of *T. baregensis*, and the original 1996 isolate has apparently been lost, the environmental geochemistry of *T. baregensis* habitats and metabolic properties of close cultivated relatives strongly suggest that *T. baregensis* is a sulfur oxidizing bacterium.

Sulfidic streams in the Frasassi Caves are colonized by biofilms with high abundances of *T. baregensis*. In similar biofilms, filamentous Sulfurovumales-group Epsilonproteobacteria and *Thiothrix* spp. were previously shown to have separate ecological niches based on dissolved sulfide to oxygen ratios (Macalady *et al.*, 2008). Given the numerical importance of *T. baregensis* populations in the biofilms, I asked whether or not *T. baregensis* dominated biofilms could similarly be described based on dissolved sulfide to oxygen ratios. I also asked how *T. baregensis* metabolism relates to biogeochemical cycling in sulfidic Frasassi Cave groundwater. If *T. baregensis* uses reduced sulfur compounds to produce sulfuric acid, which corrodes calcium carbonate cave walls, it is directly involved in cave formation. However, if *T. baregensis* incompletely oxidizes sulfide, it may produce elemental sulfur, an economically valuable chemical intermediate that can also be used as an energy source for other lithotrophic or organotrophic microorganisms.

2.3 Methods

2.3.1 Field site and geochemistry

Microbial biofilms and corresponding water samples were collected between 2006 and 2013 from Stefan Spring (SS), Cave Spring (CS), Fissure Spring (FS), Vecchio Condotto (VC), Grotta Sulfurea (GS), Acquasanta (AS), Pozzo di Cristalli (PC), and Ramo Sulfureo (RS). Biofilm samples were collected with sterile plastic pipettes, stored in sterile tubes, placed on ice, and processed within 4-6 hours of collection. Subsamples were fixed in 4% (w/v) paraformaldehyde (PFA) and stored at -20°C in preparation for fluorescent *in situ* hybridization (FISH).

Dissolved sulfide and oxygen concentrations were measured *in situ* according to Macalady *et al.* (2008). Two total dissolved hydrogen sulfide (H_2S_T) measurements were obtained for each sample with a portable spectrophotometer (Hach, Loveland, CO) using the methylene blue method (Hach method 690). Three dissolved oxygen measurements were obtained using a Hach HQ40d portable meter with a LDO101 dissolved optical probe (Hach Co., Loveland, CO, USA). Oxidation reduction potential was measured using a MTC101 ORP probe attached to a Hach HQ40d portable meter (Hach Co., Loveland, CO, USA). Water temperature, pH, and conductivity were measured using a 350i multimeter and handheld probes (WTW, Weiheim, Germany).

2.3.2 FISH analysis

FISH was performed using methods described in Amann (1995) with probes EUB338, EUB338-II, EUB-III, and Tbar581, as summarized in Table 2.1. Probe Tbar581 was designed to target *T. baregensis* strains retrieved from Italian sulfidic cave waters and excludes the original lost isolate (Macalady *et al.*, 2006; Macalady *et al.*, 2008; Jones *et al.*, 2010). The stringency of

the probe was optimized using a pure culture of *Thiofaba tepidiphila* (Mori and Suzuki, 2008), which has a 1 bp mismatch to the probe sequence. After hybridization, cells were counterstained using 4',6'-diamidino-2-phenylindole (DAPI) and mounted using Vectashield (Vectashield Laboratories Inc., Burlingame, CA, USA). A Nikon E800 epifluorescence microscope was used to view the prepared slides. The cell count and area count tools from NIS Elements AR 2.30, Hotfix (Build 312) image analysis software were used to count cells and estimate the area of *Thiobacillus baregensis* cells (Tbar581) relative to all bacteria (EUBMIX). The mean area ratio was calculated for each field of view. Multiple fields of view were analyzed until 1000 cells were counted for each sample. The final *T. baregensis* area ratio was calculated for each sample by averaging the area ratios determined from multiple fields of view. One standard deviation was calculated among the microscope fields of view. When the cellular ratios were plotted against sulfide and oxygen data, the R-squared values revealed whether or not a correlation existed between *T. baregensis* abundances and the specific chemical parameter.

2.3.3 Sample collection and enrichment culturing

Biofilm was collected from Stefan Spring 10 m downstream from the emergence on March 13, 2013 and returned to PSU for use as culture inoculum. An image of the Stefan Spring collection site is shown in Figure 2.1. Enrichment culturing was carried out in a series of 1:10 dilutions in modified sulfur oxidizing bacteria (SOB) media adapted from Kuenen *et al.* (1991). Modified SOB media contained 4.5 mM K_2HPO_4, 2.2 mM KH_2PO_4, 7.4 mM NH_4Cl, and 1.6 mM $MgSO_4.7H_2O$ under N_2/CO_2 (80:20, by volume). The medium was supplemented with 2 mL/L trace metal solution adapted from Vishniac and Santer (1957) containing 134 mM EDTA, 7.7 mM $ZnSO_4.7H_2O$, 49.7 mM $CaCl_2.2H_2O$, 12.63 mM $MnCl_2.4H_2O$, 18.0 mM $FeSO_4.7H_2O$, 2.8 mM $(NH_4)_6MoO_{24}.4H_2O$, 0.8 mM $CuSO_4.5H_2O$, and 2.1 mM $CoCl_2.6H_2O$. Electron donors

consisted of 15 mM H_2S, 0.44 g/L S^0, or 6.5 mM $S_2O_3^{2-}$. The electron acceptor was 0.74 mM NO_3^-. The medium was supplemented with 0.01 g/L erythromycin and subsequently adjusted to pH 7.2 prior to inoculation. Cultures were incubated in the dark in serum vials without shaking. Bacterial growth was evaluated using FISH by hybridizing probe Tbar581, scanning FISH slides with a fluorescence microscope, and estimating the area ratio of *T. baregensis* cells to total cells. The compositions of alternative experimental media are listed in Table 2.2.

2.3.4 16S rRNA phylogeny

A 16S rRNA gene tree was built to examine the phylogenetic position of *T. baregensis* amongst closely related organisms. The 16S rRNA gene sequences were obtained from Frasassi Cave metagenomes (Hamilton *et al.*, in prep) and the NCBI database, including three *Thiothrix* sp. selected as outgroups. MEGA 6 was used to align the sequences using Clustal W and to construct a Neighbor-Joining tree with 500 bootstrap replications.

2.3.5 Metagenomics

Metagenomic analysis was carried out in a parallel study by Hamilton *et al.* (in prep). Environmental metagenome samples designated AS07-7, FS06-10, FS08-3, GS09-5, GS10-10, PC08-3, PC08-64, and PC08-66 were sent to the Joint Genome Institute (JGI) for deep sequencing via Illumina Hi-Seq, scaffold assembly, and scaffold annotation. Annotation was completed using Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa *et al.*, 2004), Clusters of Orthologous Group of proteins (COG) (Tatusov *et al.*, 2000), Protein Family (Pfam) (Sonnhammer *et al.*, 1997), and TIGRFAM (Haft *et al.*, 2003). Scaffolds were taxonomically differentiated using tetranucleotide frequency to bin microbes by population or species (Dick *et al.*, 2009). EMIRGE, an iterative method for assembling small subunit ribosomal RNA genes

from short sequencing reads, was used to reconstruct near full-length 16S rRNA sequences for taxonomic categorization (Miller *et al.*, 2011).

The *T. baregensis* bin in sample FS06-10 was assessed for completeness by checking for the presence of conserved tRNA synthetases genes. Annotated genes were examined in order to understand how *T. baregensis* cycles C, S, and N compounds and provide information for medium construction. These included functional genes involved in chemical cycling and genes coding for antibiotic resistance.

2.4 Results

2.4.1 Tbar581 probe description

Probe Tbar581 hits 72 16S rRNA sequences in the database, all belonging to uncultured Gammaproteobacteria. These include the *T. baregensis* sequences obtained from the Frasassi Caves. Interestingly enough, Tbar581 has 1 bp mismatch to the original 1996 *T. baregensis* isolate (Hédoin *et al*., 1996).

2.4.2 Distribution of *Thiobacillus baregensis* populations in Frasassi biofilms

Since *T. baregensis* is abundant in Frasassi Cave stream biofilms and can contribute to cave formation through sulfuric acid production, I investigated its ecological niche. Specifically, I asked whether or not its distribution can be predicted based on sulfide and oxygen concentrations or ratios, as demonstrated by other abundant sulfur-oxidizing populations in similar biofilms (Macalady *et al.*, 2008). Cellular area ratios of *T. baregensis* to all bacteria are listed in Table 2.3, along with sulfide concentrations, oxygen concentrations, sulfide to oxygen concentration ratios, and oxygen to sulfide concentration ratios. Sulfide concentrations replicates are within 3% of each other, and oxygen concentrations triplicates are within 20% of each other. The area ratios of *T. baregensis* to all bacteria do not correlate with H_2S concentrations (Figure

2.2), O_2 concentration (Figure 2.3), H_2S to O_2 concentration ratios (Figure 2.4), or O_2 to H_2S concentration ratios (Figure 2.5), as indicated by the low r-squared values which ranged between 0.03 and 0.27. Furthermore, no distinct patterns were found when area ratios were plotted against total dissolved sulfide versus oxygen concentrations, as shown in Figure 2.6. This further shows the lack of relationship between *T. baregensis* abundances and sulfide to oxygen concentrations.

2.4.3 Metagenomic analysis

Metagenomic analysis was carried out in parallel to this study and enabled us to browse for *T. baregensis* associated functional genes involved in biogeochemical cycling and antibiotic resistance. Although metagenomics does not typically reveal all genes associated with a population or species, one *T. baregensis* metagenome bin was mostly complete, as evidenced by the presence of 80% of the amino acid tRNA synthetase genes (Table 2.4). C, S, O, and N functional genes are summarized in Table 2.5. Antibiotics which may hinder contaminant growth without killing *T. baregensis* are listed in Table 2.6.

2.4.4 Sample geochemistry and enrichment culturing

Stefan Spring water surrounding the biofilm used as inoculum for enrichment culturing had a pH of 7.55, temperature of 14 °C, conductivity of 2.9 mS/cm, and oxidation reduction potential of -300 mV. Based on FISH results, *T. baregensis* made up 59±23% of the biomass in the sample. An image of the inoculum stained with DAPI and hybridized with EUB338, EUB338-II, EUB338-III, and Tbar581 probes is shown in Figure 2.7. *T. baregensis* showed growth in media containing sulfide, elemental sulfur, and thiosulfate as electron donors and nitrate as the electron acceptor. Media containing the same electron donors and 1% oxygen as an electron acceptor resulted in rapid contaminant growth within three days. It is therefore difficult to ascertain whether or not *T. baregensis* is capable of using oxygen as an electron acceptor. The

most highly enriched culture contained thiosulfate, nitrate, and the antibiotic erythromycin. This culture contained 80±10% *T. baregensis* after about 168 hours of growth. Maintaining highly enriched *T. baregensis* cultures required high erythromycin concentrations (0.01 g/L).

An increase in contaminants and poor *T. baregensis* growth were associated with many failed liquid culturing attempts in modified SOB medium, especially in the absence of erythromycin. Using hydrogen as an electron donor resulted in rapid growth of a contaminant Epsilonproteobacterium described in Chapter 3. Furthermore, while *T. baregensis* is likely to be a facultative anaerobe, an increase in contaminant populations and relative lack of *T. baregensis* growth were observed when oxygen was used as the terminal electron acceptor regardless of whether sulfide, elemental sulfur, or thiosulfate served as electron donors. Increasing the concentration of nitrate did not significantly alter *T. baregensis* or contaminant growth. Although yeast extract and nutrient broth are known to increase the growth rate of some slow growing bacteria, additions of either substance yielded a high abundance of contaminants and poor *T. baregensis* growth. When thiosulfate medium was supplemented with serine, asparagine, arginine, and thiamine, dividing *T. baregensis* cells were observed after three days, but contaminants later dominated the culture. Two additional antibiotics, bleomycin and bacitracin, were tested to try to obtain an axenic *T. baregensis* culture. Adding bleomycin led to the presence of dividing *T. baregensis* cells along with some contaminant cells, not unlike the result obtained by adding erythromycin. The same result occurred after adding 0.54 μg/L bacitracin. When a higher dose of bacitracin was added to the medium (27.03 μg/L), no growth was observed after two weeks. The use of a second base liquid medium (Kodama and Watanabe, 2004) led to heavy contaminant growth in seven days regardless of whether H_2S, S^0, or $S_2O_3^{2-}$ was used as the electron donor when NO_3^- served as the electron acceptor.

Agar plating was attempted using modified SOB medium without erythromycin. Plates containing the described medium with $S_2O_3^{2-}$ and S^0 incubated under air led to the formation of contaminant colonies. These contaminants, which grew on both $S_2O_3^{2-}$ and S^0 plates, included *Halothiobacillus neapolitanus* strain CIP 104769 (accession number JN175334.1) as well as a previously uncultured Gammaproteobacterium with 99% sequence similarity to a bacterium previously described from a sulfidic cave (Porter *et al.*, 2009). The 16S sequence of the previously uncultured Gammaproteobacterium was deposited into the NCBI database (accession number KM979607). The 16S rRNA gene sequence and corresponding phylogenetic tree are shown in Figure 2.8. When the same plates were incubated under $N_2/CO_2/H_2$ (80:10:10, by volume) with H_2S and NO_3^-, the contaminant *Aeromonas hydrophila* 4AK4 (accession number CP006579.1) was isolated. No colonies were retrieved when plates with 2 g/L bicarbonate, $S_2O_3^{2-}$, and NO_3^- were incubated under N_2/H_2 (50:50, by volume). Lastly, bacteria were incapable of growth when plated in 500 mL glass bottles under $N_2/CO_2/H_2$ (40:20:40, by volume) with H_2S, $S_2O_3^{2-}$, and NO_3^-. The bottles could not be stored upside-down because the solidified medium did not hold on to the glass bottle. When the bottles were incubated in an upright position, condensation eventually fell onto the surface of the agar, disrupting the colonies and preventing further enrichment attempts.

2.4.5 *T. baregensis* phylogenetic tree

The 16S rRNA gene tree used to examine the phylogenetic position of *T. baregensis* amongst closely related organisms is shown in Figure 2.9. The bacteria within the tree show four distinct clusters, which separate the 16S rRNA genes at the genus level. These clusters include *Thiobacillus* sp., *Thiofaba* sp., *Thiovirga* sp., and three *Thiothrix* sp. used for the outgroup. Separation of the reconstructed 16S rRNA genes from the Frasassi Cave metagenomes show

how many of the sequences lie within the three genera. According to Figure 2.9, eight reconstructed sequences lie within *Thiobacillus* sp., one sequence lies within *Thiofaba* sp., and 10 sequences lie within *Thiovirga* sp.

2.5 Discussion

2.5.1 *Thiobacillus baregensis* biomass correlations with sulfide and oxygen concentrations

FISH was previously used to study niche separation between abundant sulfur-oxidizing bacterial groups in the Frasassi Caves (Macalady *et al.*, 2008). That work showed that the relative abundances of two major biofilm populations, filamentous Epsilonproteobacteria and *Thiothrix* spp., are predictable based on dissolved sulfide and oxygen ratios. In contrast, results from this study show that *T. baregensis* abundances are not correlated with dissolved sulfide and oxygen concentrations or ratios. Logical explanations for these results are that *T. baregensis* is incapable of using sulfide as an electron donor and oxygen as an electron acceptor, or that *T. baregensis* uses other redox pairs in addition to sulfide and oxygen. Culture-dependent methodology was subsequently used to explore the metabolic capabilities of *T. baregensis*.

2.5.2 Enrichment culturing and implications on biogeochemical cycling and microbial ecology

Both culture-dependent and culture-independent results show that *T. baregensis* is an autotroph. The successful enrichment medium contained no organic carbon. While contaminant populations could have served as a carbon source, the high abundance of *T. baregensis* and lack of additional sources of organic carbon strongly suggest otherwise. Aside from organic carbon, the total dissolved inorganic carbon (DIC) level in the enrichment medium was about twice the amount measured in Frasassi Cave streams. The cultures had a DIC concentration of 13.42 mM, while the Frasassi Cave streams have a DIC concentration of 6.34 ± 1.04 mM. Since these two values are comparable, it is unlikely that *T. baregensis* had to invest extra energy to obtain

carbon or adapt to high carbon levels in culture. Culture-dependent suggestions of autotrophy agrees with metagenomic analysis, which shows the presence of RuBisCo, an enzyme responsible for catalyzing the first step of carbon fixation, and carbonic anhydrase, an enzyme used in the interconversion of carbon dioxide and bicarbonate, in the *T. baregensis* bin (Hamilton *et al.*, in prep). *T. baregensis* is an autotroph that contributes organic carbon to the aphotic cave food web.

The medium that led to high enrichments of *T. baregensis* contained reduced sulfur sources as electron donors. This is consistent with functional genes involved in sulfur oxidation found in the *T. baregensis* bin. The presence of SoxAX, Sox B, SoxCD, and SoxYZ allow for full oxidation from thiosulfate to sulfate (Friedrich *et al.*, 2001; Hamilton *et al.*, in prep). Although isolation attempts were less successful when sulfide or elemental sulfur were provided as electron donors, the rapid contaminant growth I observed does not necessarily indicate that *T. baregensis* is incapable of oxidizing sulfide or elemental sulfur. It is highly likely that *T. baregensis* is capable of growing on the aforementioned electron donors, but that the contaminant populations have a competitive advantage in metabolizing those chemical species under the growth conditions I provided. In fact, genes in the *T. baregensis* bin included the complete set of SOX genes (SoxAX, SoxB, SoxCD, SoxYZ) as well as SQR, SOR, and fccC genes (Hamilton *et al.*, in prep). If expressed, the complete set of SOX genes would permit *T. baregensis* to completely oxidize reduced and intermediate sulfur compounds such as sulfide, elemental sulfur, sulfite, and thiosulfate to sulfate (Sauvé *et al.*, 2007). Aside from the SOX genes, expression of the SQR (Shahak *et al.*, 1992) and fccC (Chen *et al.*, 1994; Morris *et al.*, 1994) genes would also allow *T. baregensis* to oxidize sulfide, and expression of the SOR gene would allow it to execute a reversible reaction between sulfide and elemental sulfur (Kletzin,

1992). Overall, metagenomic analysis strongly suggests that *T. baregensis* is capable of oxidizing a variety of reduced sulfur species, including sulfide, elemental sulfur, and thiosulfate, completely to sulfate. Since *T. baregensis* has the molecular machinery to completely oxidize reduced sulfur species, it is highly likely that it contributes to the enlargement of the Frasassi Caves via sulfuric acid production.

T. baregensis enrichment culturing was most successful when nitrate was provided as the electron acceptor. This agrees with metagenomic data, which revealed dissimilatory nitrate reduction genes in the *T. baregensis* bin (Hamilton *et al.*, in prep). Although nitrate reduction genes are present, the denitrification pathway is not complete. An incomplete reduction pathway may be an artifact of the incomplete genome, or may show that *T. baregensis* can produce ammonia. However, since the genome bin is not missing many genes, as shown by the presence of amino acid tRNA synthetase genes in Table 2.4, it is likely that *T. baregensis* produces ammonia.

The quick increase in contaminant cells under microaerobic conditions does not show that *T. baregensis* is incapable of surviving in low oxygen environments. Genes found in the *T. baregensis* bin responsible for oxygen reduction include a cbb3-type cytochrome c oxidase and a bd-type cytochrome c (Sakamoto *et al.*, 1996; Pitcher *et al.*, 2002, Hamilton *et al.*, in prep). In addition to the aforementioned genes, the presence of the sulfur oxidizing gene SOR further suggests that *T. baregensis* is capable of surviving in the presence of oxygen because the molecular machinery associated with the gene is only activate when oxygen is present (Kletzin, 1992; Hamilton *et al.*, in prep).

Even though *T. baregensis* is capable of using oxygen as an electron acceptor, it is interesting that it appears to have a selective advantage when provided with nitrate in culture. In

cave streams, oxygen concentrations are up to 25 μM, while nitrate concentrations are perennially below detection limit (<0.7 μM nitrate) (Macalady *et al.*, 2008). Since *T. baregensis* is capable of using nitrate as an electron acceptor, and nitrate levels are extremely low in the Frasassi Caves, nitrate may be a limiting resource for energy generation by the autotrophic bacterial ecosystem.

The ability of *T. baregensis* to use multiple sulfur sources as electron donors and both oxygen and nitrate as electron acceptors may provide an explanation for why *T. baregensis* biomass in nature does not correlate with dissolved sulfide or oxygen concentrations, or sulfide to oxygen ratios. The lack of correlation with sulfide to oxygen ratios together with culture results suggest that *T. baregensis* does not preferentially use sulfide and oxygen to gain energy *in situ*, and that when nitrate is unavailable, *T. baregensis* is outcompeted by organisms which appear to use oxygen. Even though *T. baregensis* does not solely use the sulfide and oxygen redox pair that would allow for the greatest energy yield (supplemental calculations), the high abundance of *T. baregensis* in cave waters with a wide variety of sulfide and oxygen concentrations (Figure 2.3) suggests that its ability to use multiple energy sources is an ecological advantage.

2.5.3 Potential reasons for contaminant growth

Despite quick initial growth, it was difficult to obtain a highly enriched culture of *T. baregensis*. This shows that the base medium was not ideal for culturing *T. baregensis*. One method to create a base medium that includes all chemical species required for growth is by first using metagenomics to look for missing pathways. This can potentially reveal the amino acids, vitamins, and minerals that *T. baregensis* is incapable of synthesizing on its own. Such metagenomic analysis can be important in developing a successful enrichment medium if *T.*

baregensis has a syntrophic relationship with another microbe in the environment. In this case, gradually enriching for *T. baregensis* would simultaneously decrease the ability of *T. baregensis* to grow. Although I used results from metagenomics to inform my culturing, I was still unable to obtain an axenic culture of *T. baregensis*. Another issue that arose in the enrichment process was the quick growth of contaminant populations. Although contaminants stopped growing with a high concentration of erythromycin, this also slowed the growth of *T. baregensis*.

2.5.4 16S phylogeny of *T. baregensis* and closely related bacteria

The phylogenetic tree in Figure 2.9 shows that *T. baregensis* is closely related to *Thiofaba* and *Thiovirga* sp. The *Thiofaba* and *Thiovirga* clades each have one cultivated representative, *Thiofaba tepidiphila* and *Thiovirga sulfuroxydans*. All three species are obligate autotrophs (Ito *et al.*, 2005; Mori and Suzuki, 2008). Furthermore, all are sulfur oxidizers capable of oxidizing sulfide, elemental sulfur, and thiosulfate (Ito *et al.*, 2005; Mori and Suzuki, 2008). While *Thiofaba tepidiphila* was shown to oxidize tetrathionate, tetrathionate oxidation capabilities were not tested on *Thiovirga sulfuroxydans* (Mori and Suzuki, 2008). All three bacterial species are also capable of using oxygen as a terminal electron acceptor.

Interestingly, a major difference among the three bacterial species is the ability to use nitrate as a terminal electron acceptor. While *Thiofaba tepidiphila* and *Thiovirga sulfuroxydans* are both incapable of anaerobic growth with nitrate, *Thiobacillus baregensis* is capable of using nitrate as an electron acceptor. This is an important feature that distinguishes *T. baregensis* from its close relatives.

2.5.5 Conclusions, implications, and future work

Since *T. baregensis* is both widespread and abundant in Frasassi Cave stream biofilms, its metabolism is likely to have a significant effect on biogeochemical cycling in that environment.

As an autotroph, *T. baregensis* provides organic carbon to the aphotic ecosystem and thereby allows heterotrophs, including macrointertebrates, to survive. Its lithotrophic metabolism is versatile and links *T. baregensis* to both sulfur and nitrogen cycling. Since *T. baregensis* can oxidize multiple reduced sulfur compounds and is capable of their complete oxidation, it produces sulfuric acid as a waste product.

Properties of *T. baregensis* metabolism have implications for both science and society. Understanding *T. baregensis* metabolism is important for geoscience not only because *T. baregensis,* which has been found in multiple sulfidic groundwater environments associated with actively corroding caves (Angert *et al*., 1998; Macalady *et al*., 2006; Macalady *et al.*, 2008; Chen *et al.,* 2009; Jones *et al.,* 2010), produces sulfuric acid and is likely to be involved in cave formation through SAS, but also because its ability to reduce nitrate hints at bacterial contributions to nitrogen cycling within the Frasassi Caves. This is not easily deciphered with undetectable nitrate levels in sulfidic Frasassi Cave stream water. *T. baregensis* nitrate reduction in streams with detectable oxygen levels also leads to an implication for microbiology. The prevailing wisdom is that organisms capable of using multiple redox pairs for energy production will choose the pair that yields the most energy. The high abundance of *T. baregensis* in sulfidic Frasassi Cave streams along with its greater growth on nitrate can serve as evidence otherwise. Lastly, *T. baregensis* sulfur metabolism has implications on society. High sulfide levels can be an issue for industry, such as the tanning industry (Vannini *et al.*, 2008). Discovering and culturing more SOB can be useful for bioremediation in order to convert sulfide to its more oxidized forms.

Future work should first focus on obtaining an axenic culture of *T. baregensis*. This may be completed by continuing serial dilutions. After an axenic culture is obtained, studies should

focus on obtaining a whole genome sequence and establishing whether or not *T. baregensis* forms biofilms on calcium carbonate surfaces, potentially localizing production of sulfuric acid and increasing the rate of cave enlargement, as suggested for sulfur oxidizing microbes in other sulfidic caves (Engel *et al.*, 2004). This may be completed by measuring the amount of sulfuric acid produced by *T. baregensis* under various growth conditions, or by measuring limestone dissolution rates in cultures under various growth conditions. Lastly, its ability to use nitrate as an electron acceptor and quick contaminant growth under microaerobic conditions suggests that nitrate is present and rapidly cycled in Frasassi Cave waters regardless of the fact that it is perennially below detection limit. Future detection of *napA*, a gene responsible for nitrate reduction with greater efficiency in the presence of oxygen, could support this hypothesis (Gavira *et al.*, 2002). Obtaining a pure culture of *T. baregensis* for complete genome sequencing will be useful in further identifying how this microorganism contributes to biogeochemical cycling within the Frasassi Caves.

2.6 Tables and Figures

Table 2.1 Oligonucleotide probes used for FISH.

Probe	*Target group*	*Sequence (5' → 3')*	*Formamide (%)*	*Target site*	*Reference*
EUB338[a]	Most bacteria	GCTGCCTCCCGTAGGAGT	0-50	16S (338-355)	Amann, 1995
EUB338-II[a]	Planctomycetales	GCAGCCACCCGTAGGTGT	0-50	16S (338-355)	Daims *et al*., 1999
EUB338-III[a]	Verrucomicrobiales	GCTGCCACCCGTAGGTGT	0-50	16S (338-355)	Daims *et al*., 1999
Tbar581	*Thiobacillus baregensis* /Thiofaba clade	CCGACTGATCAAACCGCCTAC	45-50	16S (581-601)	this study

[a] Combined in equimolar amounts to make EUBMIX.

Figure 2.1 Image of biofilms from Stefan Spring collected for inoculating *T. baregensis* enrichment. Red disk for scale (22.6 cm in diameter). Photo credit: J. Macalady.

Table 2.2 Summary of media that led to overgrowth of contaminant populations. All media were adjusted between pH 6.9 and 7.2. Solidified media were incubated in plates unless stated otherwise.

Base Media (Citation)	Experimental Factor	Gases	Electron Donor	Electron Acceptor
Modified SOB Media (Liquid) (Kuenen *et al.* , 1991)	Electron Donor	$N_2/CO_2/H_2$ (40:20:40, by volume)	H_2 (40% headspace, by volume)	NO_3^- (0.74 mM)
	Electron Acceptor	N_2/CO_2 (80:20, by volume)	H_2S (15 mM) S^0 (0.44 g/L) $S_2O_3^{2-}$ (6.5 mM)	O_2 (1% headspace) NO_3^- (5 mM)
	Amino Acids Serine (0.5 g/L) Asparagine (0.5 g/L) Arginine (0.5 g/L)	N_2/CO_2 (80:20, by volume)	$S_2O_3^{2-}$ (6.5 mM)	NO_3^- (0.74 mM)
	Vitamin Thiamine (0.2 g/L)	N_2/CO_2 (80:20, by volume)	$S_2O_3^{2-}$ (6.5 mM)	NO_3^- (0.74 mM)
	Antibiotics Bleomycin (1 µg/mL) Bacitracin (0.54 µg/L) Bacitracin (27.03 µg/L)	N_2/CO_2 (80:20, by volume)	$S_2O_3^{2-}$ (6.5 mM)	NO_3^- (0.74 mM)
	Yeast Extract (0.1% w/v)	N_2/CO_2 (80:20, by volume)	H_2S (15 mM) S^0 (0.44 g/L) $S_2O_3^{2-}$ (6.5 mM)	NO_3^- (0.74 mM)
	Nutrient Broth (0.016 g/L)	N_2/CO_2 (80:20, by volume)	H_2S (15 mM) S^0 (0.44 g/L) $S_2O_3^{2-}$ (6.5 mM)	NO_3^- (0.74 mM)
Modified SOB Media (Solid) (Kuenen *et al.* , 1991)	Electron Donor	$N_2/CO_2/H_2$ (80:10:10, by volume)	H_2 (10% headspace, by volume) H_2S (15 mM)	NO_3^- (0.74 mM)
	Electron Acceptor	Air	S^0 (0.44 g/L) $S_2O_3^{2-}$ (6.5 mM)	O_2 (20.95%)
	Bicarbonate (2 g/L)	N_2/H_2 (50:50, by volume)	H_2 (10% headspace, by volume) $S_2O_3^{2-}$ (6.5 mM)	NO_3^- (0.74 mM)
	Plating in a 500 mL Bottle	$N_2/CO_2/H_2$ (40:20:40, by volume)	H_2 (10% headspace, by volume) H_2S (15 mM) $S_2O_3^{2-}$ (6.5 mM)	NO_3^- (0.74 mM)
Low Ion-Strength Medium (Liquid) (Kodama and Watanabe, 2004)	Different Base Medium	N_2/CO_2 (80:20, by volume)	H_2S (15 mM) S^0 (0.44 g/L) $S_2O_3^{2-}$ (6.5 mM)	NO_3^- (0.74 mM)

Table 2.3 Microbial samples used for FISH analysis with their corresponding H_2S concentrations, O_2 concentrations, H_2S to O_2 ratios, O_2 to H_2S ratios, and area ratios of *T. baregensis* cells based on FISH.

Sample	[H_2S] (μM)	[O_2] (μM)	[H_2S]/[O_2]	[O_2]/[H_2S]	Area ratio of *T. baregensis*
GS10-4	17.53	29.69	0.59	1.69	0.01
GS10-5	17.53	29.69	0.59	1.69	0.09
CS10-2	34.93	57.81	0.60	1.66	0.07
CS10-3	34.93	57.81	0.60	1.66	0.47
CS13-1	30.20	39.00	0.77	1.29	0.13
GS06-3	23.70	25.34	0.94	1.07	0.03
GS06-4	23.70	25.34	0.94	1.07	0.06
VC10-7	37.75	35.16	1.07	0.93	0.01
VC10-2	39.53	35.16	1.12	0.89	0.13
VC10-4	39.53	35.16	1.12	0.89	0.13
VC10-5	39.53	35.16	1.12	0.89	0.07
SS13-102	204.50	85.75	2.38	0.42	0.60
CS13-13	117.89	28.13	4.19	0.24	0.11
SS13-103	321.40	46.30	6.94	0.14	0.78
FS13-13	246.08	20.83	11.81	0.08	0.00
CS13-17	111.03	5.78	19.20	0.05	0.15
PC08-66	402.33	15.50	25.96	0.04	0.05
RS08-71	114.19	4.34	26.29	0.04	0.00
PC07-21	438.82	16.63	26.39	0.04	0.72
RS08-70	137.27	4.34	31.60	0.03	0.01
AS08-2	420.95	7.69	54.76	0.02	0.00
GS07-4	205.02	2.16	95.08	0.01	0.33
PC13-11	363.73	2.48	146.40	0.01	0.14
PC13-12	363.73	2.48	146.40	0.01	0.11
PC13-13	363.73	2.48	146.67	0.01	0.03
PC06-110	289.90	1.94	149.63	0.01	0.36
GS07-5	213.66	1.16	184.78	0.01	0.00
GS07-6	213.66	1.16	184.78	0.01	0.08
AS08-3	812.58	4.06	200.02	0.00	0.08
FS06-10	435.83	1.53	284.86	0.00	0.81
FS06-12	435.83	1.53	284.86	0.00	0.81

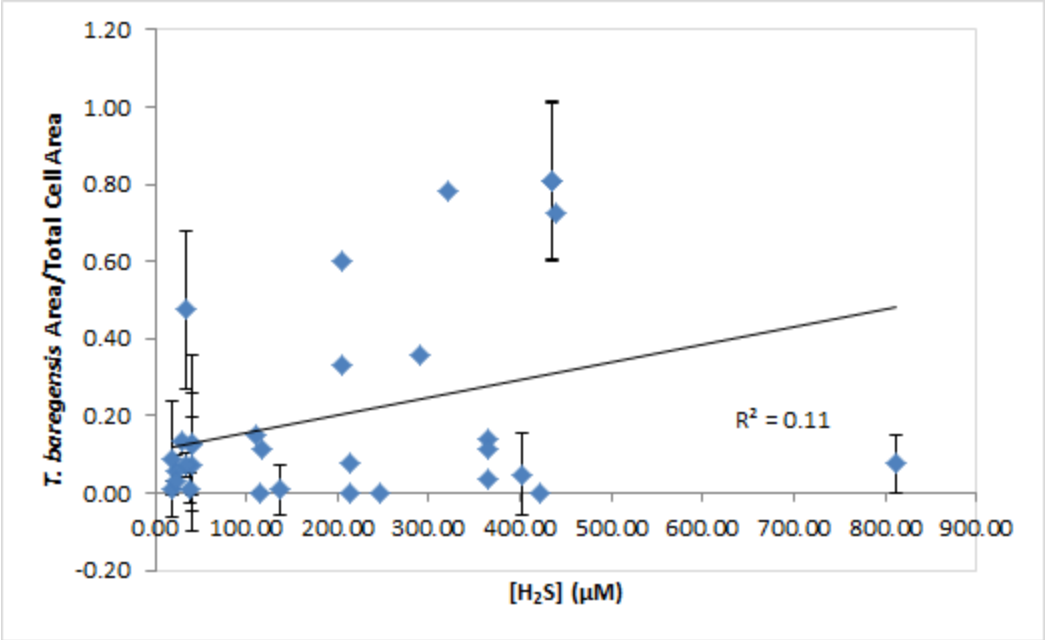

Figure 2.2 Area of *T. baregensis* to all bacteria versus H_2S concentrations. Each data point represents a total of at least 1000 counted cells. All data points are plotted with error bars, which represent one standard deviation among the microscope fields of view for each sample.

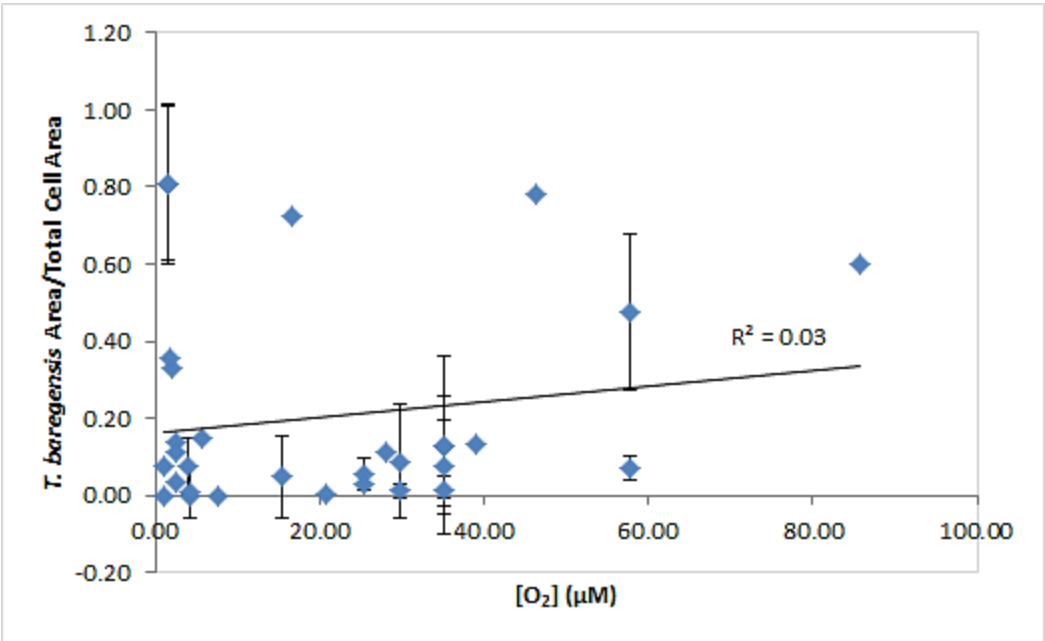

Figure 2.3 Area of *T. baregensis* to all bacteria versus *in situ* dissolved O_2 concentrations. Each data point represents a total of at least 1000 counted cells. All data points are plotted with error bars, which represent one standard deviation among the microscope fields of view for each sample.

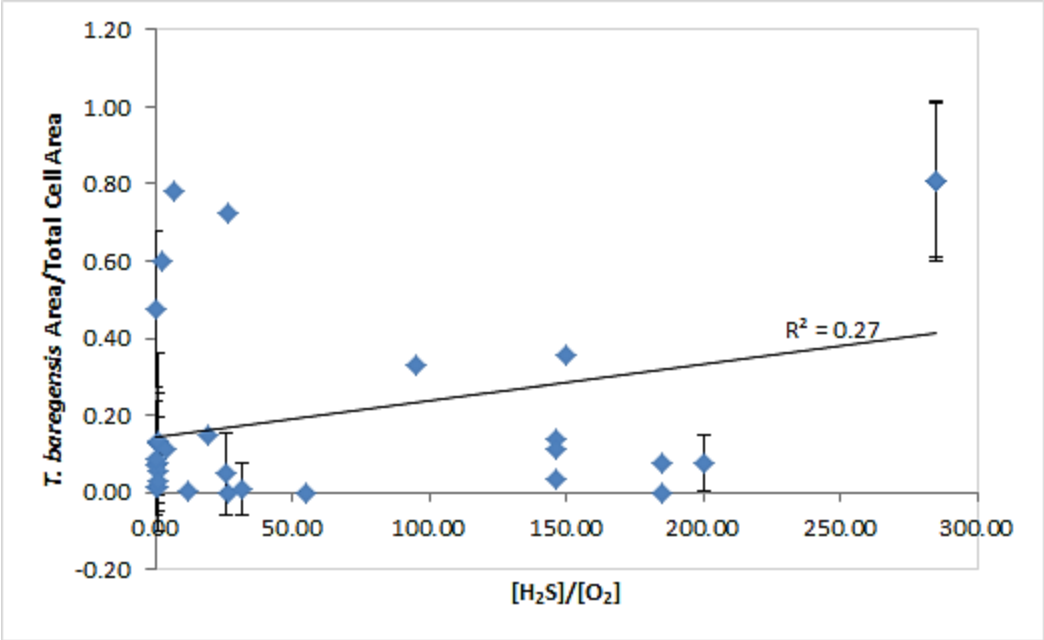

Figure 2.4 Area of *T. baregensis* to all bacteria versus H_2S to *in situ* dissolved O_2 ratios. Each data point represents a total of at least 1000 counted cells. All data points are plotted with error bars, which represent one standard deviation among the microscope fields of view for each sample.

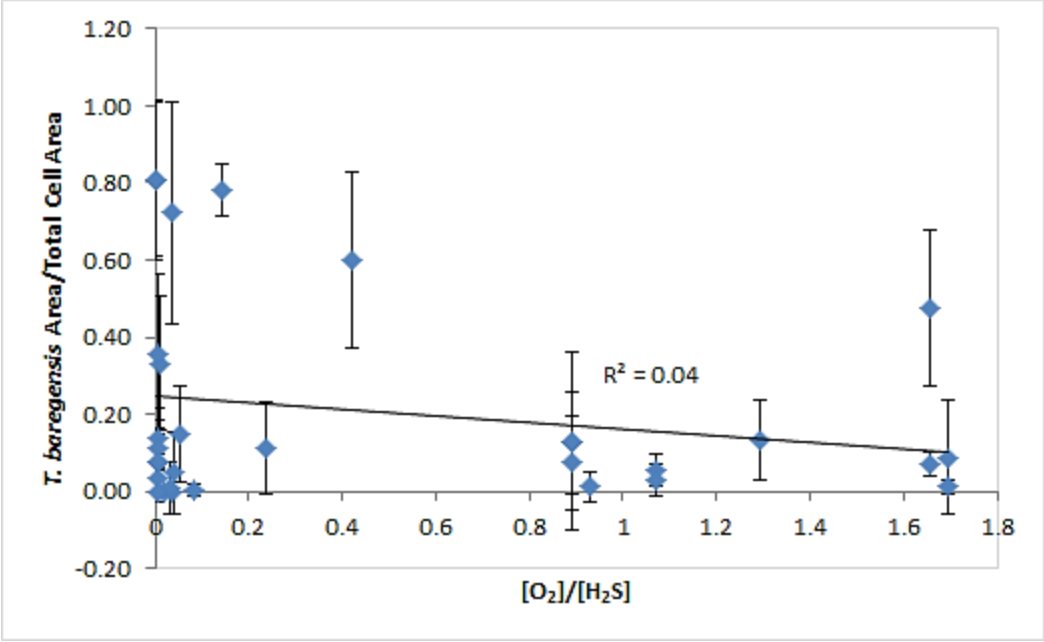

Figure 2.5 Area of *T. baregensis* to all bacteria versus *in situ* dissolved O_2 to H_2S ratios. Each data point represents a total of at least 1000 counted cells. All data points are plotted with error bars, which represent one standard deviation among the microscope fields of view for each sample.

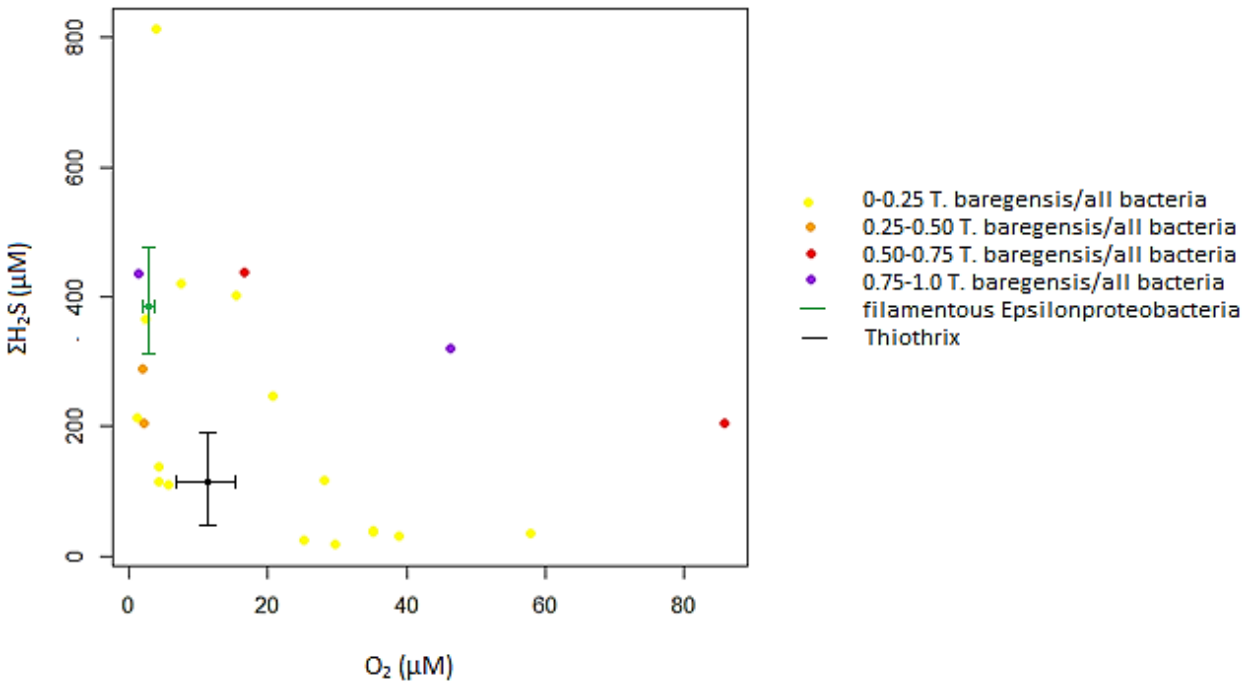

Figure 2.6 Plot of *T. baregensis* abundances with sulfide and oxygen concentrations (μM). Yellow dots indicate *T. baregensis* to all bacteria ratios between 0 and 0.25, orange dots indicate *T. baregensis* to all bacteria ratios between 0.25 and 0.5, red dots indicate *T. baregensis* to all bacteria ratios between 0.5 and 0.75, and purple dots indicate *T. baregensis* to all bacteria ratios between 0.75 and 1. Colored error bars from Macalady *et al.* show the mean and one standard deviation of major bacteria groups within Frasassi Cave biofilms (2008).

Table 2.4 Table summarizing amino acid tRNA synthetase COGs (clusters of orthologous groups of proteins) found in a nearly complete *T. baregensis* metagenomic bin (Hamilton *et al.*, in prep).

tRNA synthetase	COG ID	*T. baregensis* bin
Ala-tRNA synthetase	COG0013	0
Arg-tRNA synthetase	COG0018	5
Asp/Asn-tRNA synthetase	COG0017	1
Asp-tRNA synthetase	COG0173	1
Cys-tRNA synthetase	COG0215	1
Glu- and Gln-tRNA synthetases	COG0008	3
Gly-tRNA synthetase		
α-Subunit	COG0752	1
β-Subunit	COG0751	1
Class II	COG0423	1
His-tRNA synthetase	COG0124	1
Ile-tRNA synthetase	COG0060	0
Leu-tRNA synthetase	COG0495	4
Lys-tRNA synthetase		
Class I	COG1384	0
Class II	COG1190	0
Met-tRNA synthetase	COG0143	1
Phe-tRNA synthetase		
α-Subunit	COG0016	1
β-Subunit	COG0072	1
Pro-tRNA synthetase	COG0442	2
Ser-tRNA synthetase	COG0172	1
Thr-tRNA synthetase	COG0441	1
Trp-tRNA synthetase	COG0180	0
Tyr-tRNA synthetase	COG0162	1
Val-tRNA synthetase	COG0525	1

Table 2.5 Summary of C, S, O, and N cycling genes found in the T. baregensis bin through metagenomics (Hamilton *et al.*, in prep).

	T. baregensis
CO_2 fixation	
Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo)	Yes
Carbonic anhydrase	Yes
S-oxidation	
Sulfide:quinone reductase (SQR)	Yes
SOX system	Yes (soxAX, CD, YZ, B)
soxCD	Yes
Thiosulfate:quinol reductase (TQO)	No evidence
Tetrathionate hydrolase (TTH)	No evidence
Sulfur oxygenase reductase (SOR)	Yes
Flavocytochrome C (fccC)	Yes
Dissimilatory sulfite reductase (dsrAB)	No evidence
O-metabolism	
cbb3-type cytochrome c oxidase	Yes
bd-type cytochrome c	Yes
N-metabolism	
N_2-fixation (nifH, D, K)	No evidence
Ammonia assimilation	Yes
Ammonia permease	No evidence
Glutamine synthetase	Yes
Nitrate reduction	Yes (partial pathway)

Table 2.6 Summary of metagenomics results for antibiotic resistance in the *T. baregensis* bin with corresponding COG, Pfam, and KO database identification codes (Hamilton *et al.*, in prep). Clusters of Orthologous Groups of proteins (COGs) groups proteins based on phylogeny (Tatusov *et al.*, 2000), Pfam groups protein domain families (Sonnhammer *et al.*, 1997), and KEGG Orthology (KO) annotates genes based on experimental evidence of protein interactions (Kanehisa *et al.*, 2004).

Antibiotic	Description	COG	Pfam	KO
Bleomycin	Bleomycin resistance protein	COG0346	pfam00903	K01759
Erythromycin	Erythromycin resistance leader peptide	-	pfam08057	-
Methylenomycin A	Methylenomycin A resistance protein	-	pfam07690	K08166
Bacitracin	Bacitracin resistance protein BacA	COG1968	pfam02673	K06153

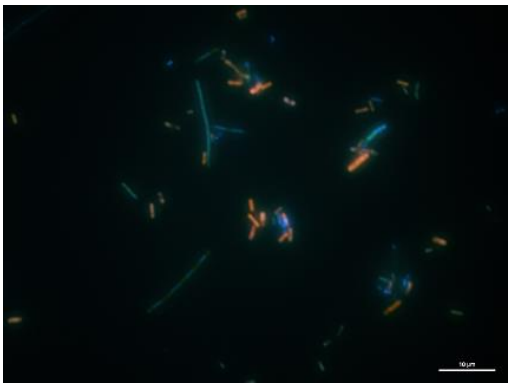

Figure 2.7 FISH images of the inoculum used in culturing. Blue DAPI stain shows all DNA, green EUBMIX probes shows all bacteria, and red Tbar581 probe shows *T. baregensis*. Scale bar is 10 μm.

A.

>Frasassi_Isolate
CACCGUGGCAAUGCUGAUCCACGAUUACUAGCGAUUCCGACUUCAUGCAG
UCGAGUUGCAGACUGCAAUCCGGACUAAGAUCGGCUUUAUGGGAUUGGCU
CCACCUCGCGGUUUGGCUACCCUCUGUACCGACCAUUGUAGCACGUGUGU
AGCCCUGGCCAUAAGGGCCAUGAUGACUUGACGUCAUCCCCACCUUCCUC
CGGUUUGUCACCGGCAGUCUCUUUAGAGUUCCCACCAUAACGUGCUGGCA
ACUAAAAGAUAAGGGUUGCGCUCGUUGCGGGACUUAACCCAACAUCUCACG
ACACGAGCUGACGACAGCCAUGCAGCACCUGUCUCAGAGUUCCCGAAGGC
ACCAAGUCAUCUCUGACGAGUUCUCUGGAUGUCAAGGCCAGGUAAGGUUU
UUCGCGUUGCAUCGAAUUAAACCACAUGCUCCACCGCUUGUGCGGGCCCC
CGUCAAUUCCUUUGAGUUUCAACCUUGCGGCCGUACUCCCCAGGCGGUCU
ACUUAACGCGUUAGCUCCGUCACCGAAAGNNUUAGCCCUCCCGACGACAA
GUAGACAUCGUUUAGGGCGUGGACUACCAGGGUAUCUAAUCCUGUUUGCU
CCCCACGCUUUCGUACCUCAGCGUCAAUGUUGGCCCAGGUGGCUGCCUUC
GCCAUUGGUGUUCUUUCUGAUAUCUACGCAUUUCACCGCUACACCAGAAA
UUCCGCCACCCUCUACCACAUUCUAGAUGUUCAGUAUCCAAUGCAAUUCC
CAGGUUGAGCCCGGGGAUUUCACAUCAGACUUAAACGUCCGCCUACGCAC
GCUUUACGCCCAGUAAUUCCGAUUAACGCUUGCACCCUCCGUAUUACCGC
GGCUGCUGGCACGGAGUUAGCCGGUGCUUUUUCUUUGAGUAACGUCAAAC
AGUCAGACUAUUAACCUAACUGCGUUCUUCCUCAACAAAAGUGCUUUACA
ACCCGCAGGCCUUCUUCACACACGCGGCAUUGCUGGAUCAGGCUUUCGCC
CAUUGUCCAAUAUUCCCCACUGCUGCCUCCCGUAGGAGUCUGGGCCGUGU
CUCAGUCCCAGUGUGGCCGUACACCCUCUCAGGCCGGCUACUGAUCGCAG
CCUUGGUGAGCCAUUACCUCACCAACUAGCUAAUCAGACAUGGGCUCAUC
CUUUGGCACGUGGUCCGAAGAUCCCACGCUUUGCUCCGAAGAGGUUAUGC
GGUAUUAGCGUUAGUUUCCCAACGUUAU

B.

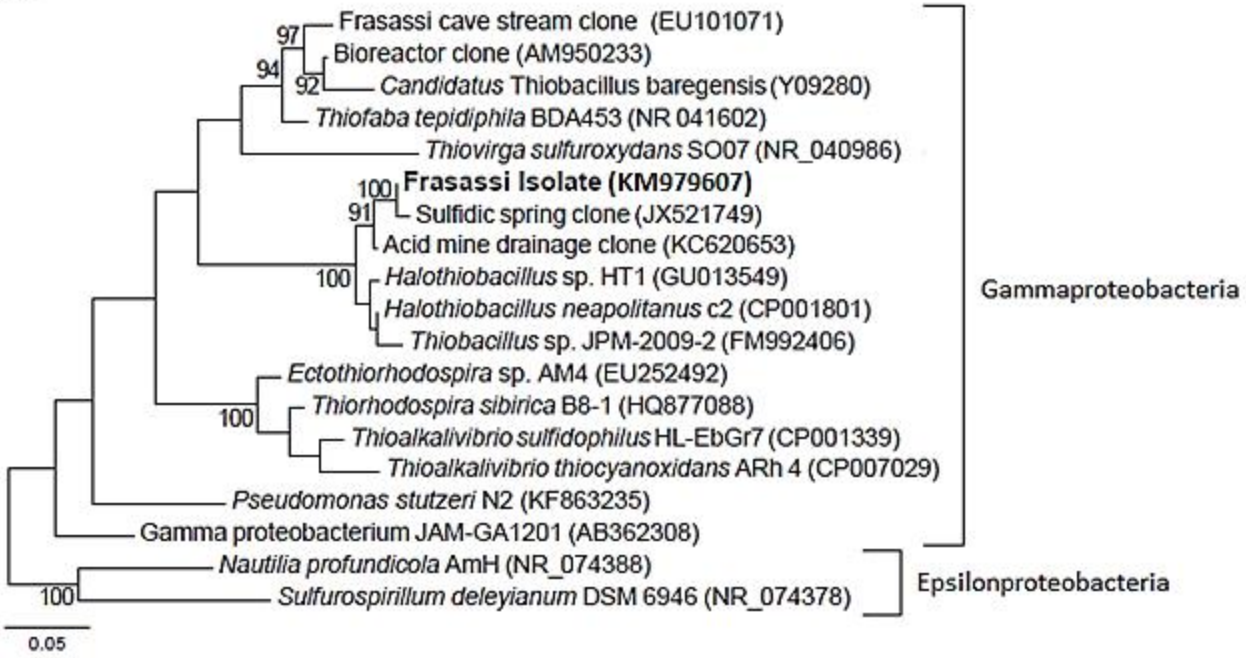

Figure 2.8 16S rRNA sequence information of a previously uncultured Gammaproteobacterium isolated from $S_2O_3^{2-}$ and S^0 plates with O_2. (A) The 16S rRNA sequence of the isolated bacterium. (B) A 16S rRNA tree showing the phylogenetic position of the isolated bacterium (in bold) among closely related organisms with sequences obtained from the NCBI database. Accession numbers are given in parentheses.

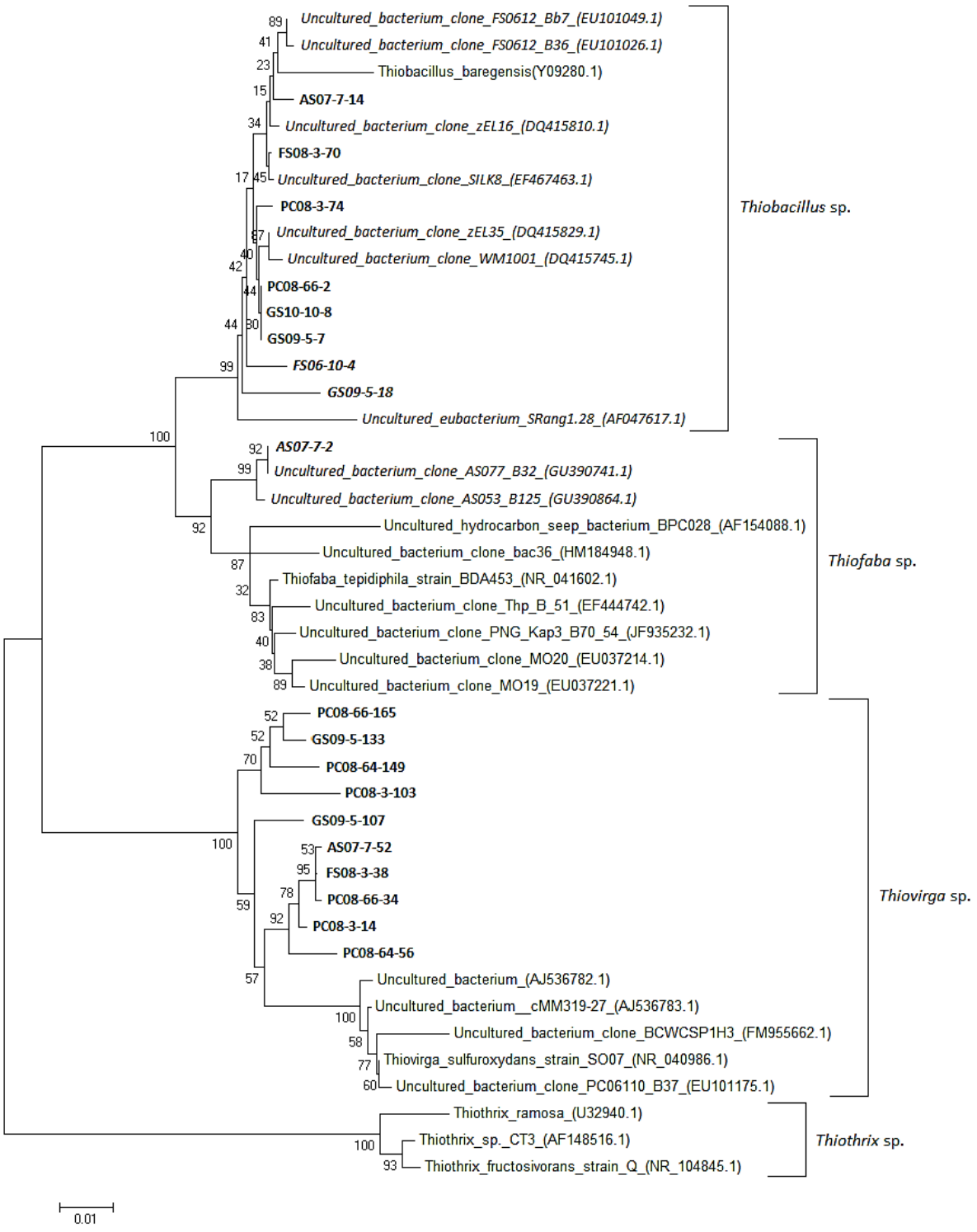

Figure 2.9 Maximum likelihood phylogeny of 16S rRNA sequences from Frasassi metagenomes, shown in bold, and the NCBI database. Accession numbers are given in parentheses. 16S rRNA sequences that hybridize to the Tbar581 probe are shown in italics.

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2.8 Supplemental calculations

Gibbs free energy calculations for six environmental conditions with the following set parameters:

Temperature	14°C (287.15 K)
pH	7.0
Concentrations	1 M

Equations:

$\Delta G° = \sum \Delta G^{\circ}_{fproducts} - \sum \Delta G^{\circ}_{freactants}$

$\Delta G = \Delta G° + 2.303 RT \log Q$

$Q = \frac{A^a B^b}{C^c D^d}$

1. Sulfide and oxygen

$HS^- + 2\ O_2 \rightarrow SO_4^{2-} + H^+$

$\Delta G° = \left(\left(-744.6 \frac{kJ}{mol}\right) + 0\ \frac{kJ}{mol}\right) - \left(12.05\ \frac{kJ}{mol} + 2\left(0\ \frac{kJ}{mol}\right)\right) = -756.65\ \frac{kJ}{mol}$

$\Delta G(\text{per reaction}) = -756.65\ \frac{kJ}{mol} + (2.303)\left(0.008314 \frac{kJ}{molx\ K}\right)(287.15\ K)(\log(10^{-7})) = -795.14 \frac{kJ}{mol}$

$\Delta G(\text{per mol S}) = -795.14 \frac{kJ}{mol}$

2. Elemental sulfur and oxygen

$2\ S^0 + 3\ O_2 + 2\ H_2O \rightarrow 2\ SO_4^{2-} + 4\ H^+$

$\Delta G° = \left(2\left(-744.6 \frac{kJ}{mol}\right) + 4\left(0\ \frac{kJ}{mol}\right)\right) - \left(2\left(0\ \frac{kJ}{mol}\right) + 3\left(0\ \frac{kJ}{mol}\right) + 2\left(-237.17 \frac{kJ}{mol}\right)\right) = -1014.86\ \frac{kJ}{mol}$

$\Delta G(\text{per reaction}) = -1014.86\ \frac{kJ}{mol} + (2.303)\left(0.008314 \frac{kJ}{molx\ K}\right)(287.15\ K)(\log(10^{-28})) = -1168.81 \frac{kJ}{mol}$

$\Delta G(\text{per mol S}) = -584.40 \frac{kJ}{mol}$

3. Thiosulfate and oxygen

$S_2O_3^{2-} + 2\ O_2 + H_2O \rightarrow 2\ SO_4^{2-} + 2\ H^+$

$\Delta G° = \left(2\left(-744.6 \frac{kJ}{mol}\right) + 2\left(0\ \frac{kJ}{mol}\right)\right) - \left(\left(-513.4\ \frac{kJ}{mol}\right) + 2\left(0\ \frac{kJ}{mol}\right) + \left(-237.17 \frac{kJ}{mol}\right)\right) = -738.63\ \frac{kJ}{mol}$

$\Delta G(\text{per reaction}) = -738.63\ \frac{kJ}{mol} + (2.303)\left(0.008314 \frac{kJ}{molx\ K}\right)(287.15\ K)(\log(10^{-14})) = -815.60 \frac{kJ}{mol}$

$\Delta G(\text{per mol S}) = -407.80 \frac{kJ}{mol}$

4. Sulfide and nitrate

$HS^- + NO_3^- + H_2O + H^+ \rightarrow SO_4^{2-} + NH_4^+$

$\Delta G° = \left(\left(-744.6 \frac{kJ}{mol}\right) + \left(-79.37\ \frac{kJ}{mol}\right)\right) - \left(\left(12.05\ \frac{kJ}{mol}\right) + \left(-111.34\ \frac{kJ}{mol}\right) + \left(-237.17\ \frac{kJ}{mol}\right) + \left(0\ \frac{kJ}{mol}\right)\right) = -487.51\ \frac{kJ}{mol}$

$\Delta G(\text{per reaction}) = -487.51\ \frac{kJ}{mol} + (2.303)\left(0.008314 \frac{kJ}{molx\ K}\right)(287.15\ K)(\log(10^{7})) = -449.02 \frac{kJ}{mol}$

$\Delta G(\text{per mol S}) = -449.02 \frac{kJ}{mol}$

5. Elemental sulfur and nitrate

$4\ S^0 + 3\ NO_3^- + 7\ H_2O \rightarrow 4\ SO_4^{2-} + 3\ NH_4^+ + 2H^+$

$\Delta G° = \left(4\left(-744.6\ \frac{kJ}{mol}\right) + 3\left(-79.37\ \frac{kJ}{mol}\right) + 2\left(0\ \frac{kJ}{mol}\right)\right) - \left(4\left(0\ \frac{kJ}{mol}\right) + 3\left(-111.34\ \frac{kJ}{mol}\right) + 7\left(-237.17\ \frac{kJ}{mol}\right)\right) = -1222.3\ \frac{kJ}{mol}$

$\Delta G(\text{per reaction}) = -1222.3\ \frac{kJ}{mol} + (2.303)\left(0.008314\frac{kJ}{molx\ K}\right)(287.15\ K)(\log(10^{-14})) = -1299.27\frac{kJ}{mol}$

$\Delta G(\text{per mol S}) = -324.82\frac{kJ}{mol}$

6. Thiosulfate and nitrate

$S_2O_3^{2-} + NO_3^- + 2\ H_2O \rightarrow 2\ SO_4^{2-} + NH_4^+$

$\Delta G° = \left(2\left(-744.6\ \frac{kJ}{mol}\right) + \left(-79.37\ \frac{kJ}{mol}\right)\right) - \left(\left(-513.4\ \frac{kJ}{mol}\right) + \left(-111.34\ \frac{kJ}{mol}\right) + 2\left(-237.17\ \frac{kJ}{mol}\right)\right) = -469.49\ \frac{kJ}{mol}$

$\Delta G(\text{per reaction}) = -469.49\ \frac{kJ}{mol} + (2.303)\left(0.008314\frac{kJ}{molx\ K}\right)(287.15\ K)(\log(1)) = -469.49\frac{kJ}{mol}$

$\Delta G(\text{per mol S}) = -234.75\frac{kJ}{mol}$

CHAPTER 3: ENRICHMENT AND CHARACTERIZATION OF A NOVEL STRAIN OF *SULFURICURVUM KUJIENSE*

3.1 Abstract

Epsilonproteobacteria are the least studied class of Proteobacteria. Within the class lies the *Sulfuricurvum*, a genus with one cultivated representative, *Sulfuricurvum kujiense*. This study describes the enrichment and growth characteristics of a previously undescribed strain of *S. kujiense*, the first Epsilonproteobacterium enriched from the Frasassi Caves. The *S. kujiense* in this study shares 99% sequence similarity with *S. kujiense* strain YK-1 and groups within *S. kujiense* clusters in both 16S rRNA and *napA* gene phylogenies. Growth experiments conducted over a period of 194 hours show that the new *S. kujiense* strain is metabolically similar to previously isolated *S. kujiense* strains. All strains are capable of growth on sulfide, elemental sulfur, thiosulfate, and hydrogen with nitrate or oxygen (1% headspace). The ability to grow on sulfide, elemental sulfur, and thiosulfate makes *S. kujiense* metabolically similar to the dominant *Sulfuricurvum* members within the cave, which have been shown in parallel metagenomic studies to have the molecular machinery to oxidize reduced sulfur compounds. However, the new *S. kujiense* strain is distinct from *Candidatus* Sulfuricurvum sp. RIFRC-1 in that it is capable of oxidizing thiosulfate. Contaminants within the enriched culture were present under all culture conditions but showed the least growth under hydrogen. Future work should focus on isolating the novel strain by plating or serial diluting enrichment cultures under $H_2/N_2/CO_2$ and nitrate, using whole genome sequencing to browse for functional genes, and determining whether or not the strain produces sulfuric acid for limestone dissolution and cave formation.

3.2 Introduction

The Italian Frasassi Caves are a sulfidic subterranean ecosystem teeming with microbial life. While the aphotic ecosystem does not contain organic carbon from surface environments, the presence of both reduced and oxidized compounds allow for an ecosystem based on microbial autotrophy through chemically derived energy (Jones *et al.*, 2008; Porter *et al.*, 2009). Many bacterial populations in sulfidic Frasassi Cave streams generate energy through the oxidization of reduced sulfur compounds (Macalady *et al.*, 2006; Macalady *et al.*, 2008). In the process of energy formation, bacteria capable of complete sulfur oxidation produce sulfuric acid, which corrodes calcium carbonate cave walls and leads to cave enlargement (Hubbard *et al.*, 1990; Hose *et al.*, 2000; Engel *et al.*, 2001; Engel *et al.*, 2004a; Macalady *et al.*, 2006). This

differs from bacteria that incompletely oxidize sulfide, a process which may produce the economically important chemical species elemental sulfur. Since biotic processes affect geochemical cycling, it is of interest to determine how microbial redox transformations alter both biotic and abiotic factors, such as substrate availability for neighboring organisms and the rate of cave development.

Among the bacteria within the Frasassi Caves are Epsilonproteobacteria, the least characterized class of Proteobacteria (Engel *et al.*, 2004b; Campbell *et al.*, 2006). Epsilonproteobacteria are common in Frasassi biofilms, with most found within the Sulfurovumales clade. Other Epsilonproteobacteria representatives in the biofilms group with *Arcobacter* as well as the Sulfuricurvales and 1068 groups (Macalady *et al.*, 2008). Clones within the Sulfuricurvales group lie within *Sulfuricurvum* (Macalady *et al.*, 2008), a genus with only one isolated species, *S. kujiense* (Kodama and Watanabe, 2003; Kodama and Watanabe, 2004). There are currently four isolated strains of *S. kujiense*, designated strain YK-1, YK-2, YK-3, and YK-4 (Kodama and Watanabe, 2003; Kodama and Watanabe, 2004). Of the four isolated strains, the metabolic capabilities of strain YK-1 have been described both experimentally and through complete genome annotation. These studies have determined that strain YK-1 is a mesophilic lithoautotroph capable of oxidizing sulfide, elemental sulfur, thiosulfate, and hydrogen under microaerobic or anaerobic conditions with nitrate as the electron acceptor (Kodama and Watanabe, 2003; Kodama and Watanabe, 2004; Han *et al.*, 2012).

This study describes the enrichment of a novel strain of *S. kujiense*, the first Epsilonproteobacterium successfully enriched from the Frasassi Caves, and focuses on determining the conditions that optimize growth in order to understand potential contributions to biogeochemical cycling. If the novel *S. kujiense* strain is capable of complete oxidation of

reduced sulfur species, it is likely to contribute to cave formation (Hubbard *et al.*, 1990; Hose *et al.*, 2000; Engel *et al.*, 2001; Engel *et al.*, 2004a; Macalady *et al.*, 2006). However, if it only partially oxidizes reduced sulfur compounds, it may form elemental sulfur. Better understanding the metabolic properties associated with this cultivated strain will potentially allow for a deeper understanding of metabolic diversity within the ecologically successful but poorly characterized *Sulfuricurvum* genus.

3.3 Methods

3.3.1 Enrichment culturing

Inoculum was retrieved from Stefan Spring as described in Chapter 2 and transferred to modified SOB medium. The modified SOB medium contained 4.5 mM K_2HPO_4, 2.2 mM KH_2PO_4, 7.4 mM NH_4Cl, and 1.6 mM $MgSO_4.7H_2O$ with a headspace of N_2/CO_2 (80:20, by volume). Medium was supplemented with 2 mL/L trace metal solution adapted from Vishniac and Santer (1957) containing 134 mM EDTA, 7.7 mM $ZnSO_4.7H_2O$, 49.7 mM $CaCl_2.2H_2O$, 12.63 mM $MnCl_2.4H_2O$, 18.0 mM $FeSO_4.7H_2O$, 2.8 mM $(NH_4)_6MoO_{24}.4H_2O$, 0.8 mM $CuSO_4.5H_2O$, and 2.1 mM $CoCl_2.6H_2O$. The medium contained 6.5 mM $S_2O_3^{2-}$ to serve as the electron donor and 0.74 mM NO_3^- to serve as the electron acceptor. Cultures were incubated in the dark without shaking. Hydrogen was provided as an alternative electron donor by culturing under $N_2/CO_2/H_2$ headspace (40:20:40, by volume).

Both liquid and solid media variations were made on the modified SOB medium. An alternative liquid medium contained both H_2 and $S_2O_3^{2-}$ as electron donors, NO_3^- as the electron acceptor, 2 g/L bicarbonate to enhance CO_2 availability, and a N_2/H_2 headspace (50:50, by volume). An alternative solid medium contained H_2 as the electron donor, NO_3^- as the electron acceptor, and 2 g/L bicarbonate. Plates were incubated under N_2/H_2 (50:50, by volume).

3.3.2 FISH and microscopy

Cellular growth was monitored using fluorescent *in-situ* hybridization (FISH) and quantitative cell counting. Probe EP656 (CATCTGCCCCTTCTGAAC) was designed using methods described in Hugenholtz *et al.* (2001). EP656 was designed to hit representatives within the *Sulfuricurvum* genus and targets nucleotides 656-673 on the 16S rRNA gene. The stringency was optimized using a pure culture of *Sulfurimonas denitrificans* (Hoor, 1975; Takai *et al.*, 2006), which has 5 bp mismatches to the probe sequence.

For acridine orange staining, 100 µL of undiluted sample was placed on a 0.2 µm Isopore^{TM} polycarbonate membrane filters (Millipore) for vacuum filtration. After washing the filters with 1 mL sterile water, 0.5 mL of dye (0.01% (w/v) acridine orange solution in 20 mM TRIS buffer, pH 7.2) was added to the filter and incubated for 1 minute. The dye was then filtered, and the filter was placed on a microscope slide. Slides were immediately viewed with a Nikon E800 epifluorescence microscope. In order to obtain cell counts, 10 fields of view were randomly selected and counted using a counting grid (Olympus). The cell count values for 10 fields of view were determined for each culture and used to calculate cell density in the cultures (cells/mL).

After determining the cell density, FISH was used to find the ratio of *Sulfuricurvum* sp. to total cells. FISH was performed on 4% paraformaldehyde (PFA) fixed subsamples using methodology described in Amann (1995) with probe EP656. Prior to microscopy, cells were counterstained using 4',6'-diamidino-2-phenylindole (DAPI) and mounted using Vectashield (Vectashield Laboratories Inc., Burlingame, CA, USA). Completed slides were viewed with the Nikon E800 epifluorescence microscope, and the cell count tool from NIS Elements AR 2.30, Hotfix (Build 312) image analysis software was used to quantify cells. After 10 images were

captured per sample, the total number of Epsilonproteobacteria cells that fluoresced under EP656 and the total number of cells (or inactive, low ribosome-copy cells) that fluoresced under DAPI were recorded.

3.3.3 Phylogenetic analysis with 16S rRNA and *napA* genes

Colony PCR was used to test the degree of enrichment in the culture and to determine the 16S rRNA and *napA* gene sequences of the Epsilonproteobacterium. To obtain template DNA, bacteria were concentrated by centrifuging 1 mL of culture at room temperature for 4 minutes at 16.1 rcf. Colony PCR was performed on the concentrated culture with a 16S rRNA bacteria-specific amplification primer set. The PCR reaction contained 1-150 ng DNA template, 1 x buffer (TaKaRa Bio Inc., Siga, Japan), 0.2 mM each dNTP, 1.5 units high-affinity ExTaq DNA polymerase, 0.2 μM forward primer 27F (AGAGTTTGATCCTGGCTCAG), and 0.2 μM reverse primer 1492r (GGTTACCTTGTTACGACTT). After an initial denaturation period of 15 minutes at 99°C followed by 5 minutes at 80°C, thermal cycling consisted of 35 cycles of denaturation at 95°C for 30 seconds, stepwise annealing from 60°C to 50°C in 2°C intervals for 60 seconds, elongation at 72°C for 90 seconds, and a final elongation at 72°C for 20 minutes. PCR products were tested for successful amplification by loading 5 μL PCR products and 5 μL DNA marker (Bionexus Hi-Lo^{TM} DNA marker) with 1 μL loading dye into 1% agarose gels. Gel electrophoresis ran for 45 minutes at 95 V. PCR products with 16S rRNA amplification were subsequently sequenced at the Penn State Nucleic Acid Facility with 1 μM 27F primer and 1 μM 1492R primer. Sequences were manually checked for quality and assembled using CodonCode Aligner v.2.0.4 (CodonCode Corporation, USA). BLASTN was used to retrieve highly similar sequences for comparison (Altschul *et al.*, 1990). A phylogenetic tree was constructed from the 16S rRNA sequences through approximate likelihood-ratio tests in PhyML 3.0 (Guindon and

Gascuel, 2003; Anisimova and Gascuel, 2006). The dataset included closely related sequences identified through BLASTN and ARB (Ludwig *et al.*, 2004), as well as 16S rRNA sequences previously retrieved from the Frasassi and Acquasanta Terme cave systems (Hamilton *et al.*, 2014). All sequences were aligned with MEGA 6.0, and the best evolutionary model was determined with jModeltest 2.1.1 (Darriba *et al.*, 2012; Tamura *et al.*, 2013). For maximum likelihood trees, the General Time Reversible substitution model and gamma-distributed rate variation with a proportion of invariable sites were used. Phylograms were rate-smoothed using the multidimensional version of Rambaut's parameterization in PAUP 4.0 (Swofford, 2001).

NapA is a gene coding for nitrate reductase, an enzyme responsible for nitrate reduction in the presence of oxygen (Lloyd, 1993; Berks *et al.*, 1995). Methods for *napA* amplification are described in Flanagan *et al.* (1999). After 1 mL of the enrichment culture was centrifuged at room temperature for 4 minutes at 16.1 rcf, the pellet was boiled for 15 minutes at 99°C prior to being heated for 5 minutes at 80°C. The first PCR round consisted of 2.5 μL of 20 pmol μL^{-1} V16 (GCNCCNTGYMGNTTYTGYGG) and V17 (RTGYTGRTTRAANCCCATNGTCCA) primer, 0.75 μL ExTaq DNA polymerase, 10 μL 10X buffer, 3mM $MgCl_2$, and 2 μL of 10 mM dNTP mix added to PCR tubes for 100 μL reactions. Thermal cycling consisted of an initial denaturation period for 2 minutes at 94°C, 30 cycles of denaturation at 94°C for 1 minute, attachment at 50°C for 1 minute, and elongation at 72°C for two minutes, and a final elongation at 72°C for 10 minutes. The second round of PCR contained the same reactants as the first round, except for 2.5 μL of 20 pmol μL^{-1} V66 (TAYTTYYTNHSNAARATHATGTAYGG) and V67 (DATNGGRTGCATYTCNGCCATRTT), a lower $MgCl_2$ concentration of 1.5 mM, and 2 μL template of PCR product from round one. Thermal cycling conditions were the same as round one. PCR products were separated using gel electrophoresis as described above and purified with

a QIAEX II Gel Extraction Kit (QIAGEN). After the purified DNA was re-amplified with the reagents and methods used in the second round of PCR, the PCR product was sent for sequencing with 1 μM V66 and 1 μM V67. Sequences were manually checked for quality control using CodonCode Aligner v.2.0.4 (CodonCode Corporation, USA). In order to construct a *napA* phylogenetic tree, the enrichment sequence was translated and used as a query to mine closely related *napA* sequences from the NCBI database using BLASTP. Amino acid sequences were aligned with ClustalX (version 2.1) using the Gonnet 250 protein substitution matrix and default gap extension along with opening penalties (Larkin *et al.*, 2007). Alignments were manually curated, and the best evolutionary model was determined using ProtTest (version 3, Darriba *et al.*, 2011). Phylogeny was evaluated using PhyML with one thousand bootstrap replicates using the best evolution model as identified by ProtTest (Guindon and Gascuel, 2003).

3.3.4 Growth experiments

A highly enriched culture was subcultured with various electron donors and acceptors to test growth conditions and metabolic characteristics. Electron donors included H_2S (2 mM), S^0 (0.44 g/L), $S_2O_3^{2-}$ (2 mM), and H_2 (40% headspace). Electron acceptors included O_2 (1% headspace using autoclaved air) and NO_3^- (0.74 mM). Culture vials with H_2S, S^0, and $S_2O_3^{2-}$ paired with O_2 and NO_3^- contained N_2/CO_2 headspace (80:20, by volume), while culture vials with H_2 paired with O_2 and NO_3^- contained $N_2/CO_2/H_2$ headspace (40:20:40, by volume). For each growth condition, pH was adjusted between 6.9 and 7.2 with NaOH (1 M). Finally, 0.5 mL inoculum was injected into 50 mL media. Triplicate cultures were made for each growth condition.

Sampling occurred immediately after inoculation and approximately once every 24 hours thereafter for a total of 194 hours. To sample cultures, 0.50 mL was removed from each culture,

vortexed, fixed in 4% (w/v) PFA, and stored at -20°C for acridine orange staining and fluorescent *in situ* hybridization (FISH) analysis. The average ratio of *S. kujiense* to all bacteria was calculated for each culture. The amount of *Sulfuricurvum* cells per volume was found by multiplying the value of *Sulfuricurvum* to all cells, obtained through FISH counts, with the number of cells per volume, obtained through acridine orange staining. Lastly, the mean value and one standard deviation of *Sulfuricurvum* to all cells were calculated for each environmental condition.

The number of contaminant cells was also monitored for each environmental condition. To calculate the number of contaminant cells within each culture, the ratio of *S. kujiense* to all bacteria obtained through FISH was subtracted from one and multiplied by the total number of cells obtained through AO counts for each culture. The three values from triplicate cultures were then averaged, and one standard deviation was calculated from the average.

In order to test whether rotary shaking during incubation would encourage filament formation, two cultures with H_2 and NO_3^- were set up for a parallel experiment. One culture was incubated in the dark without shaking, and the other culture was incubated in the dark with shaking at 90 rpm. Both cultures were checked daily for filamentous growth over a period of 14 days using light microscopy. The ratio of filaments to rods was determined by scanning 10 fields of view and estimating percentages of cells with each morphology.

3.4 Results

3.4.1 EP656 probe description

Probe EP656 has a stringency of 45% and hybridizes to a total of 221/245 (90%) of *Sulfuricurvum* sequences currently available in public databases. EP656 has no mismatches to the *S. kujiense* strain enriched in this study as well as *Candidatus* Sulfuricurvum sp. RIFRC-1.

While EP656 has no mismatches to the majority (86.36%) of the sequences described from Frasassi metagenomes (Hamilton *et al.*, 2014), the probe has 3 bp mismatches to PC08_66_Sulfuricurvum_Genome_bin (accession number KM410848), 7 bp mismatches to FS06_10_10 (accession number KM410359), and 3 bp mismatches to FS08_3_87 (accession number KM410413), as shown in Figure 3.1.

3.4.2 Enrichment culturing

A highly enriched culture inoculated with biofilm from Stefan Spring (geochemistry described in Chapter 2) grown under hydrogen and nitrate was tested using 16S rRNA colony PCR. The 16S rRNA sequence was deposited to the NCBI database (accession number KM979608). The top BLAST hit was to an uncultured Epsilonproteobacterium (99% sequence identity, accession AB478659.1), and the second best hit was to *Sulfuricurvum kujiense* strain DSM 16994 (99% sequence identity, accession NR_074398.1). A list of top BLAST hits is shown in Table 3.1. The acquisition of a single Epsilonproteobacterial 16S rRNA sequence is consistent with a highly enriched culture. I will subsequently refer to the highly enriched strain as *Sulfuricurvum* sp. strain Frasassi.

Less successful enrichments resulted after altering the composition of the liquid medium described above or plating on media solidified with agarose. Although the original inoculum was obtained from a culture that used $S_2O_3^{2-}$ as the sole electron donor, serial dilution experiments showed that cultures provided with both H_2 and $S_2O_3^{2-}$ had a greater amount of contaminants than cultures provided with H_2 alone. Similar results occurred when HCO_3^- was added to the medium. Furthermore, plates with added HCO_3^- incubated under N_2/H_2 (50:50, by volume) resulted in pure cultures of a contaminant. BLAST results indicated that the contaminant had

99% sequence identity with the uncultured Gammaproteobaterium *Shewanella* sp. GC-4-d (accession number FJ159436).

3.4.3 Phylogenetic analysis

The 16S rRNA tree shown in Figure 3.1 gives the phylogenetic position of strain Frasassi in relation to sequences retrieved from Frasassi metagenomes (Hamilton *et al.*, 2014), and the 16S rRNA tree shown in Figure 3.2 gives the phylogenetic position of strain Frasassi in relation to a greater diversity of Epsilonproteobacterial genera. According to Figure 3.1, strain Frasassi is evolutionarily distant to most 16S rRNA sequences obtained from Frasassi metagenomes (Hamilton *et al.*, 2014). Comparison of the 16S rRNA genes shows 99% sequence similarity between strain Frasassi and *Sulfuricurvum kujiense* strain YK-1 (accession number NR_074398), and 98% sequence similarity between strain Frasassi and *Candidatus* Sulfuricurvum sp. RIFRC-1 (accession number CP003920). As shown in Figure 3.2, strain Frasassi lies in the same cluster as *Sulfuricurvum kujiense* strain YK-1 (63% bootstrap support), but also groups with *Candidatus* Sulfuricurvum sp. RIFRC-1 (99% bootstrap support).

A phylogenetic tree constructed using *napA* gene sequences from Frasassi and Acquasanta metagenomes and the NCBI database is shown in Figure 3.3. *NapA* sequence comparison shows 87% sequence similarity between strain Frasassi (accession number KP004245) and *S. kujiense* YK-1 (accession number YP_004059557), and 62% sequence similarity between strain Frasassi and *Candidatus* Sulfuricurvum sp. RIFRC-1 (accession number YP_007528244). According to Figure 3.3, the *napA* gene of strain Frasassi is most evolutionarily close to *S. kujiense* YK-1 (99% bootstrap value), but is also close to *Sulfuricurvum* from the Frasassi Caves (accession numbers 1000435711, 10016071, 100010353, 99% bootstrap

value). *Candidatus* Sulfuricurvum sp. RIFRC-1 had uncertain placement in the *napA* gene tree (50% bootstrap value).

3.4.4 Growth experiments

Sulfuricurvum exhibited growth when sulfide, elemental sulfur, thiosulfate, and hydrogen were used as electron donors, regardless of whether small amounts of oxygen or nitrate were used as electron acceptors (Table 3.2 and Figures 3.3 and 3.4). During exponential growth phase, strain Frasassi shows the greatest increase in cell numbers under hydrogen paired with oxygen or nitrate. A similar increase in cell numbers were observed when strain Frasassi was grown under sulfide, elemental sulfur, and thiosulfate paired with oxygen or nitrate.

Contaminant growth calculated by difference is shown in Table 3.3 and Figures 3.5 and 3.6. While contaminants grew under all growth conditions, less contaminant growth relative to strain Frasassi growth was observed in cultures grown on hydrogen as an electron donor and either oxygen or nitrate as an electron acceptor. Contaminants in the cultures were rods ranging between 2 to 3 μm long.

3.4.5 Morphology

While single *Sulfuricurvum* sp. strain Frasassi cells tend to range between 2 and 3 μm in length, they display various morphologies based on the electron donor and acceptor provided in the medium. Morphologies include cocci, rods, and filaments, as shown in Figure 3.7. Cultures grown on sulfide tend to form clusters consisting of both single rods and filaments and contain brightly lit circular particles. Cultures grown on hydrogen as an electron donor also consist of single rods and filaments, but less clumping is observed until cells reach high density. The colonies formed in hydrogen cultures do not contain bright particles. Cultures grown on oxygen typically have more rods, while cultures grown on nitrate tend to have a greater number of

filaments. Although I hypothesized that shaking would increase the amount of filamentous growth, nitrate cultures incubated on a shaker did not have a significantly greater number of filaments than cultures incubated without shaking.

3.5 Discussion

3.5.1 Assessing probe EP656

Prior to the development of probe EP656, probe EP404 was routinely used to assess the abundance of Epsilonproteobacteria within environmental samples and enrichments (Macalady *et al*., 2006). However, EP404 is a broad probe that targets most genera within Epsilonproteobacteria, making it unsuitable as a tool to evaluate the abundance of *Sulfuricurvum* in enrichments that may contain multiple Epsilonproteobacterial genera. Since the EP656 probe is specific to and has no mismatches to most 16S rRNA sequences within *Sulfuricurvum*, as shown in Figure 3.1, it is a highly useful probe for determining the abundance of *Sulfuricurvum* while excluding other Epsilonproteobacteria.

The EP656 probe appears to have mismatches to three 16S rRNA *Sulfuricurvum* sequences obtained from the Frasassi metagenomes (Figure 3.1). However, these sequences lie in a separate cluster from the majority of the *Sulfuricurvum*, suggesting that the three deviant sequences are representatives of a novel genus.

3.5.2 Phylogeny

Two genes, 16S rRNA and *napA*, were used to decipher the evolutionary relationships between strain Frasassi and its close relatives. Since 16S rRNA is found in all prokaryotes and is slow to undergo evolutionary change, it is a marker gene for understanding evolutionary relationships (Woese and Fox, 1977; Coenye and Vandamme, 2003). According to Figure 3.2, strain Frasassi is closely related to *S. kujiense* strain YK-1 and *Candidatus* Sulfuricurvum sp.

RIFRC-1. The high bootstrap value shows the reliability of the clustering. Closer analysis of the cluster shows that strain Frasassi is more closely related to *S. kujiense* strain YK-1, but the low bootstrap value shows low confidence in the grouping. Nonetheless, the higher sequence similarity between strain Frasassi and *S. kujiense* strain YK-1 along with the high bootstrap value for the cluster of *Candidatus* Sulfuricurvum sp. RIFRC-1 with a Frasassi Cave clone in Figure 3.1 shows that strain Frasassi groups with *S. kujiense*.

Furthermore, the distance between the 16S rRNA sequences between strain Frasassi and sequences from metagenomic analysis (Figure 3.1) suggests that strain Frasassi is not an abundant species within the cave. However, strain Frasassi is a novel strain of *S. kujiense*, originally isolated from an underground oil storage cavity (Kodama and Watanabe, 2004), and is also evolutionarily close to the ecologically successful *Candidatus* Sulfuricurvum sp. RIFRC-1 described from subsurface aquifer sediment (Handley *et al.*, 2014). Since strain Frasassi is a close ancestor of bacteria found in terrestrial subsurface environments, its ability to grow on multiple reduced sulfur sources can have an impact on subsurface biogeochemical cycling.

NapA is a gene found in a diverse group of bacteria responsible for nitrate reduction in microoxic environments using a periplasmic nitrate reductase enzyme complex (Flanagan *et al.*, 1999; Smith *et al.*, 2007). The *napA* phylogeny provides an additional perspective on the phylogenetic position of strain Frasassi (Figure 3.3). The high bootstrap values supporting the cluster for strain Frasassi with *S. kujiense* YK-1 provides additional evidence to support the conclusion that strain Frasassi is most closely related to *S. kujiense*. A difference in the *napA* tree (Figure 3.3) compared to the 16S tree (Figure 3.1) is the closer evolutionary distance between strain Frasassi and *Sulfuricurvum* from the Frasassi Caves. Finally, the low confidence assigned

to the placement of *Candidatus* Sulfuricurvum sp. RIFRC-1 shows the difference in the *napA* gene between sp. RIFRC-1 and closely related bacteria.

Although both the 16S rRNA and *napA* gene trees show that strain Frasassi is a novel strain of *S. kujiense,* there are potential disagreements in the evolutionary relationship between strain Frasassi and other related *Sulfuricurvum*. The position of strain Frasassi in the 16S rRNA tree shows that it is evolutionarily distant from the more abundant *Sulfuricurvum* within the Frasassi Caves, while the *napA* tree suggests that the bacteria are closely related. There is also a lack of consensus on the evolutionary relationship between strain Frasassi and *Candidatus* Sulfuricurvum sp. RIFRC-1 between the two trees due to the uncertain position of RIFRC-1 in the *napA* tree. Determining the *napA* sequences of more *Sulfuricurvum* spp. can help resolve the issue by allowing for more sequence comparisons.

3.5.3 *S. kujiense* strain Frasassi growth characteristics

S. kujiense strain Frasassi is a facultative anaerobe capable of lithoautotrophy. Although the strain's heterotrophic properties were not tested, its ability to replicate quickly when grown in the absence of organic carbon sources shows that it is capable of carbon fixation. Furthermore, its ability to use hydrogen and reduced sulfur as electron donors indicates that strain Frasassi is a lithotroph. Strain Frasassi is a facultative anaerobe due to its ability to use oxygen (1%) and nitrate as terminal electron acceptors.

Strain Frasassi has metabolic flexibility due to its ability to use multiple electron donors. While cultures with sulfide, elemental sulfur, and thiosulfate led to slow growth of strain Frasassi, clear growth was still exhibited, as shown in Figures 3.3 and 3.4. The ability to oxidize multiple reduced sulfur sources suggests that strain Frasassi may produce sulfuric acid. Since the Frasassi Caves develop as sulfuric acid corrodes the calcium carbonate cave walls, strain Frasassi

may play a role in cave formation. Additional testing is required to determine whether or not strain Frasassi oxidizes sulfide to sulfuric acid. Furthermore, although strain Frasassi is capable of growth on multiple sulfur sources, the greatest amount of biomass is generated when hydrogen is supplied as the electron donor, as shown in Figures 3.3 and 3.4. This establishes strain Frasassi as a bacterium primarily involved in hydrogen oxidation, a metabolism commonly found in other cultured Epsilonproteobacteria (Campbell *et al.*, 2006).

Strain Frasassi is also notable in its ability to use both oxygen and nitrate as electron acceptors. The fact that strain Frasassi is capable of using nitrate as an oxidizing agent and that the *napA* gene was successfully amplified from the enrichment culture both serve as evidence for the presence of nitrate in Frasassi Cave waters. This is an interesting finding considering how nitrate levels are perenially below detection limits (Macalady *et al.*, 2006; Macalady *et al.*, 2008).

Sulfuricurvum remains an enigmatic genus with few characterized representatives. Strain Frasassi enriched in this study has similarities and differences to other representatives within the genus. *Sulfuricurvum kujiense* YK-1 is currently the only isolated member within the *Sulfuricurvum* genus. Strain Frasassi described in this study is metabolically similar to YK-1 in that both organisms are capable of oxidizing sulfide, elemental sulfur, thiosulfate, and hydrogen with nitrate or small amounts of oxygen (Kodama and Watanabe, 2003; Kodama and Watanabe, 2004). These metabolic properties also make strain Frasassi similar to uncultured *Sulfuricurvum* from the Frasassi Caves based on metagenomic data. The *Sulfuricurvum* population abundant in the PC metagenome has the genetic machinery required to completely oxidize reduced sulfur compounds (Hamilton *et al.*, 2014). However, strain Frasassi differs from the recently described *Candidatus* Sulfuricurvum sp. RIFRC-1 based on metagenomic data. *Candidatus* Sulfuricurvum

sp. RIFRC-1 is capable of oxidizing sulfide, elemental sulfur, sulfite, and hydrogen with nitrate, nitric oxide, and small amounts of oxygen (Handley *et al.*, 2014). While the ability of *S. kujiense* to use sulfite and nitric oxide has not been tested, *S. kujiense* strains YK-1 and Frasassi are capable of oxidizing thiosulfate, while *Candidatus* Sulfuricurvum sp. RIFRC-1 appears to lack the molecular machinery to do so. Such differences highlight the metabolic diversity that exists within the genus (Kodama and Watanabe, 2004; Han *et al.*, 2012).

3.5.4 Contaminants

The low yet persistent contaminant populations found in all tested growth conditions (Figures 3.5 and 3.6) show the difficulty in separating contaminants from strain Frasassi. Although *Shewanella* sp. GC-4-d was identified as a contaminant, others may exist. Members of *Shewanella* are characterized by their ability to reduce sulfur compounds, especially thiosulfate, to sulfide under aerobic conditions (Venkateswaran *et al.*, 1999). Thus, future plans for obtaining a pure culture should include serial dilutions using sulfide or hydrogen as electron donors, and nitrate as the electron acceptor. It is not known whether or not there are more contaminants in the culture.

3.5.5 Conclusions, implications, and future work

The lithoautotrophic Epsilonproteobacterium described in this study is a new strain of *Sulfuricurvum kujiense* with similar metabolic properties to previously described strains. Although strain Frasassi is capable of sulfur oxidization, it is primarily involved in hydrogen oxidation, which leads to the greatest increase in biomass. Electron acceptors include oxygen (1%) and nitrate. Since strain Frasassi is capable of reducing nitrate, and the *napA* gene was successfully amplified from a highly enriched culture, it is likely that nitrate exists in Frasassi cave waters, even though it is perennially below detection limits.

Isolating *Sulfuricurvum* spp. and understanding the metabolic properties of more representatives within the genus have implications on geoscience, microbiology, and society. *Sulfuricurvum* sp. strain Frasassi and its close relatives have been described from various subsurface terrestrial freshwater environments (Kodama and Watanabe, 2003; Kodama and Watanabe, 2004; Handley *et al.*, 2014), suggesting that their shared metabolic properties can contribute to biogeochemical cycling in more terrestrial subsurface environments. Aside from providing organic carbon to the subsurface, their ability to oxidize sulfur compounds can potentially lead to sulfuric acid production for cave formation and provide both lithotrophs and organotrophs with energy sources to allow complex microbial ecosystems to exist. Isolating more representatives within *Sulfuricurvum* also has implications on microbiology because the genus contains only one isolate, and Epsilonproteobacteria remains the least studied class of Proteobacteria. Increasing our knowledge of microbial diversity will allow for a more thorough understanding of the role between microbes and their environments. Lastly, since representatives of *Sulfuricurvum* have all been capable of sulfide oxidation (Kodama and Watanabe, 2003; Kodama and Watanabe, 2004; Handley *et al.*, 2014), isolating more *Sulfuricurvum* spp. can allow for bioremediation application to decrease the concentration of industrially produced sulfide (Vannini *et al.*, 2008).

Future studies should focus on isolating strain Frasassi and describing its metabolism. Isolation is most likely to be successful by serial diluting or plating the medium with hydrogen and nitrate because these conditions that lead to quick strain Frasassi growth and may prevent contaminant growth. To browse for multiple functional genes within the bacterial genome, the whole genome of the pure culture can be sequenced and annotated. The culture can also be used to determine whether or not and how quickly strain Frasassi can contribute to SAS by measuring

the amount of calcium carbonate dissolution under various environmental conditions. Obtaining a pure culture of strain Frasassi can potentially lead to more discoveries on how the strain influences biogeochemical cycling.

3.6 Tables and Figures

Table 3.1 Table of top BLAST hits.

Description	Max score	Total score	Query cover	E value	Ident	Accession
Uncultured epsilon proteobacterium gene for 16S rRNA, partial sequence, clone: 019-Cadma	2292	2292	100%	0.0	99%	AB478659.1
Sulfuricurvum kujiense strain DSM 16994 16S ribosomal RNA gene, complete sequence	2276	2276	100%	0.0	99%	NR_074398.1
Uncultured bacterium clone M17-10-B13 16S ribosomal RNA gene, partial sequence	2276	2276	100%	0.0	99%	JQ088389.1
Sulfuricurvum kujiense DSM 16994, complete genome	2276	6823	100%	0.0	99%	CP002355.1
Sulfuricurvum kujiense gene for 16S rRNA, partial sequence, strain:YK-4	2276	2276	100%	0.0	99%	AB080645.1

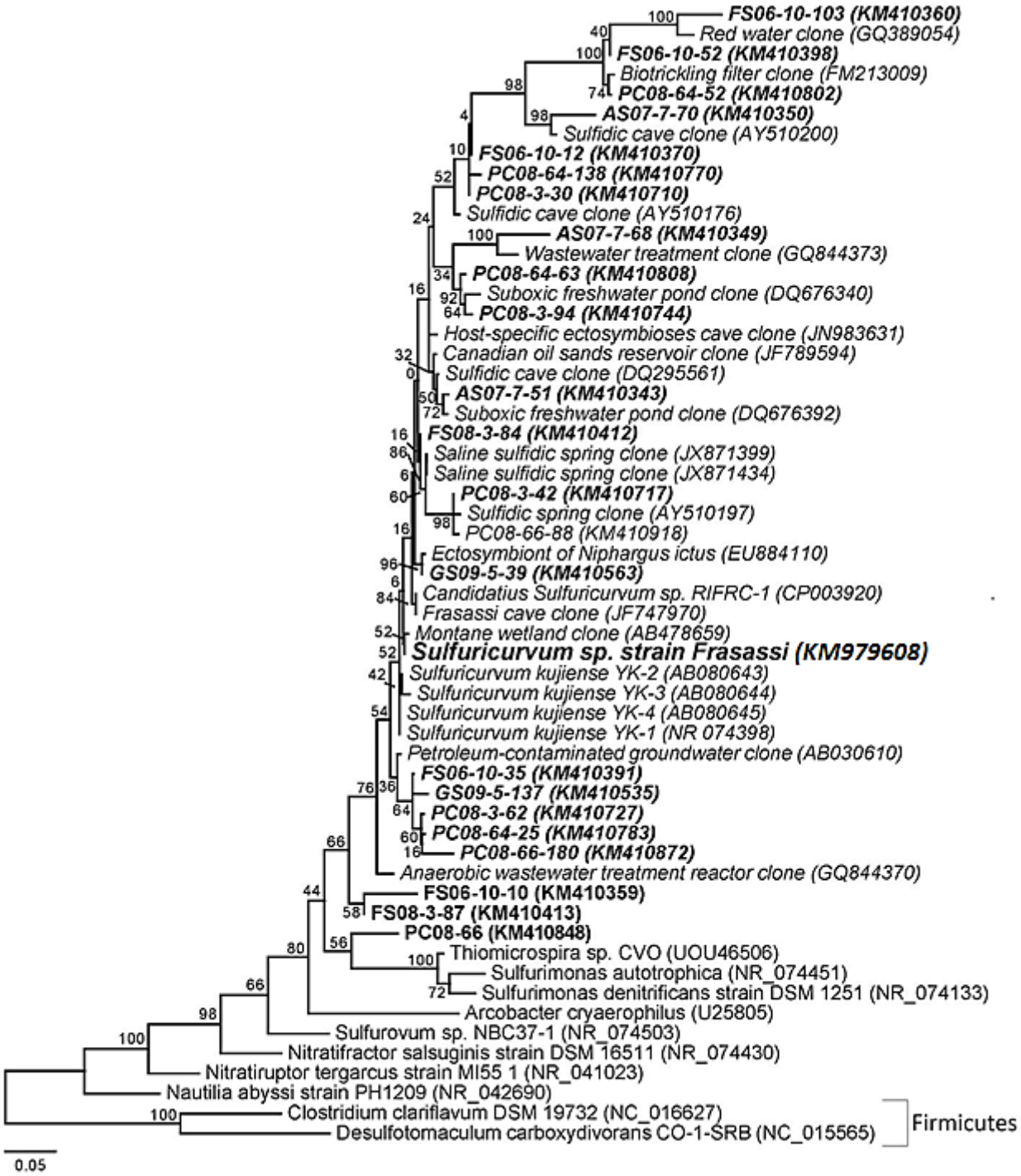

Figure 3.1 Maximum likelihood based phylogenetic 16S rRNA gene tree of Epsilonproteobacteria, including *Sulfuricurvum* sp. strain Frasassi. Accession numbers are provided in parentheses. Bootstrap support values based on 1000 bootstrap samplings are noted. Sample site designations for EMIRGE-reconstructed metagenomic 16S rRNA sequences (in bold): Grotta Sulfurea (GS), Pozzo dei Cristalli (PC), Fissure Spring (FS), and Grotta Nuova di Rio Garrafo in Acquasanta (AS).

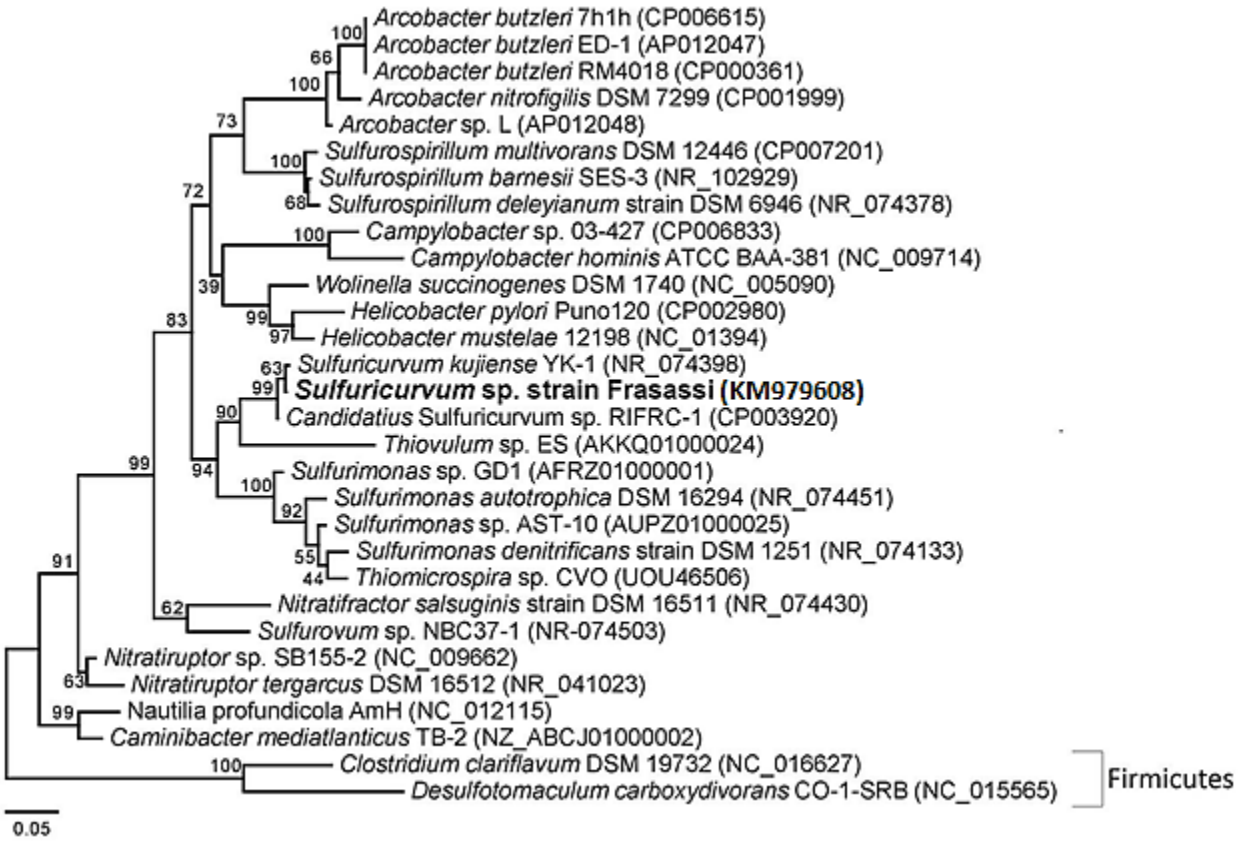

Figure 3.2 Maximum likelihood based phylogenetic 16S rRNA gene tree of Epsilonproteobacteria, including *Sulfuricurvum* sp. strain Frasassi. Accession numbers are provided in parentheses. Bootstrap support values based on 1000 bootstrap samplings are noted.

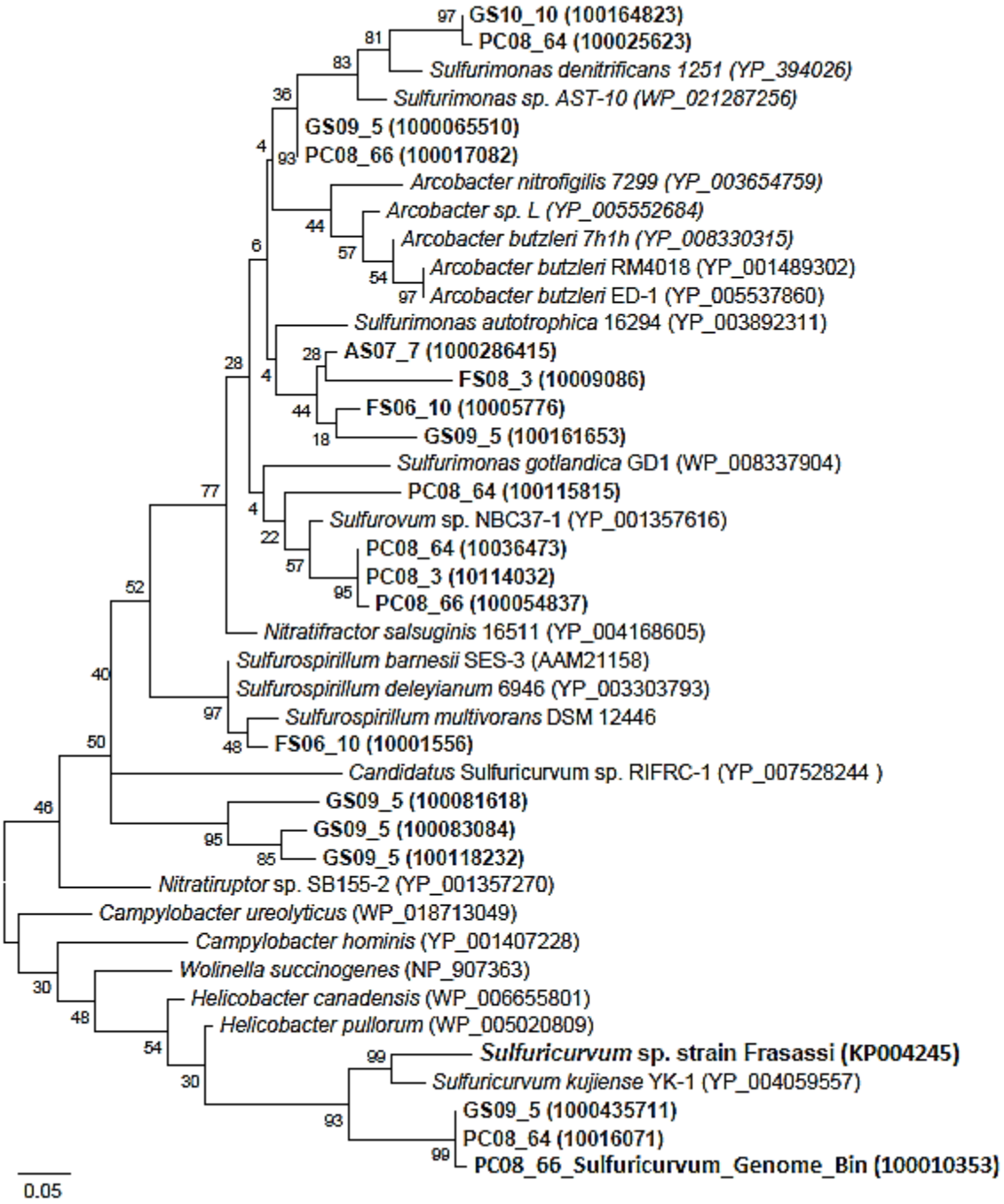

Figure 3.3 Maximum likelihood phylogeny of the *napA* sequence from the *Sulfuricurvum* described in this study (*Sulfuricurvum* sp. strain Frasassi) along with closely related sequences from Frasassi and Acquasanta metagenomes (in bold) and the NCBI database. Accession numbers or IMG designations are given in parentheses. Bootstrap support values based on 1000 bootstrap samplings are noted.

Table 3.2 Raw data for the average number of *Sulfuricurvum kujiense* cells in triplicate cultures over a period of 194 hours. Standard deviation values show one standard deviation.

Growth Condition	Time (Hours)								
	0	17	42	66	90	113.5	137.5	164.5	194
Hydrogen and Oxygen Average	5.03E+05	4.33E+04	6.80E+05	1.76E+06	3.65E+06	5.32E+06	8.64E+06	9.55E+06	7.33E+06
Hydrogen and Oxygen Standard Deviation	5.45E+05	7.50E+04	4.19E+05	9.18E+05	1.09E+06	2.66E+06	3.48E+06	1.37E+06	2.55E+06
Hydrogen and Nitrate Average	4.53E+05	2.28E+05	6.28E+05	1.20E+06	3.33E+06	5.64E+06	6.87E+06	7.08E+06	4.34E+06
Hydrogen and Nitrate Standard Deviation	1.72E+05	3.96E+05	5.22E+05	1.33E+05	1.41E+06	2.04E+06	2.28E+06	2.41E+06	1.21E+06
Sulfide and Oxygen Average	2.14E+05	1.79E+05	5.45E+05	7.90E+05	8.06E+05	4.17E+05	1.85E+05	1.37E+06	1.98E+06
Sulfide and Oxygen Standard Deviation	1.87E+05	1.83E+05	8.78E+05	2.45E+05	3.59E+05	4.09E+05	1.63E+05	1.73E+06	8.44E+05
Sulfide and Nitrate Average	7.54E+05	2.32E+05	3.56E+05	6.47E+05	1.75E+06	1.29E+06	7.46E+05	2.48E+06	2.49E+06
Sulfide and Nitrate Standard Deviation	4.04E+05	5.93E+04	2.17E+05	5.84E+05	4.21E+05	6.15E+05	5.43E+05	1.61E+06	8.83E+05
Sulfur and Oxygen Average	4.15E+05	1.34E+05	3.95E+05	6.70E+05	3.07E+05	1.47E+05	8.61E+05	6.68E+05	2.12E+06
Sulfur and Oxygen Standard Deviation	9.81E+04	1.29E+05	2.91E+05	4.37E+05	1.14E+05	2.08E+05	8.01E+04	6.07E+05	7.60E+05
Sulfur and Nitrate Average	4.63E+04	1.41E+05	4.02E+05	4.46E+05	7.49E+05	2.64E+05	6.02E+05	1.42E+06	1.80E+06
Sulfur and Nitrate Standard Deviation	8.02E+04	1.68E+05	1.16E+05	7.04E+04	3.67E+05	2.09E+05	4.61E+05	3.42E+05	6.16E+05
Thiosulfate and Oxygen Average	2.10E+05	1.54E+05	2.03E+05	6.95E+05	7.42E+05	5.14E+05	3.02E+05	1.45E+06	2.41E+06
Thiosulfate and Oxygen Standard Deviation	2.14E+05	1.26E+05	2.73E+05	4.40E+05	4.45E+05	2.06E+05	2.55E+05	2.32E+05	5.79E+05
Thiosulfate and Nitrate Average	5.90E+05	2.61E+04	1.22E+05	5.02E+05	3.43E+05	2.89E+05	1.69E+05	1.26E+06	1.13E+06
Thiosulfate and Nitrate Standard Deviation	1.02E+06	2.37E+04	2.11E+05	3.50E+05	1.32E+05	2.18E+05	1.58E+05	3.01E+05	5.03E+05

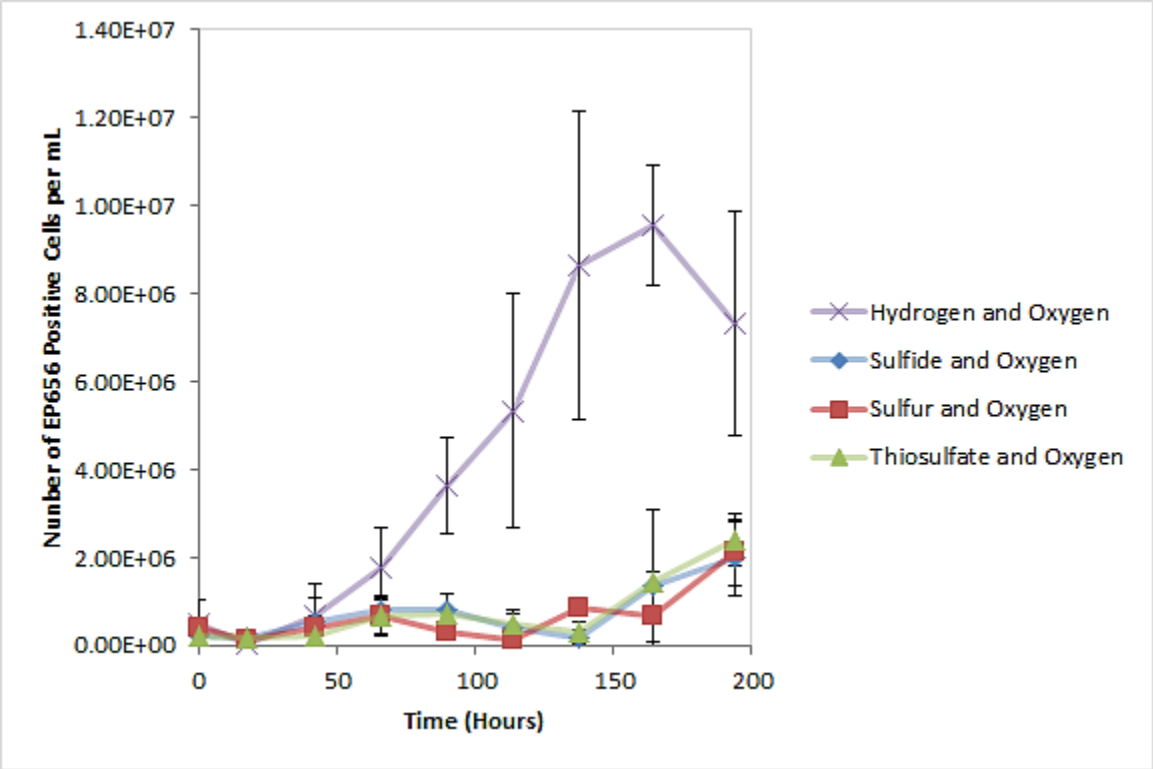

Figure 3.4 Growth curves showing the average number of *S. kujiense* cells in triplicate cultures determined by cell counts, DAPI staining, and EP656 probe hybridization under four different growth conditions: hydrogen and oxygen, sulfide and oxygen, sulfur and oxygen, and thiosulfate and oxygen. Error bars represent one standard deviation.

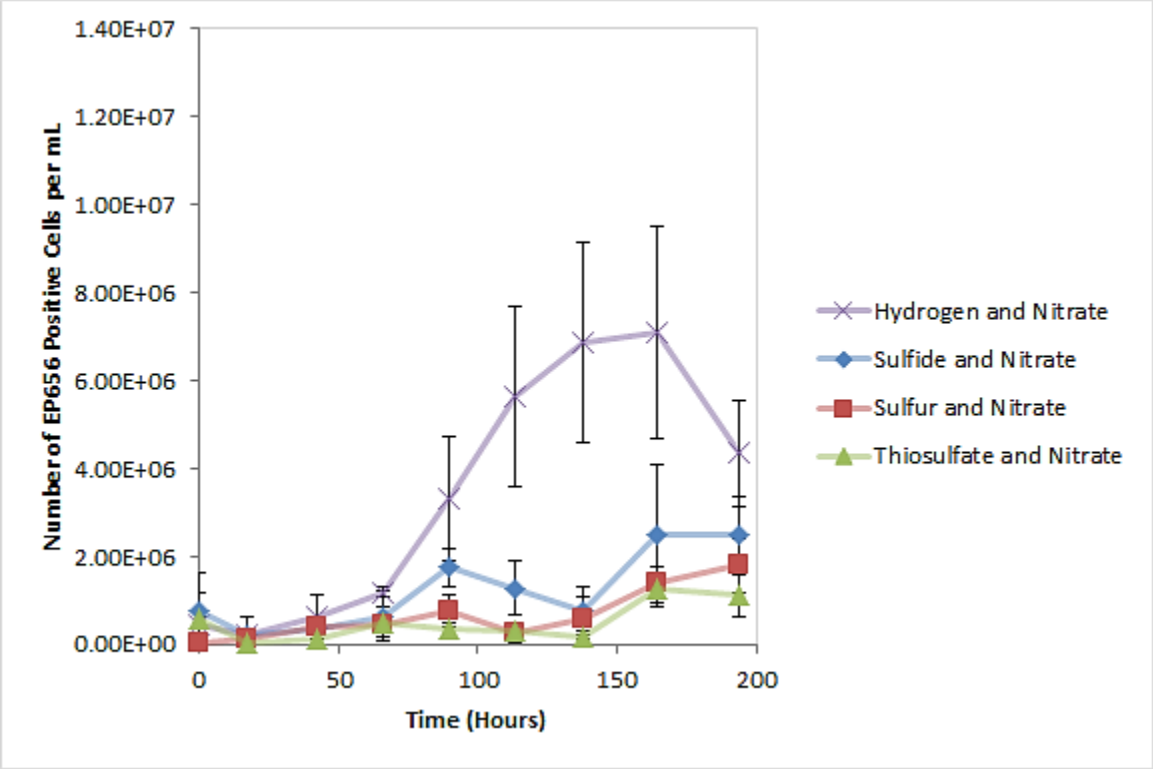

Figure 3.5 Growth curves showing the average number of *S. kujiense* cells in triplicate cultures determined by cell counts, DAPI staining, and EP656 probe hybridization under four different growth conditions: hydrogen and nitrate, sulfide and nitrate, sulfur and nitrate, and thiosulfate and nitrate. Error bars represent one standard deviation.

Table 3.3 Raw data for the average number of EP656 negative cells in triplicate cultures over a period of 194 hours. Standard deviation values show one standard deviation.

Growth Condition	Time (Hours)								
	0	17	42	66	90	113.5	137.5	164.5	194
Hydrogen and Oxygen Average	1.81E+06	1.30E+06	1.39E+06	7.82E+05	7.65E+05	6.41E+05	1.19E+06	2.41E+06	1.84E+06
Hydrogen and Oxygen Standard Deviation	3.10E+05	6.31E+05	8.32E+05	1.43E+05	3.57E+05	1.94E+05	8.91E+05	1.62E+06	1.42E+06
Hydrogen and Nitrate Average	1.39E+06	4.74E+05	5.10E+05	9.56E+05	2.11E+05	2.52E+05	5.69E+05	2.23E+06	9.44E+05
Hydrogen and Nitrate Standard Deviation	3.19E+05	1.91E+05	5.03E+04	5.64E+05	1.91E+05	3.56E+05	6.83E+05	1.88E+05	3.34E+05
Sulfide and Oxygen Average	1.86E+06	8.93E+05	1.05E+06	9.65E+05	1.26E+06	1.58E+06	1.03E+06	2.86E+06	9.31E+05
Sulfide and Oxygen Standard Deviation	5.64E+04	1.20E+05	1.08E+05	5.56E+04	3.20E+05	1.18E+06	2.97E+05	7.74E+05	2.76E+05
Sulfide and Nitrate Average	1.72E+06	1.05E+06	8.48E+05	5.16E+05	8.55E+05	6.34E+05	1.45E+06	2.33E+06	1.66E+06
Sulfide and Nitrate Standard Deviation	5.99E+05	2.33E+05	2.67E+05	1.65E+05	4.91E+05	2.95E+05	1.08E+06	1.11E+06	5.59E+05
Sulfur and Oxygen Average	1.71E+06	8.40E+05	1.34E+06	7.48E+05	9.25E+05	1.09E+06	1.49E+06	2.33E+06	1.69E+06
Sulfur and Oxygen Standard Deviation	1.89E+05	4.72E+05	4.38E+05	1.85E+05	3.62E+05	7.14E+05	4.38E+05	3.68E+05	8.89E+05
Sulfur and Nitrate Average	1.62E+06	1.06E+06	1.39E+06	9.09E+05	1.39E+06	1.25E+06	1.24E+06	2.85E+06	2.27E+06
Sulfur and Nitrate Standard Deviation	1.03E+06	1.37E+05	2.96E+05	6.35E+05	1.66E+05	7.75E+04	2.64E+05	3.29E+05	4.97E+05
Thiosulfate and Oxygen Average	2.64E+06	8.87E+05	5.70E+05	2.65E+05	2.26E+05	2.87E+05	1.77E+05	9.72E+05	5.56E+05
Thiosulfate and Oxygen Standard Deviation	5.70E+05	1.31E+05	2.09E+05	1.80E+05	2.38E+05	2.26E+05	1.98E+05	3.38E+05	4.11E+05
Thiosulfate and Nitrate Average	1.92E+06	2.56E+06	5.26E+05	2.13E+05	2.28E+05	8.33E+05	7.61E+05	2.67E+06	5.32E+05
Thiosulfate and Nitrate Standard Deviation	2.84E+05	3.57E+05	7.09E+05	1.64E+05	9.94E+04	3.19E+04	5.70E+04	2.61E+06	3.18E+05

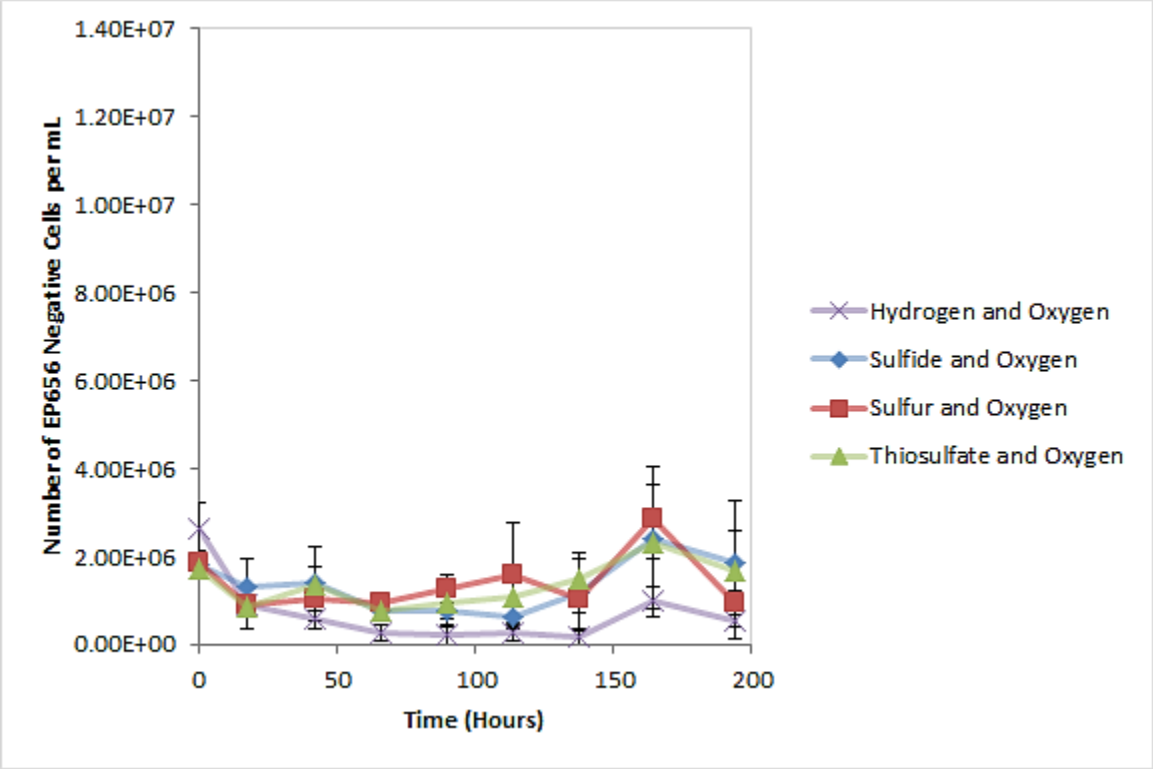

Figure 3.6 Growth curves showing the average number of contaminant cells in triplicate cultures determined by cell counts, DAPI staining, and EP656 probe hybridization under four different growth conditions: hydrogen and oxygen, sulfide and oxygen, sulfur and oxygen, and thiosulfate and oxygen. Error bars represent one standard deviation.

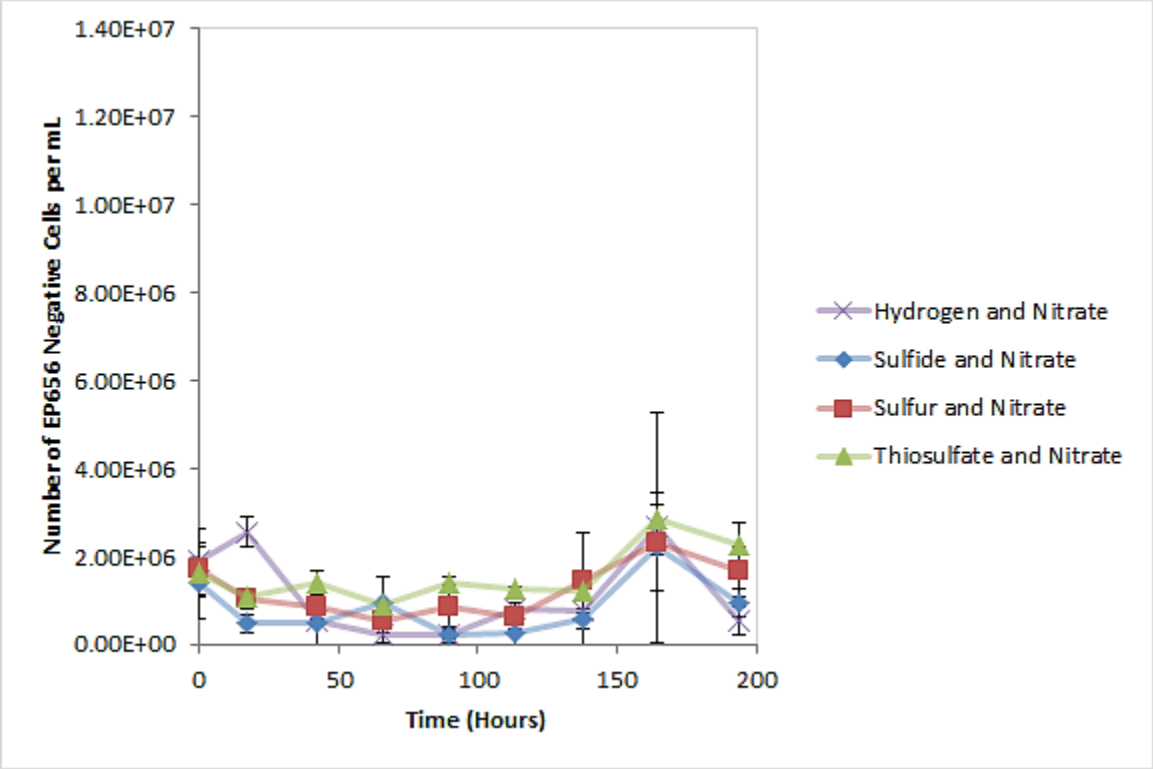

Figure 3.7 Growth curves showing the average number of contaminant cells in triplicate cultures determined by cell counts, DAPI staining, and EP656 probe hybridization under four different growth conditions: hydrogen and nitrate, sulfide and nitrate, sulfur and nitrate, and thiosulfate and nitrate. Error bars represent one standard deviation.

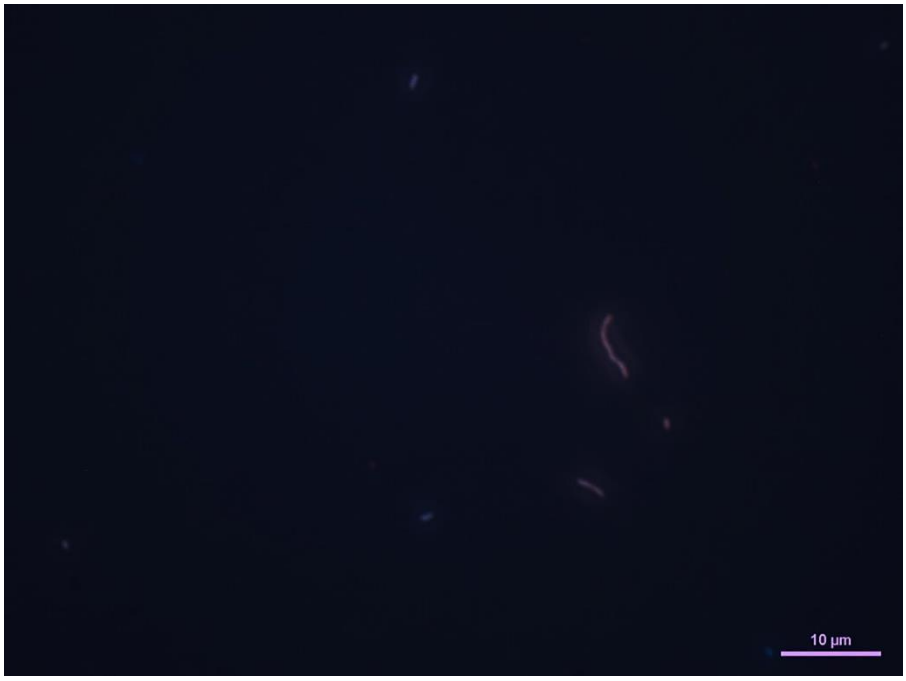

Figure 3.8 Monograph of various morphologies of *Sulfuricurvum*. Blue (DAPI stain) shows all DNA, and red (probe EP656) shows *Sulfuricurvum*.

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