Barite Crusts From A Brine Pool In The Gulf of Mexico And The Role Of Sulfur Oxidizing Bacteria In The Precipitation Of Barite ($BaSO_4$)

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Abstract

Barite (BaSO4) is found in certain benthic marine settings, sometimes in proximity to microbial mat communities. However, barite precipitation is typically thought to form from a mixing of sulfate and barium rich fluids. Barite mineral crusts collected from a brine pool in the Gulf of Mexico contain filamentous mineral structures of grossly similar morphology to filamentous surrounding sulfur-oxidizing *Beggiatoa* mats. Molecular analyses of DNA preserved in the Gulf of Mexico barite crusts suggest that microbial sulfur-oxidation could play a role in the formation of these authigenic precipitates. Laboratory experiments using several strains of benthic marine bacteria show that sulfide-oxidizing bacteria have the capability to mediate barite precipitation via the oxidation of reduced sulfur compounds to sulfate. The results of this study suggest that sulfide-oxidizing bacteria may play a role in the precipitation of certain marine barite deposits, and expands the potential role of bacteria in marine barite formation to include their potential to generate sulfate under sulfate-limited conditions, such as some brine fluids.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

GOM	Gulf of Mexico
SOB	Sulfur-Oxidizing Bacteria
SRB	Sulfur-Reducing Bacteria
SEM	Scanning Electron Microscope
XRD	X-ray Diffraction
EDS	Energy Dispersive Spectroscopy

1. Introduction

The mineral barite (BaSO4) is known from many depositional environments and is associated with a variety of different geologic processes and pressure-temperature conditions (review by Griffith and Paytan, 2012). Natural waters are most commonly undersaturated with respect to barite (Chow and Goldberg, 1960; Church and Wolgemuth, 1972; Griffith and Paytan, 2012; Monnin et al., 1999; Rushdi et al., 2000). Therefore, barite precipitation generally occurs when fluids enriched in barium (Ba^{2+}) encounter those containing sulfate (SO_4^{-2}) (Aloisi et al., 2004):

$$Ba^{2+} + SO_4^{2-} <=> BaSO_4$$

This reaction can lead to the precipitation of authigenic barite in a variety of depositional settings. In marine systems, these include at the seafloor, or within sediments (Aloisi et al., 2004; Torres et al., 1996), and diffuse precipitates are also known to form in the water column (Bertram and James, 1997; Stroobants et al., 1991). While barite precipitation can occur without the aid of biology, a number of recent studies have explored the role of microbes in facilitating barite precipitation (Bertram and James, 1997; Bonny and Jones, 2007; Bonny and Jones, 2008; Gonzalez-Muñoz et al., 2012; Griffith and Paytan, 2012; Rasmussen, 2000; Sanz-Montero et al., 2009; Senko et al., 2004). As with other systems in which microbes are thought to play a role in mineral precipitation, identifying the specific mechanism(s) by which microbes influence the process can be difficult. Acting in a passive role, microbes may act as nucleation sites for precipitation (Gonzalez-Muñoz

et al., 2012). Other potential mechanisms involve the enrichment of SO_4^{-2} through redox transformations or remobilization (Phillips, 2001; Senko et al., 2004). Bonny and Jones (2008) suggest that intracellular barium enrichment and/or metabolic sulfur oxidation may be important for barite precipitation at Flybye Springs in Canada's Northwest Territories. Additionally Bonny and Jones (2008) showed through experimentation with streamer-forming sulfur-oxidizing bacteria from Jasper Cold Sulfur Springs in Alberta, that sulfur-oxidizing bacteria and associated extracellular polymeric substances (EPS) can serve as nucleation substrates for barite precipitation, and that barite precipitation is only restricted to microbial substrates when sulfate is limited. Similarly, a recent study by Gonzalez-Muñoz et al. (2012) showed through experimentation with pelagic bacterial organisms that bacteria have the capability to passively precipitate barite by providing nucleation sites and enhancing crystal growth. Gonzalez-Muñoz et al. (2012) suggest that the role of microbes in sulfate production is likely of little importance in the marine water column where seawater sulfate is abundant. The studies above generally suggest that the role in which bacteria play in barite formation is that of a passive nucleation site.

However, active microbial processes may also be important for the precipitation of barite under certain environmental conditions. A study by Senko et al. (2004) reports on barite precipitation in a spring in the Anadarko Basin of southwestern Oklahoma. Senko et al. (2004) propose a model that suggests that soluble barium can be released from barite by sulfate-reducing bacteria and then reprecipitated as barite by metabolic processes that generate sulfate, such as sulfide oxidation or sulfur disproportionation.

Barite is often found in close association with benthic microbial communities at cold seeps, such as in the Gulf of Mexico and the Derugin Basin, Sea of Okhotsk (Aloisi et al., 2004; Castellini et al., 2006; Feng and Roberts, 2011; Greinert et al., 2002; Griffith and Paytan, 2012; Joye, 2005; Torres et al., 2003). The anaerobic oxidation of methane at seep sites results in increases to porewater alkalinity that drive the precipitation of authigenic carbonates, as well as the production of hydrogen sulfide that fuels ecosystems of sulfide-oxidizing bacteria and associated macrofauna. Barium enriched fluids at certain seep settings may result in the precipitation of barite over carbonate minerals (Aloisi et al., 2004; Feng and Roberts, 2011; Greinert et al., 2002; Orcutt et al., 2005; Torres et al., 1996). Barite crusts and chimneys are observed at many cold seep associated brine pool sites, including in the Gulf of Mexico (GOM) (Feng and Roberts, 2011). These barite crusts commonly co-occur with mats of sulfide-oxidizing bacteria. Typical geochemistry of GOM brine pool sites have been described as having low sulfate concentrations less than 1 mM whereas typical surrounding waters contain ~ 28 mM SO_4^{2-} (Joye, 2005). Feng and Roberts (2011) suggest that GOM barium rich fluids originated either from basinal brine and/or meteoric waters or a deeper origin that has been influenced by dissolved evaporites during transport to the seafloor.

The seepage of sulfate-poor brine fluids into sediments characterized by high rates of bacterial sulfide-oxidation introduces the possibility that sulfide oxidation may be important for the precipitation of barite in brine pool margin sediments. Additionally,

polyphosphate metabolism by sulfide-oxidizing bacteria is thought to play a role in the precipitation of apatite in modern sediments (see review by Crosby and Bailey 2012), and filamentous sulfide-oxidizing bacteria are known to become encrusted in apatite (Bailey et al., 2013)

During exploration of brine pool sediments in the Gulf of Mexico (GOM), barite crusts were recovered that exhibit filamentous mineral features that resemble *Beggiotoa,* a type of filamentous sulfide-oxidizing bacteria that are abundant in the microbial mats found on and around the barite precipitates. Here we investigate the role of sulfide-oxidizing bacteria in the precipitation of barite mineral crusts from the Gulf of Mexico. First we recovered and sequenced DNA from within the barite crusts in order to assess the composition of the bacterial community present at the time of precipitation in order to determine whether or not sulfide-oxidizing organisms were present. Second, we used laboratory experiments to study the potential mechanisms by which sulfide-oxidizing bacteria might influence the precipitation of barite under marine brine pool conditions.

2. Methods

Geological context and sampling method: Green Canyon Block 246 is located on the upper mid-continental slope southwest of the Mississippi Delta in the Gulf of Mexico. The area surrounding the GC246 site is characterized by a complex terrain that includes active mud volcanoes and brine seepage features. The barite crusts examined in this study were collected from the shoreline of a 15-25 cm deep brine lake known as Dead Crab Lake (27º42.1985'N, 90º39.0112'W; 867 m water depth). Sediments surrounding Dead

Crab Lake host authigenic mineral crusts and chimneys, as well as orange and white microbial mats rich in *Beggiatoa*. Barite samples collected for molecular analysis were frozen and stored at -80ºC, while additional samples collected for mineralogical analysis were stored at 25ºC.

2.1. Imaging and mineralogical analysis

Initial observations and imaging were made during sample collection and while onboard the RV-Atlantis using an Olympus SZX16 stereo microscope. Bulk mineralogical analysis of the dried GOM crust was performed using a Rigaku Miniflex powder X-ray diffraction (XRD). The x-ray source was a Cu anode operated at 30 kV and 15 mA using CuKα radiation. Scans were taken at 2º per minute and covered an angular region of 15º≤ 2θ ≤65º. Mineral spectra were identified using MDI's XRD software JADE. GOM crust was examined using a low vacuum Hitachi T-1000 SEM. Semi-quantitative element abundances were measured using energy-dispersive x-ray spectroscopy (EDS) running Bruker Quantax 50 software. Observations were made with an acceleration voltage of 15 kV, with acquisition times of 90 seconds for EDS spectra.

2.2. Amplicon and Clone Library Construction

In order to differentiate between genetic material from surface-attached cells and genetic material contained within the mineral crust, barite crust samples selected for microbial analysis went through a series of washing steps before sample homogenization and DNA extraction (Mason et al., Unpublished). Washing steps included rinsing the sample in 0.2

μm filtered 1x phosphate buffered saline (PBS), followed by sonication at 160 watts for 15 seconds. Samples were then centrifuged for 5 min at 4000 x g. Supernatant was removed and fresh 1x PBS was added. Washing was performed a total of nine times, with the 3rd, 6th, and 9th washes collected and tested with polymerase chain reaction (PCR) amplification for the presence of amplifiable DNA. DNA extraction was performed on the barite crust after the 9th wash. Between 0.2 -0.6 grams of barite crust was processed during DNA extraction.

DNA was extracted using the MoBio Powersoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) following the manufacturer's protocol, with a few modifications. Specifically, following addition of the first solution, samples were incubated at 65ºC for 5 min, vortexed briefly, and returned to 65º for 5 min (Mason et al., Unpublished). Additionally, bead beating was performed in three steps, with one third of the supernatant removed at minute 5, minute 8, and the rest at minute 10, in order to maximize DNA recovery. After extraction, concentrations were measured using a Thermo Scientific Nanodrop 2000C Spectrophotometer. Bacterial 16S rRNA genes were amplified using PCR in order to confirm successful DNA extraction. 25 μl reaction mixtures contained GoTaq Green Master Mix 2X (Promega, Madison, WI), DNA template, 0.2 μM EUB 27F, and 0.2 μM EUB 1492R (Table 1). Reactions were incubated in a Bio Rad C1000 Thermal Cycler as follows: 3 min at 95ºC for initial denaturation, followed by 30 cycles of PCR consisting of 1 min denaturation at 95ºC, annealing for 90 sec, and elongation for

2 min at 75ºC, with a final elongation for 10 min at 72ºC. PCR products were checked for amplification using gel electrophoresis.

Two 16S rRNA clone libraries were generated in order to characterize the diversity of bacterial DNA preserved within the mineralized crust. General characterization of the bacterial community used universal primers, 27F and 1492R with the same thermal cycler protocol as described previously. A second clone library was generated using EUB primer 341F and *Beggiatoa* specific primer VSOXBr (Table 1) in order to target representatives of the Beggiatoaceae that are commonly missed by universal primers. Amplification using 341F and VSOXBr required a nested PCR of a touchdown PCR protocol. 25 µl reactions were incubated as follows: initial denaturation of 4 min at 95ºC, 6 cycles of touchdown PCR consisting of 45 sec of denaturation at 95ºC, annealing for 45 sec at temperatures of 59ºC, 57ºC, 55ºC, and elongation for 45 sec at 72ºC, followed by 24 cycles of PCR at an annealing 53ºC for 45 sec, with a final elongation step at 72ºC for 7 min.

PCR products were then cloned into the pCR2.1-TOPO plasmid, and used with the chemical competent One Shot Mach1-T1 *E. coli* cells as specified in the Topo TA cloning kit (Invitrogen, Carlsbad, CA). Colonies were isolated by streaking on LB plates with 40 mg/ml of x-gal eluted in dimethylformamide and incubated for 8 hours at 37ºC. Colonies were then picked directly for colony PCR using the M13F and M13R primer set as specified in the Topo TA cloning kit. Additionally picked colonies were also

inoculated in liquid LB broth with 10 mg/ml of ampicillin and subsequently preserved and stored in 25% (wt/vol) sterile glycerol at -80ºC. Colony PCR products were checked via gel electrophoresis, cleaned up using DNA Clean and Concentrator -5 kit (Zymo Research), and concentrations measured with a Nanodrop spectrophotometer. Additionally the *Beggiatoa* specific clone library sequences were further analyzed through a restriction enzyme digest.

Sanger sequencing was performed using a 96 capillary ABI 3730xl that uses ABI BigDye Terminator versions 3.1 chemistry. Sequencing reactions for general bacterial analysis consist of both forward and reverse reactions, whereas *Beggiatoa*-specific sequencing used the forward reaction only. Partial sequences were assembled using Sequencher (Bromberg et al., 1995) with final sequence lengths of >1300 bp reads for the general bacterial library and >500 bp reads for the *Beggiatoa* specific library.

16S rRNA sequences from the general bacterial and *Beggiatoa* specific clone libraries were aligned using the NAST aligner at greengenes (DeSantis et al., 2006). Sequences from the five isolates used in the precipitation experiments were aligned with the ARB aligner (Ludwig et al., 2004). Sequences were added to a database of nearly 480,000 representative bacterial species. Manual refinement was performed in ARB using the ARB_Edit4 sequence editor. Alignments were trimmed so that all sequences were of equal length, and nucleotide positions with less than 50% base-pair conservation were masked. The final alignment lengths were 1320 and 512 nucleotide positions for the

general bacteria library and *Beggiatoa* specific library respectively. The analyses included the top BLAST hits (Altschul et al., 1990), the top three closest relatives in the ARB database, and representatives of major divisions with an emphasis on sulfur metabolism sequences. Neighbor joining analyses were performed in PAUP* version 4b10 (Swofford, 1999) with Jukes-Cantor (JC) corrected distance matrix and 2000 bootstrap replicates.

Sequencing of amplicon libraries targeting the V3 hypervariable region of the 16S rRNA gene was also performed using an approach similar to that of Bartram et al. (2011), using Qiagen's HotStar Taq plus DNA Polymerase Kit (Valencia, CA), 50 µl reaction mixtures contained 2.5 µM HotStar taq polymerase, 10x loading buffer, 0.2 µM dNTP's, and 0.2 µM of V3 specific primers (Table1). Reactions were incubated as follows: 5 min at 95ºC for initial denaturation, followed by 25 cycles of PCR consisting of 1 min denaturation at 95ºC, 1 min annealing at 50ºC, and 1 min elongation at 72°C with a final extension step at 72°C for 7 min.

Gel electrophoresis was used to separate DNA products from primers and primer dimers using a 2% agarose gel. Appropriate bands were cut out and processed using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Orange, CA)

V3 specific primers were tagged with a unique barcode (see Table 1), which allows for multiplexing and pooling of 13 total samples on a single lane for paired end Illumina

MiSeq analysis. Resulting data was then processed using Galaxy (Blankenberg et al., 2010; Giardine et al., 2005; Goecks et al., 2010) through the Minnesota Supercomputing Institute (MSI). Sequences were trimmed to remove the inserted "N" degenerate bases from the reverse primer (see Table 1). Forward and reverse sequences were assembled using PAired-eND Assembler (Masella et al., 2012). Alignment, using a SILVA bacteria database as a template, filtering removing any gaps and chimeras were performed using Mothur (Schloss et al., 2009). Additionally Mothur was used to taxonomically classify the resulting dataset. Rarefaction analyses of both the general 16S rRNA clone library and the 16S rRNA Illumina amplicon data sets were computed using Mothur.

2.3. Precipitation Experiments

In order to investigate the mechanisms by which sulfur bacteria might induce barite precipitation, experiments were conducted using five environmental isolates: strain thiotaurens (JN882289), *Roseobacter* sp. strain LH4, *Halomonas* sp. strain BM23, and *Maribacter* sp. strain LM2. All bacteria where isolated from sediment collected in the Gulf of Mexico, site GC233 near the site of barite crust collection, with exception of strain thiotaurens (JN882289) collected off the coast of Massachusetts and *Thiomicrospira crunogena* (NR_074329) which was isolated from the East Pacific Rise (Jannasch et al., 1985). PCR amplification using *sox*B specific primers, soxB693F and soxB1446B (Table 1;(Petri et al., 2001)), was performed in order to assess potential sulfur-oxidizing pathways for each of the five selected bacteria. *Roseobacter*, strain *Thiotaurens,* and *Thiomicrospira crunogena* showed amplification for *sox*B, with

Halomonas showing a very faint amplification. No *sox*B gene amplification was observed for *Maribacter*.

PCR amplification was performed as a two-step PCR modified from Petri et al. (2001). 25 µl reactions were incubated as follows: initial denaturation of 2 min at 95ºC, 10 cycles consisting of 30 sec of denaturation at 95ºC, annealing for 40 sec at temperatures of 55ºC, and elongation for 60 sec at 72ºC, followed by 24 cycles of PCR with an annealing temperature of 47ºC for 45 sec, with a final elongation step at 72ºC for 5 min.

All isolates were initially cultured in liquid BD Diffco Marine Broth 2216 expect for *Thiomicrospira crunogena*, which was cultured in liquid TMS media (Jannasch et al., 1985), and incubated for 72 hours at 25º ± 1º before inoculation of the precipitation experiments.

Precipitation experiments were conducted using a TMS agar base with known ingredients as follows: 430mM NaCl, 3.3 mM NH4Cl, 6.0 mM MgCl2 6H20, 1.5% agar (w/v), phenol red, 2.0 mM CaCl2, 10 mM Tris.Cl, and 3.1 mM K2HPO4. Phenol Red was used in the media as a pH indicator and base media was adjusted to pH 7.92. Four variations of plates were used during precipitation experiments consisting of the TMS agar base supplemented with different concentrations of barium and thiosulfate ($S_2O_3^{2-}$): 1) 2mM Ba^{2+} and 10 mM $S_2O_3^{2-}$; 2) No Ba^{2+} and 10 mM $S_2O_3^{2-}$; 3) No Ba^{2+} and no $S_2O_3^{2-}$; 4) 2mM Ba^{2+} and no $S_2O_3^{2-}$. 20 µl aliquots of the liquid culture inocula were pipetted onto

the experimental plates. Petri dishes were stored on the bench top at 25º ± 1º. Strain *Thiotaurens* was grown in a hypoxic chamber due to its sensitivity to oxygen – a characteristic that it shares with some closely related sulfide-oxidizing gammaproteobacteria such as various marine *Beggiatoa* strains. Experiments were monitored daily for cell growth, mineral precipitation, and pH change.

TMS plates with 2mM BaCl2 and ~10 mM thiosulfate were used as a base for control tests without cells. Four sets of control experiments were also constructed including experiments: 1) with paraformaldehyde (PFA) fixed cells; 2) without cells; 3) with added mineral grains; and 4) PFA-fixed cells without the addition of barium. Controls 1 and 2 were sub-divided into three sets - each of which included the addition of sulfate solution. Sulfate solutions consisted of sea salt solution at pH 7.7, sea salt solution at pH 4.0, and 10mM SO_4^{2-} solution at 7.7 pH. Sea salt solutions were made from Sigma Aldrich Sea Salts mixed to 40 g/L and consist of 2660 mg/L of sulfate. Set 3 used three separate inorganic mineral substrates, kaolinite, montmorillonite (bentonite), and quartz sand, placed on top of the TMS plates. Mineral substrate plates were tested with the sea salt solution at pH 7.7 and 10mM $NaSO_4$ at 7.7 pH. Before experimental testing inorganic substrates were washed using a three-step process that included, rinsing in molecular grade ethanol, centrifuging for 3 min at 1000 x g, and removal of the supernatant. Washing was performed a total of three times. Set 4 contained plates with PFA-fixed cells without the addition of sulfate solution. All experiments conducted with PFA-fixed

cells used approximately 200 µl of fixed cell solution consisting of *Roseobacter* sp. fixed in a 4% paraformaldahyde solution in seawater and incubated at 4ºC for 3 hours.

Experimental precipitates were analyzed using a Bruker-AXS microdiffractometer with 2.2 kW Sealed Cu X-ray Source and a low vacuum Hitachi T-1000 SEM. Experimental precipitate samples selected for SEM analysis were cut out from the Petri plate and attached to an aluminum SEM stub with a piece of carbon tape. Samples were then dried in an incubator at 40ºC overnight to prevent unwanted halite precipitation. Samples selected for microdiffractometer analysis were cut out from the Petri plate and placed directly on to a quartz stub with no subsequent drying procedure. Sample scans consisted of two runs at 5 min each at 2θ 28º and 2θ 58º. Spectra were integrated and merged using GADDS and JADE software (Bruker-AXS; Materials Data, Inc).

Abiotic precipitation was quantified by analysis of media plates using ImageJ (Abràmoff et al., 2004). Plates were photographed using a digital camera and then converted to an RGB (Red-Green-Blue) stack. The threshold of the green image of the stack was then adjusted so that mineral precipitates were specifically isolated to stand out compared to the background (growth media) portion of the image. Measurements for the amount of plate area occupied with precipitation as seen in photographs were calculated using the analyze > measure function of ImageJ, added by selecting and de-selecting the "limit to threshold" icon in the set measurements dialog box in order to calculate total area vs. area limited to the threshold (i.e. the mineral precipitates).

3. Results

3.1. GOM crust mineral characterization

Light microscopy images of the barite crust show areas of bacteria-like filaments coated or entombed by mineral precipitates. When observed immediately upon collection, living filamentous sulfur bacteria could be observed on the exteriors of the mineral crusts. Electron microscopy images show that individual filaments can be traced from areas where the filament is completely entombed to areas where the organic material is exposed (Fig. 1). Crystal morphology, as imaged by ESEM observations, show a spherical and dumbbell shaped habit associated with the entombed filaments (Fig. 2). Powder XRD analysis shows that the mineralized crust is homogeneously $BaSO_4$ (Fig. 3). A slight quartz (SiO2) peak is visible at ~ 2θ 26.5º and an unidentified peak is visible at ~ 2θ 45º.

3.2. Phylogenetic analysis of DNA recovered from barite crust

Thirty-eight sequences were assembled from the bacterial clone library using primer set 27f and 1492r. Figure 4 shows the general distribution of sequences at the phylum/class rank found within the sample that are greater then 1% of the community composition. The most abundant sequences belong to the Proteobacteria, with gammaproteobacteria comprising 39.5% of the community and alphaproteobacteria comprising 15.8%. The next most-abundant sequences fall within the Bacteroidetes, the Epsilonproteobacteria,

and unclassified bacteria each of which comprise 7.9% of the community. Firmicutes are the next most abundant at 5.3% of the community.

Figure 4 also shows the community differences between the general bacterial 16S rRNA clone library and the 16S rRNA Illumina amplicon library. The Illumina MiSeq amplicon library generated using primers to the V3 hypervariable region of the 16S rRNA gene resulted in 102,664 sequences. Broad phylogenetic trends observed in the 16S rRNA gene amplicon library are very similar to those observed in the bacterial clone library. Again, the most abundant sequences fall within the Proteobacteria, with gammaproteobacteria sequences comprising 37.7% of the community and alphaproteobacteria with 16.2%. The biggest difference between the two data sets is the percentage of bacteroidetes within the community as analyzed in the amplicon library, comprising 16.5%, up from 7.9% in the clone library. Another noticeable difference is seen within the unclassified bacteria, which comprise 2% of the community in the amplicon library, down from 7.9% in the clone library.

Figure 5 shows the distribution at the ranks of order and genus of sequences in the 16S rRNA Illumina amplicon library. Of the Gammaproteobacteria sequences, 20% were of the order Thiotrichales, with the genera *Leucothrix, Beggiatoa,* and *Thioploca* comprising 2%, 0.1%, and 0.3% of the community respectively. Of the Alphaproteobacteria sequences, *Roseobacter* was the most abundant genus, comprising 38% of the community. The most abundant sequences within the Epsilonproteobacteria fall within

the genera *Sulfurovum, Sulfuricurvum, Sulfurospirillum,* and *Sulfurimonas* comprising 58%, 18%, 16%, and 4.6% of the community respectively. Of the Deltaproteobacteria sequences 32%, 26%, and 5% were of the order Desulfobacterales, Desulfuromonadales, and Desulfovibrionales respectively.

Neighbor joining trees constructed using a Jukes-Cantor (JC) corrected distance matrix, show phylogenetic relationships of the general bacterial 16S rRNA clone library in addition to the five isolates from pure cultures used in the precipitation experiments (Fig. 6). Bootstrap values for 2000 replicates are shown. Clone library sequences were selected specifically to show the potential sulfur metabolisms associated with the GOM crust. Additionally sequences within the *Bacteroidetes* were selected to show their relationship to the *Maribacter* sp. used during the precipitation experiment. Seven sequences fell within the Alphaproteobacteria and are grouped with the family *Rhodobacteraceae*, which include the genera *Roseobacter, Sulfitobacter,* and *Roseovarius*. Four sequences fell within the Epsilonproteobacteria, two of which are grouped with *Sulfurospirillum* and two of which are grouped to *Sulfurovum.* Unlike with the clade-specific clone library results,there were no matches for sequences from known sulfide-oxidizing bacteria within the Gammaproteobacteria using the general bacterial primers. Results from the *Beggiatoa* specific clone library using primers 341f and vsoxbr show that two out of nine sequences cluster with *Candidatus* Maribeggiatoa vulgaris (Fig. 7). The other seven sequences BLAST results show top matches to unknown marine sediment bacteria.

Phylogenetic analysis for the two clone sequences was modeled after the *Thiotrichaceae* tree from Salman et al. (2011).

The results of rarefaction analysis of the 16S rRNA Illumina amplicon library are shown in Figure 4.

3.3. Mineral precipitation experiments with pure cultures

Bacterial growth on mineral precipitation experiment plates was observed for 34 days and the pH of each plate was measured at the end of the experiment. *Roseobacter* sp. showed the most variability in its influence on the pH of the media between the different mixtures: 2mM Ba^{2+} with 10mM $S_2O_3^{2-}$ at pH ~5.8; No Ba^{2+} with 10mM $S_2O_3^{2-}$ at pH ~5.0, and no $S_2O_3^{2-}$ with and without 2mM Ba^{2+} at pH ~7.7. *Halomonas* sp. showed a slight change to more basic conditions on the third day observed by the pink to purple color change of the Phenol Red, however a final pH ~7.4 was recorded. *Maribacter* sp. showed a final pH of ~6.8 for all mixes. The pH of *Thiomicrospira crunogena* was ~4.5 for media with 10mM $S_2O_3^{2-}$ with and without 2mM Ba^{2+}, whereas a final pH of ~7.8 was measured for media with no $S_2O_3^{2-}$ with and without 2mM Ba^{2+}. Strain *Thiotaurens* showed a final pH of ~6.5 for media with 10mM $S_2O_3^{2-}$ with and without 2mM Ba^{2+} and a final pH of ~7.8 for mixes of no $S_2O_3^{2-}$ with and without 2mM Ba^{2+}.

Roseobacter sp., *Halomonas* sp., and *Maribacter* sp. exhibited growth in colony biomass within two days of the experiment for all plate mixtures. *Thiomicrospira crunogena*

exhibited visible colony growth on Day 2 for the mix with no Ba^{2+} with 10mM $S_2O_3^{2-}$ and Day 3 for 2mM Ba^{2+} with 10mM $S_2O_3^{2-}$. No growth was exhibited for mixes of no $S_2O_3^{2-}$ with and without 2mM Ba^{2+} Strain *Thiotaurens* showed signs of growth for mixes of 10mM $S_2O_3^{2-}$ with and without 2mM Ba^{2+} by Day 3. Little to no growth was exhibited for No Ba^{2+} with no $S_2O_3^{2-}$, however 2mM Ba^{2+} with no $S_2O_3^{2-}$showed small amounts of growth by the end of the experiment.

All five bacterial isolates showed growth on media mixtures with 2mM thiosulfate, however the media mixture without thiosulfate supported growth of only *Roseobacter* sp., *Halomonas* sp., and *Maribacter* sp.

The development of observable mineral precipitates varied between the five bacterial isolates. Crystal growth was observed for *Roseobacter* sp. within the microbial lawn on Day 3, isolated crystal growth within the colonies was visible between Days 5 and 7. *Thiomicrospira crunogena* showed mineral precipitation approximately by Day 4, followed by strain *Thiotaurens,* which exhibited precipitates by Day 10 of the experiment. Density and concentration of mineral precipitates varied slightly between *Roseobacter* sp., *Thiomicrospira crunogenes*, and strain *Thiotaurens*; however by Day 34, precipitation was localized on and around microbial colonies radiating outward (Fig. 8). Mineral precipitates developed by Day 12 on plates with *Maribacter* sp., however the location of precipitates differed from the other isolates in that mineral precipitates formed a halo ~5mm away from the colony, with no precipitates observed within the actual

colony. *Halomonas* sp. exhibited no mineral precipitation until 45 days after inoculation. Mineral precipitates were observed on *Halomonas* sp. and mineral density, concentration, and location were similar to those exhibited by *Roseobacter* sp.

Typical experimental mineral precipitates exhibited spherical shapes of approximately 3 to 6 μm in length. Figure 8 shows the general relationship between mineral precipitates and colony growth. SEM analysis shows entombed cells within several of the spherical precipitates (Fig. 10).

Some experiments lacking cells, and those containing only experimentally-fixed cells, did show mineral precipitation. Mineral precipitation was observed on both PFA-fixed and non-PFA fixed cell plates when sulfate-containing solutions were added. No precipitation was observed for plates that contained PFA-fixed cells on media that contained thiosulfate and no supplemental sulfate . The area occupied by mineral precipitates was calculated from photographs using ImageJ. Percents calculated show variation between plates with and without PFA-fixed cells based on specific added sulfate solution (Fig. 11).

Minimal mineral precipitation was observed for plates with sea salt solutions at pH 4 and 7.7 for both plates with and without PFA-fixed cells. Higher amounts of precipitation were calculated for plates with the 10mM Na_2SO_4 solution than plates that contained sea salt solutions at pH 4 and 7.7 for both plates with and without PFA fixed cells (Fig. 11).

Little to no mineral precipitation was observed on the plates that contained kaolinite, montmorillonite (bentonite), or quartz sand with either the Sea Salt pH 7.7 or 10mM $NaSO_4$ solutions.

Mineral precipitates were analyzed using a XRD microdiffractometer. Uninocculated control plates were analyzed and show no known associated mineral peaks. However, a high intensity signal can be seen from 2θ 24º - 46º that is thought to be an artifact of the agar when mounted to the microdiffractometer.

Several mineral precipitates from each sample plate were chosen for mineral characterization. Mineral analysis of all five bacterial isolates show peaks with close resemblance to that of BaSO4 (Fig. 12) Samples of *Halomonas* sp. and *Thiomicrospira crunogena* each additionally contained halite peaks. Analyses of *Roseobacter* sp., *Halomonas* sp., strain *Thiotaurens,* and *Thiomicrospira crunogena* were all measured directly on colonies. Mineral precipitates from *Maribacter* sp. were not associated directly with bacterial growth. Observed halite peaks were the result of dehydration-induced crystal growth during analysis. Variations between the spectra readings of the BaSO4 from sample to sample could be due to such variables as sample placement, sample thickness, and shape of the agar placed on the sample holder.

Abiotic precipitation experiments were also analyzed using a XRD microdiffractometer.

Mineral analysis of the abiotic precipitation experiments shows that mineral precipitates from all of the experiments are $BaSO_4$ (Fig. 12).

4. Discussion

Barite crusts collected from sediments surrounding brine pools in the Gulf of Mexico (GOM) contain filamentous features that resemble *Beggiotoa,* a filamentous sulfide-oxidizing bacteria. Extraction of DNA from the rinsed barite crust followed by sequencing of the 16S rRNA gene was used to gain insights into the community composition present during mineral precipitation. This approach does not differentiate between DNA from living organisms within the crust (if present) versus undegraded relict DNA from past life. Community analysis using the 16S rRNA gene consisted of a thirty-eight sequence bacterial clone library, nine sequence *Beggiatoa*-specific clone library, and a 16S rRNA Illumina amplicon library containing approximately 102,664 sequences, all produced from material extracted from one barite crust sample. General trends in 16S rRNA community composition suggest the most dominant 16S rRNA gene contributions come from representatives of the Gammaproteobacteria, Alphaproteobacteria, and Epsilonproteobacteria.

4.1. Microbial analyses of barite crust

Within the bacterial clone library, gammaproteobacteria comprise >37% of the community composition. No sequences from sulfide-oxidizing bacteria were present in the clone library produced with universal primers. However, two of the nine sequences

generated from the library generated using *Beggiatoa*-specific primers grouped most closely with *Ca* Maribeggiatoa vulgaris. The absence of sequences representing sulfide-oxidizing gammaproteobacteria in the clone library produced using general eubacterial primers versus that produced using Beggiatoaceae clade-specific primers could be the result of primer bias and the relatively small size of the clone library. Amplicon libraries targeting the V3 region of the 16S rRNA gene were used to supplement the general and clade-specific clone libraries. Despite the small number of sequences (n=38) in the clone library versus the amplicon library (n=102,664), similar diversity patterns are observed (Fig. 4a). The results of rarefaction analysis of the 16S rRNA Illumina amplicon analysis suggest that the major bacterial populations have been adequately sampled (Fig. 4b).

The amplicon library shows that 20% of the Gammaproteobacteria-related OTUs (7740) are representatives of the order Thiotricales, which includes chemoautotrophic sulfide-oxidizing bacteria such as *Beggiatoa*, *Thioploca*, *Thiomargarita*, and *Leucothrix* (Larkin and Strohl, 1983; Salman et al., 2011; Teske et al., 1995; Teske et al., 1999 (Ahmad, 2006; Sievert, 2007).

Alphaproteobacteria are thought to be the most abundant of marine cellular organisms (Giovannoni et al., 2005; Williams et al., 2007) but represent 16.2% percent of the community in the amplicon library and 15.6% percent of the clone library. Phylogenetic analysis of the bacterial clone library show several sequences within the Alphaproteobacteria group within the family *Rhodobacteraceae*, which include the

genera *Roseobacter, Sulfitobacter,* and *Roseovarius.* 16S rRNA Illumina amplicon analysis suggests that within the *Rhodobacteraceae,* 38% of the sequence composition falls within the genus *Roseobacter*. The *Roseobacter* are found in many natural environments (Brinkhoff et al., 2008; Buchan et al., 2005; Wagner-Döbler and Biebl, 2006) and the species is thought to be capable of metabolic sulfur oxidation, using inorganic sulfur compounds such as sulfide and thiosulfate (Buchan et al., 2005; González et al., 1999; Moran et al., 2003).

The Epsilonproteobacteria comprise between 7.9% and 6.9% of the clone library and 16S rRNA Illumina amplicon sequences respectively. 16S rRNA Illumina amplicon analysis suggests that 99% of the Epsilonproteobacteria sequences (7083) belong to representatives of the Camplyobacterales, 58% of which belong to the *Sulfurovum clade*, 18% to *Sulfuricurvum,* 16% to *Sulfurospirillum,* and 4.6% to *Sulfurimonas*. *Sulfurovum, Sulfuricurvum*. *Sulfurimonas* are all consider sulfur-oxidizing bacteria, whereas the *Sulfurospirillum* are sulfur-reducing bacteria (Campbell et al., 2006).

Studies have demonstrated that sulfur-oxidizing Gammaproteobacteria and Epsilonproteobacteria bacteria are commonly found in habits enriched in inorganic sulfur compounds (Campbell et al., 2006; Inagaki et al., 2003; Stewart et al., 2005; Yamamoto and Takai, 2011).

The presence of Deltaproteobacteria suggests the GOM crust potentially includes sulfate-reducing bacteria. Clone library and 16S rRNA Illumina amplicon analysis suggests deltaproteobacteria compose 2.6% and 4.8 % of the overall community respectively. 16S rRNA Illumina amplicon analysis suggest that 32%, 26% and 15% of the sequences cluster within the Desulfobacterales, Desulfuromonadales, and Sh765B-TzT-29 respectively. Members of the Desulfobacterales and Desulfuromonadales are known sulfate-reducing bacteria (Miller et al., 2013). Evidence suggests that Sh765B-TzT-29 is possibly linked to the anaerobic oxidation of methane using iron and manganese as an electron acceptor (Siegert et al., 2011).

4.2. Precipitation experiments

Precipitation experiments were performed to investigate the potential mechanisms by which sulfide-oxidizing bacteria might influence the precipitation of barite under marine conditions. The molecular analyses described above demonstrate that sulfide-oxidizing bacteria were abundant (~29% of totally community composition) in the microenvironment in which the barite crusts precipitated. Moreover, extant *Beggiatoa* mats are abundant in the sediments surrounding the mineral crusts, mineralized filaments that resemble *Beggiatoa* are found within the crust, and molecular evidence shows specifically the presence of *Beggiatoa* DNA with the crusts. The remains of microorganisms preserved within a mineral deposit do not necessarily indicate that those organisms were involved in the mineral precipitation. However, bacteria are known to encourage the precipitation of barite under a variety of conditions and sulfide-oxidizing

bacteria are thought to be actively involved in the precipitation of barite by producing sulfate under certain low sulfate conditions. Bonny and Jones (2007, 2008) observed barite precipitation associated with microbial EPS and filaments, including filaments of sulfide-oxidizing bacteria within a sulfidic spring at Flybye and Jasper Cold Springs in Canada's Northwest Territories. Based on the geochemistry of the system and the association of certain barite crystal morphologies with microbial biomass, Bonny and Jones (2008) suggested that some types of barite in the spring deposits formed primarily though abiotic reactions, whereas other barite precipitates formed via reaction of barium adsorbed to microbial cells and/or EPS with sulfate derived from the microbial oxidation of sulfide. Senko et al. (2007) suggested that a combination of bacterial sulfate reduction and bacterial sulfide oxidation were primarily responsible for barite precipitation in a terrestrial hot spring, with sulfate-reduction acting to remobilize barium from barite, and sulfide-oxidation acting to provide sulfate for its re-precipitation.

In marine systems, the role of microbes in barite precipitation is most commonly attributed to the ability of the cells to act as mineral nucleation sites. Unlike certain terrestrial systems, sulfide oxidation is thought to be unimportant because sulfate is abundant in seawater (e.g. Gonzalez-Muñoz et al. (2012). However, brine pools, including the site in this study, and brine pool sulfate concentrations can be substantially lower than seawater (Joye, 2005). Sulfur flux within GOM brine sites often varies. In GOM brine pool site GC233, the transition of overlying seawater to brine occurred gradually between 30 and 150 cm, and reported brine fluids show concentrations of little

to no SO_4^2 (0 to 1mM) (Joye et al., 2005). Brine sulfate concentrations for GOM sites GC233 and GB425 are much lower compared to other brine pool sites, Orca Basin (GOM; 20-47 mM SO_4^{-2}), Uraina Basin (Mediterranean; 85 mM SO_4^{-2}) and Libeccio Basin (Mediterranean; 99 mM SO_4^{-2}) (Joye et al., 2005). Core samples of *Beggiatoa* mats associated with brine flows were collected by Joye et al. (2004) from GOM sites AT340 and MC853. H_2S and SO_4^{-2} concentrations for site AT340 were 0.00 mM H_2S and 28.50 mM SO_4^{-2}, whereas site MC853 showed 6.30 mM H_2S and 14.10 mM SO_4^{-2}. In low sulfate brine environments, bacterial sulfide-oxidation may indeed serve as a source of sulfate for barite precipitation.

As part of this study, barite precipitation experiments were performed with and without bacterial cells in order to better understand the influence of bacteria on the precipitation of barite and to look for mineral characteristics that might be used to differentiate between abiotic precipitates and those that are influenced by the activities of microorganisms. Substitutions were made for compounds containing sulfate during base media construction to simulate barite precipitation under conditions that are initially depleted in sulfate. Thiosulfate ($S_2O_3^{2-}$) was chosen over hydrogen sulfide (H_2S) because H_2S is harder to work with experimentally due to its rapid abiotic oxidation.

Experiments were conducted using five environmental isolates: Strain Thiotaurens (JN882289), *Thiomicrospira crunogena* (NR_074329), *Roseobacter* sp. strain LH4, *Halomonas* sp. strain BM23, and *Maribacter* sp. strain LM2. Isolates were specifically

chosen in order to test different sulfur-oxidation pathways in the role of barite precipitation (Fig. 9). Strain Thiotaurens (JN882289), *Thiomicrospira crunogena* (NR_074329), and *Roseobacter* sp. strain LH4 were selected because they are known thiosulfate-oxidizing bacteria. *Halomonas* sp. strain BM23 was chosen because its metabolism generates sulfate, but at a slower rate than the other sulfide-oxidizing strains selected here. *Maribacter* sp. strain LM2, a heterotroph, was chosen as a negative control because it does not have the capability to oxidize thiosulfate.

Strain *Thiotaurens* is a gammaproteobacteria. Through the PCR analysis with *soxB* specific primers we have shown that Strain *Thiotaurens* most likely has the *sox*B gene, which allow it to oxidize thiosulfate and generate sulfate. *Thiomicrospira crunogena* is a colorless sulfur-oxidizing bacterium that can oxidize reduced sulfur compounds (sulfide, thiosulfate, and elemental sulfur) as an energy source in chemolithoautotrophic growth (Jannasch et al., 1985). Results of PCR with *sox*B primer set shows amplification for *Thiomicrospira crunogena*, confirming what is already known about its ability to oxidize thiosulfate and serve as a positive control for *soxB* screening of other strains. *Roseobacter* sp. is an alphaproteobacteria and is grouped within the family *Rhodobacteraceae*. *Roseobacter* are known to possess the *sox*CD pathway, allowing them to oxidize thiosulfate to sulfuric acid without the production of elemental sulfur intermediates (Teske et al., 2000). Additionally PCR results with *sox*B primer set shows amplification of the *sox*B gene, confirming their potential to oxidize thiosulfate. *Halomonas* sp. is a gammaproteobacteria that has shown to be a base-producing thiosulfate-oxidizing

bacterium (Teske et al., 2000). Teske et al. (2000) observed that after 20 days of growth on sulfate-free thiosulfate agar slants, sulfate production was found. Additionally a slight amplification was seen during PCR analysis with *sox*B specific primers. *Maribacter* sp. is a member of the *Flavobacteriaceae,* is a heterotroph, and is known for its ability to degrade agar. Additionally, some species are known to produce acidic compounds via heterotrophic metabolism (Nedashkovskaya et al., 2004). PCR amplification with *sox*B specific primer was checked, but not observed in *Maribacter* sp.

4.3. Experimental Precipitation

Mineral precipitation development varied between the five bacterial isolates. *Roseobacter* sp., *Thiomicrospira crunogena*, and strain *Thiotaurens* showed localized barite precipitation on and around microbial colonies radiating outward from colony growth. *Halomonas* sp. exhibited late mineral precipitation in comparison to *Roseobacter* sp, *Thiomicrospira crunogena*, and strain *Thiotaurens*, which can possibly be explained by observations made by Teske et al. (2000) stating that *Halomonas* sp. produces sulfate after approximately 20 days of growth. The results of barite precipitation for *Roseobacter* sp., *Thiomicrospira crunogena*, strain *Thiotaurens*, and *Halomonas* sp. suggest that all four influenced the precipitation of barite through the production of sulfate via the oxidation of thiosulfate. One explanation for colony concentrated mineral precipitation could be due to the diffusion of microbial produced sulfate. As microbes metabolized reduced sulfur compounds (thiosulfate, H_2S, S_0), metabolically- produced sulfate would quickly react with free barium ions within the media and begin to locally precipitate

barite. This would be visible as barite precipitation directly on colony biomass as observed with *Roseobacter* sp., *Thiomicrospira crunogena*, strain *Thiotaurens*, and *Halomonas* sp. As microbes produce sulfate, diffusion would extend beyond the colony growth, resulting in the outward radiation of barite precipitation. This process seemed to be particularly evident with individually colony growth of *Roseobacter* sp. that exhibited rapid precipitation. This rapid precipitation is consistent with the metabolism of sulfide-oxidizing *Roseobacter*, which oxidize thiosulfate directly to sulfuric acid without the production of sulfur intermediates that result from incomplete *sox*-based sulfide oxidation pathways.

Barite precipitation associated with *Maribacter* sp. was visibly different from that observed with the sulfide-oxidizing strains, forming a halo-shaped pattern around colony growth. Similar halo production has previously been described due to sulfatase activity of several *Vibrio* species grown on sulfate-containing agar medium (Kitaura et al., 1983). Additionally agar-degrading bacteria have the potential to form pits in agar-based media and members of the Bacteroidetes, more specifically *Flavobacteria,* are known to degrade agar. These organisms can potentially hydrolozye carrageenan, which is a sulfated polysaccharides found in the red algae from which the agar is derived (Michel et al, 2006). If *Maribacter* degraded the carrageenans in the agar, releasing the bound sulfate, this could potentially explain the halo-shaped barite precipitation observed in our experiments. However, more investigation as to whether carrageenases are found in *Maribacter* sp. strain LM2 will be needed to test this hypothesis.

Spherical and dumbbell shaped aggregates were the most common morphotype of precipitate produced in our experiments. These morphologies are very similar to those barite precipitates produced experimentally by Gonzalez-Munoz et al. (2012) and resemble those associated with the *Beggiatoa*-like filaments in the brine pool crusts examined in this study. Scanning electron and optical microscopy of experimental precipitates shows a close spatial association between barite precipitation and colony growth. Additionally cells can be observed entombed within several of the spherical barite precipitates (Fig. 10). The observed entombment of sulfide-oxidizing cells within the experimental precipitates is potentially analogous to the entombment of *Beggiatoa* filaments observed in the GOM barite crust.

Unlike the live cell experiments, no barite precipitation was observed on plates with PFA-fixed cells that were not supplemented with sulfate, illustrating the potential role of sulfide-oxidizing bacteria to facilitate barite precipitation under sulfate impoverished conditions. Abiotic barite precipitation only occurred on plates with the addition of a sulfate solution. Both plates with and without PFA-fixed cells showed precipitation when amended with each of the three of the sulfate source solutions. Plates with PFA-fixed cells visibly appeared to have higher yields of mineral precipitates when compared with plates that contained no cells. However image analysis of precipitation area (Fig. 11) show no quantifiable differences between mineral precipitation between plates with and without PFA-fixed cells. These findings suggest that, under the conditions of the

experiment, metabolically-inactive cells had little effect on the areal extent of barite precipitation.

Barite precipitation was shown associated with *Roseobacter* sp., *Thiomicrospira crunogenes*, strain *Thiotaurens,* and *Halomonas* sp.colony biomass, while no precipitation localization was observed in the control experiments with and without dead cells.

Experimental results suggest that bacterial production of sulfate can greatly influence the precipitation of barite as seen in terrestrial environments by Senko et al. (2007). Although precipitation in bacterial growth experiments is spatially associated with colony growth we attribute this primarily to microbial sulfate production. Nucleation of barite on metabolically-inactive cells did not appear to be a significant factor. Based on results from Bonny and Jones (2008) and Gonzalez-Muñoz et al. (2012) we suggest that in addition to cells acting as favorable mineral nucleation sites, bacterial sulfate production can play a significant role in barite precipitation in brine pool systems with low sulfate concentrations, such as GC246. . Sulfur isotopes approaches will allow us to test this hypothesis in the future.

4.4. Sulfate sources

Previous studies have identified three potential sulfate sources in the precipitation of cold seep barites as suggested by the sulfur isotope compositions of the precipitates.

Suggested sulfate sources are: 1) seawater sulfate; 2) a pool of sulfate enriched in ^{34}S resulting from the anaerobic oxidation of methane and bacterial sulfate reduction; 3) sulfate resulting from the oxidation of the reduced sulfur generated by the anaerobic oxidation of methane and bacterial sulfate reduction (Fig. 13). Feng and Roberts (2011) conclude that the majority of barite collected during their study had $\delta^{34}S$ and $\delta^{18}O$ values higher than those of modern seawater sulfate, suggesting that barite was formed from seawater sulfate that had been isotopically fractionated to varying degrees by biological sulfate reduction. However several barite samples analyzed by Feng and Roberts (2011) show isotopes values close to or slightly below seawater sulfate. We hypothesize these ^{34}S-depleted barites could result from the oxidation of biologically fractionated sulfide that influenced localized barite precipitation through sulfate production.

5. Conclusions

Barite crusts collected from a brine pool in the Gulf of Mexico contain filamentous mineral structures of grossly similar morphology to filamentous sulfur-oxidizing *Beggiatoa* that form extensive mats in the vicinity of the barite crusts. Investigation of the role of sulfide-oxidizing bacteria in the precipitation of GOM barite mineral crusts was performed using molecular analyses to assess the composition of the bacterial community present at the time of barite precipitation followed by laboratory experiments to study the potential mechanisms by which sulfide-oxidizing bacteria may influence barite precipitation. Molecular biology analyses of the DNA preserved in the barite crust targeting the 16S rRNA gene show that sulfide-oxidizing bacteria were abundant in the

environment that precipitated the barite crust, comprising approximately 29% of the community composition. Precipitation experiments show the ability for sulfur-oxidizing bacteria to influence barite precipitation through the production of sulfate via the oxidation of thiosulfate. The barite precipitation experiments showed the entombment of sulfide-oxidizing bacterial cells in a manner reminiscent of the barite-encrusted filaments from the brine pool, suggesting that under high barium, low sulfate conditions, sulfate produced by chemolithotrophic sulfide-oxidation rapidly reacts with barium to precipitate barite in the immediate vicinity of the cell.

Although previous studies have suggested the sulfate source for certain Gulf of Mexico barite crusts stems from the residual sulfate pool fractionated by anaerobic oxidation of methane and microbial sulfur reduction, our experimental results suggest that microbial sulfur-oxidizing could play a role in localized microbial barite precipitation. Further analysis is currently underway to characterize the sulfur and oxygen isotopes associated with the entombed filaments found in the GOM barite crust, as well as additional 16S rRNA Illumian amplicon analyses that will compare microbial communities associated with the barite crust to those of brine pool sediments and brine pool fluids, in order to better characterize the sulfide-oxidizing bacterial community that is specific to the barite precipitates. Ultimately it is the goal of this investigation, combined with ongoing research, to determine the role of bacteria in the precipitation of barite in Gulf of Mexico brine pool settings.

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7. Table Captions

Table 1. Primer sequences used for 16S rRNA bacterial clone library, *Beggiatoa* specific clone library, 16S rRNA Illumina amplicon analysis, and *soxB* analysis. Illumina amplicon V3_7R primer is unique with regards to the highlighted barcode region used to distinguish sample origin from a pooled data set, and the number of 'N' degenerate bases within the sequence.

Primer	Sequence	Reference
EUB 27F	AGAGTTTGATCMTGGCTCAG	
EUB 1492R	GGTTACCTTGTTACGACTT	
EUB primer 341F	CCTACGGGAGGCAGCAG	
VSOXBr	GGATYAATYTCCCCCAACAT	
V3_7R	caagcagaagacggcatacgagatGATCTGgtgactggagttcagacgtgtgctc ttccgatctATTACCGCGGCTGCTGG	Bartram 2011
V3_F modified2	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctNN NNCCTACGGGAGGCAGCAG	Bartram 2011
soxB693F	ATCGGNCARGCNTTYCCNTA	Petri 2001
soxB1164B	AARTTNCCNCGNCGRTA	Petri 2001

8. Figure Captions

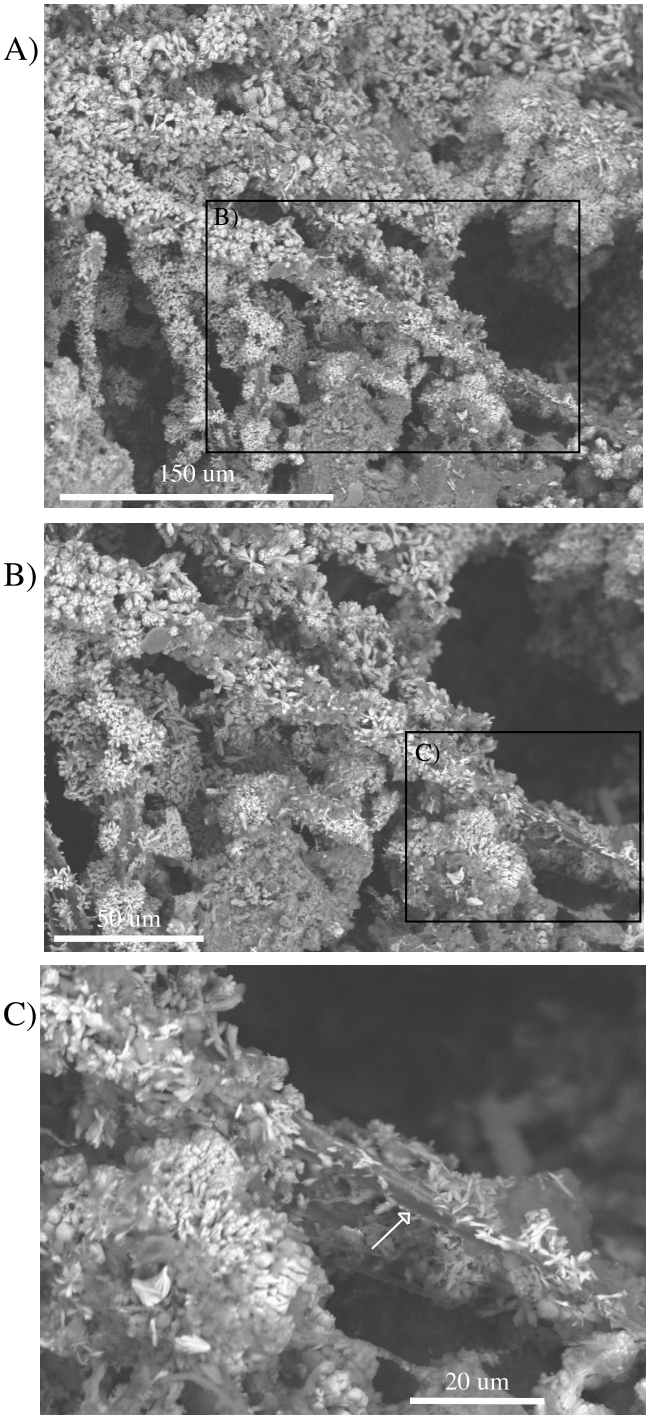

Figure 1. A: Typical barite entombed filament found within the Gulf of Mexico barite crust. B: Magnified portion of crust shows barite entombed filament from A. C: Portion of crust shows barite entombed filament from B. Arrow points to exposed organic filament with barite precipitation crystal development.

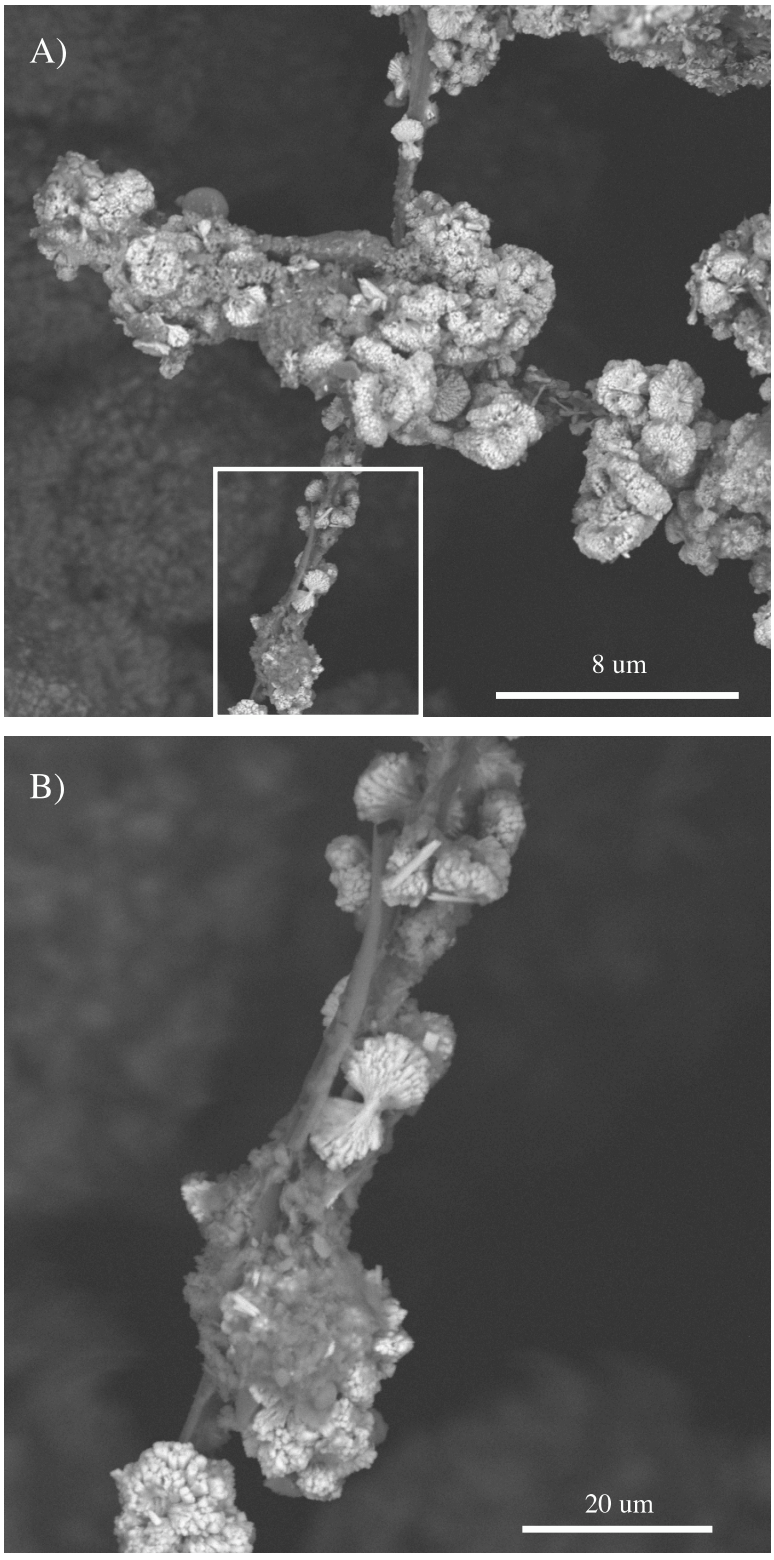

Figure 2. Spatial relationship of barbell barite morphology associated with filaments found in the Gulf of Mexico barite crust, A. B: magnified portion of box in A.

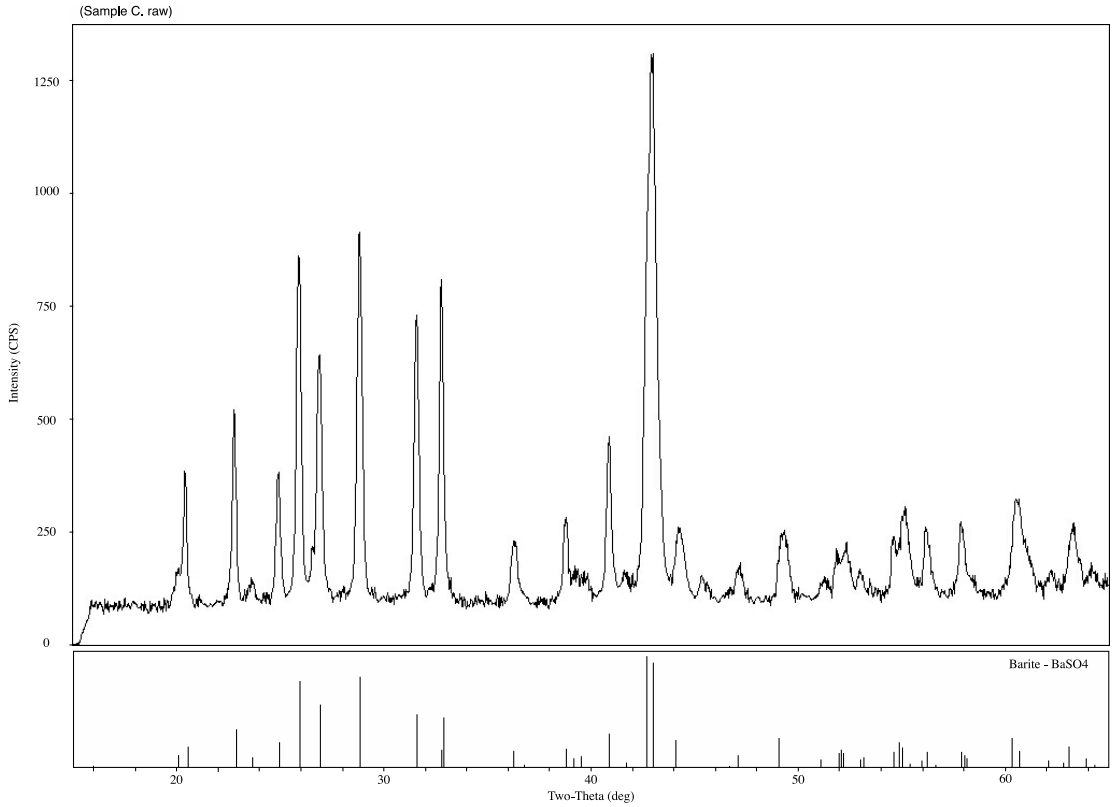

Figure 3. X-ray diffraction diagram of precipitates found in GOM mineral crust. Typical barite (BaSO4) peaks are indicated below GOM spectra. A slight quartz (SiO2) peak is visible at ~ 2θ 26.5º and an unidentified peak is visible at ~ 2θ 45º.

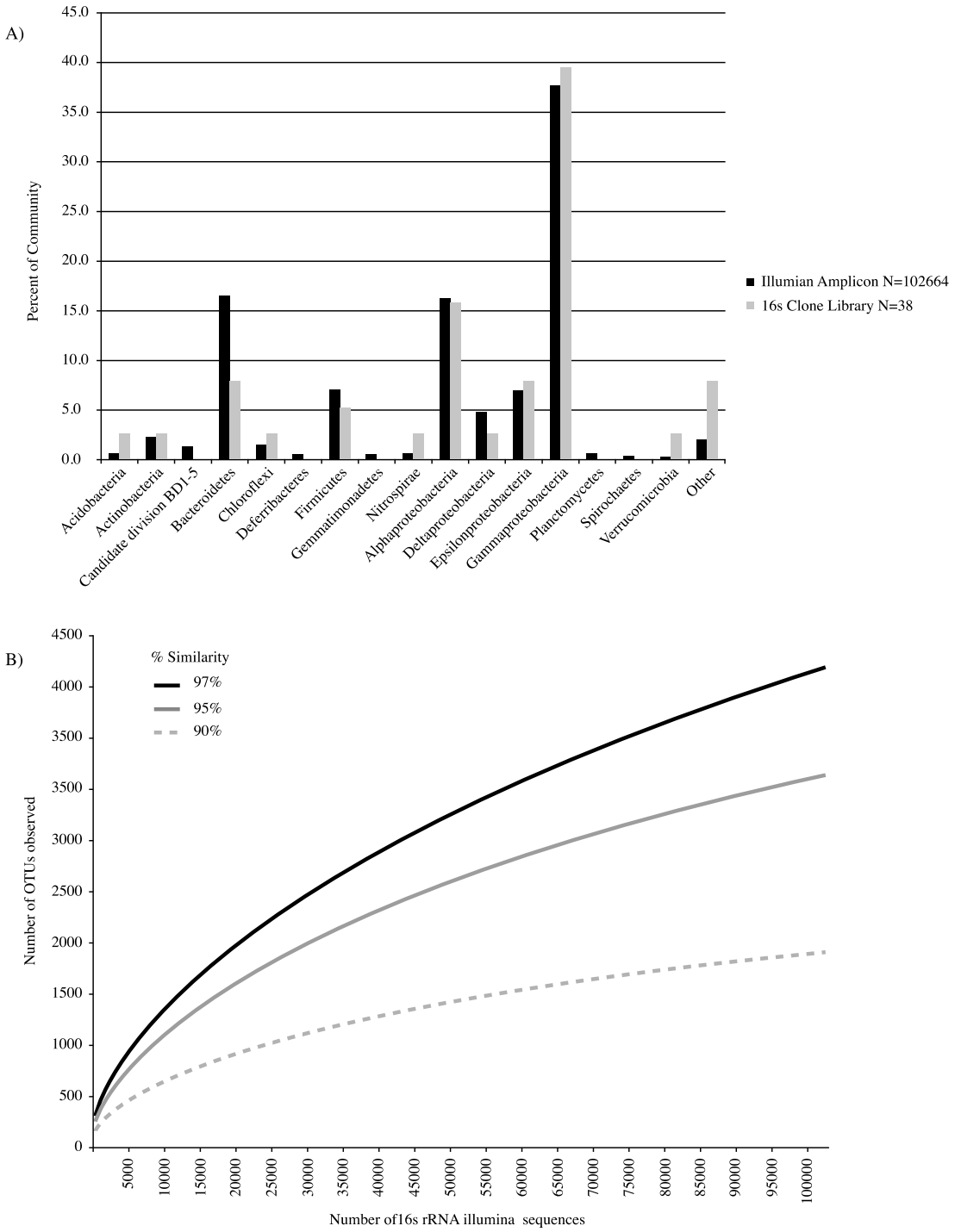

Figure 4. A: Taxonomic composition comparison between general 16S rRNA clone library and 16s rRNA Illumina amplicon library from GOM barite crust. Both libraries were comprised from the same extraction. B: Rarefaction of 16S rRNA Illumina amplicon library from the GOM barite crust. OTUs are defined at 98%, 95%, and 90% sequence identity.

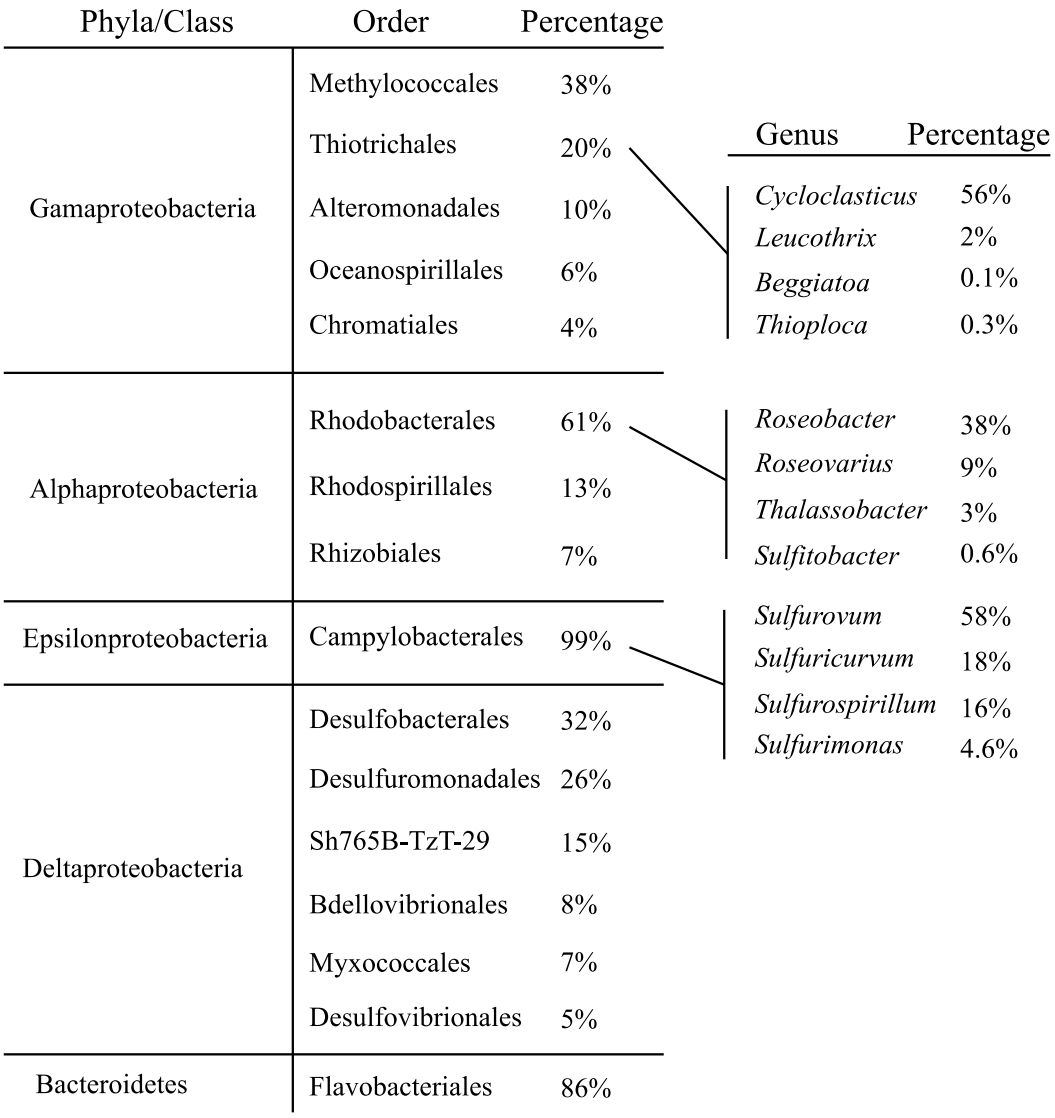

Figure 5. Percent of sequences designated at the rank of order and genus of known sulfur metabolizing bacterias from the 16S rRNA Illumina amplicon analysis. Phlyum/class references are included to clarify lineage of genera; percentages are based on the total number of sample sequences.

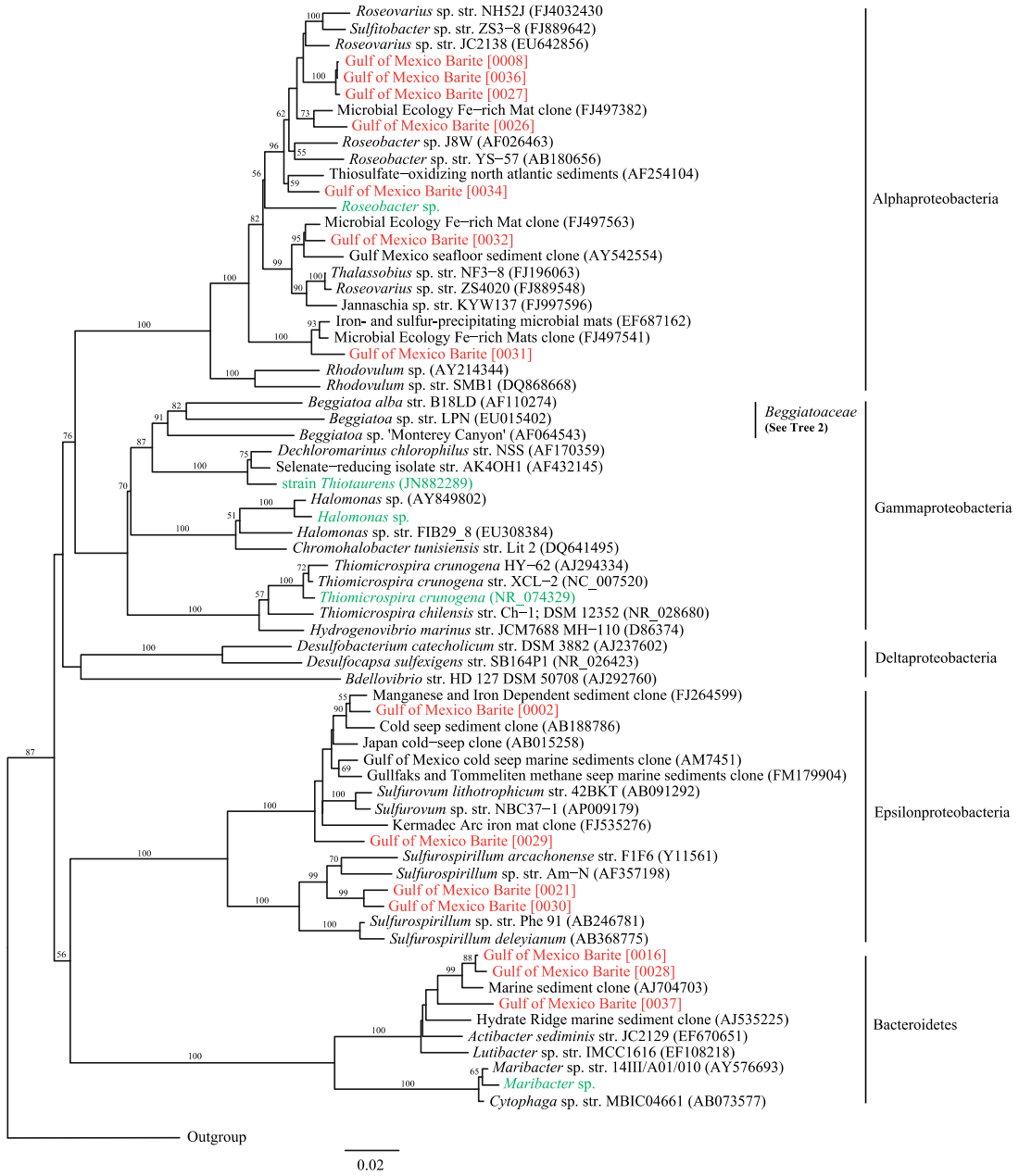

Figure 6. Neighbor-joining phylogram of 16S rRNA gene sequences from the Gulf of Mexico barite crust bacterial clone library (highlighted in red) and five bacteria used in the precipitation experiments (highlighted in green). Sequences from the bacterial clone library were chosen to highlight sulfur metabolisms, specifically sulfur-oxidation. Bootstrap values greater than 50 are shown for each node.

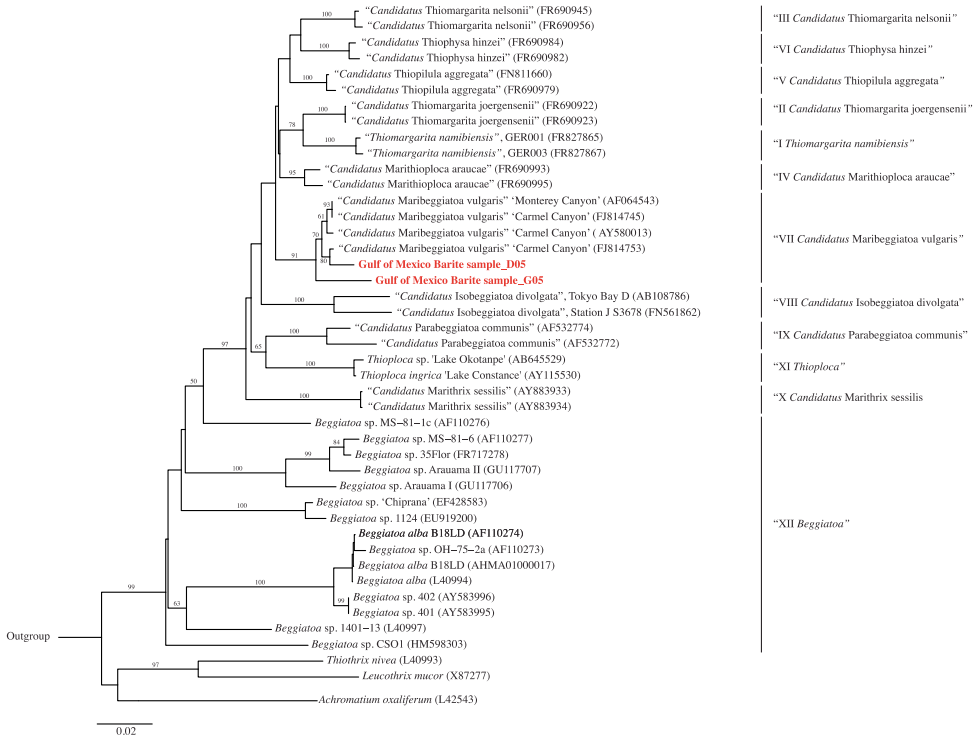

Figure 7. Neighbor-joining phylogram of 16S rRNA gene sequences of Gulf of Mexico Barite crust with *Beggiatoa*-specific primers shown in relation to sequences from other large colorless sulfur bacteria. Bootstrap values greater than 50 are shown for each node. Clade lettering and nomenclature is from Salman et al. (2011). Barite crust sequences from this study are highlighted in red.

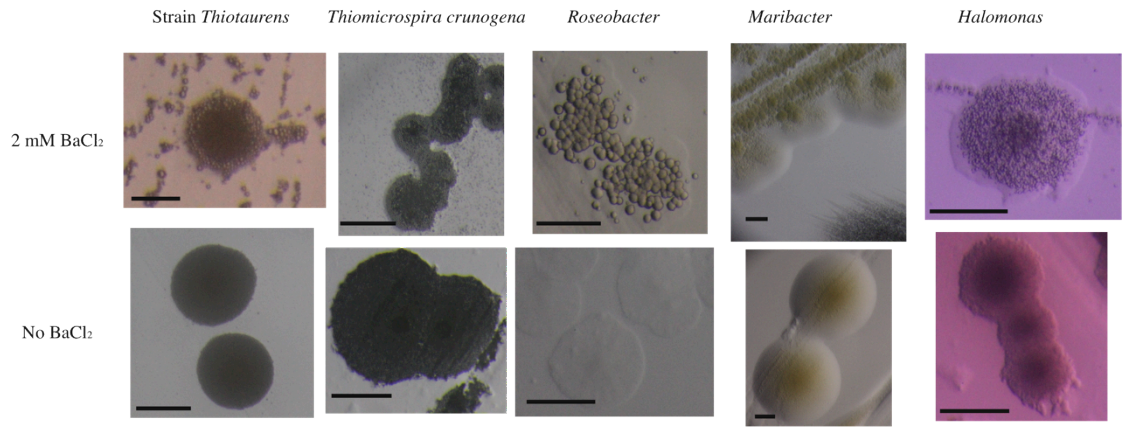

Figure 8. Light microscopy images of experimental precipitation for each of the five selected bacteria. Images show precipitation results of microbial growth on media containing 10 mM thiosulfate ($S_2O_3^{2-}$) with and without 2mM Ba^{2+}. Barite precipitation can be seen associated with colony growth.

Organisim	Sulfur Oxidation?	Chosen For
Maribacter	No	Negative control (organoheterotroph)
Roseobacter	Yes ($H_2S \rightarrow SO_4^{2-}$, no S^o production?)	Sulfate producer
Thiomicrospira crunogena	Yes $H_2S \rightarrow S^0 \rightarrow SO_4^{2-}$	Sulfate producer
strain *Thiotaurens*	Yes $H_2S \rightarrow S^0 \rightarrow SO_4^{2-}$	Sulfate producer
Halomonas	Yes, slowly. (C_{org} requirement?)	Slower sulfate producer

Figure 9. Selected bacterial isolates used during precipitation experiments. Bacteria were specifically selected in order to test the potential role of different sulfur-oxidation pathways in the precipitation of barite.

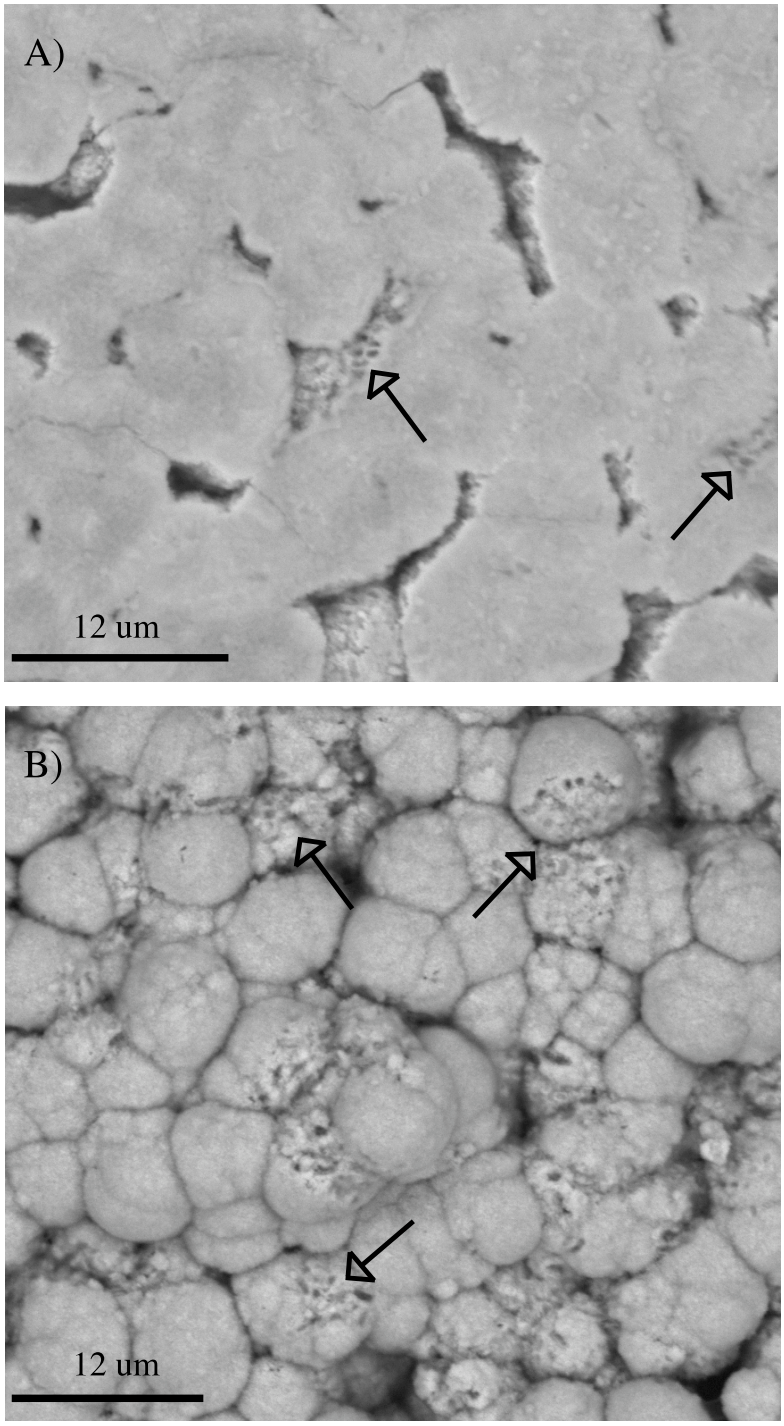

Figure 10. Scanning electron microscopy photographs showing morphologies of barite precipitates. A: Barite precipitates as seen on colony growth (*Halomonas* sp strain BM23). B: Globular barite precipitates. Arrows indicate areas of probable bacterial cells entombed within barite precipitates in both A and B. Other bacterial experiments show similar barite morphologies.

		Percent precipitation
PFA-fixed cells	Sea salt solution pH 7.7	1.2% ± 0.5%
	Sea salt solution pH 4	0.04% ± 0.01%
	10mM Na_2SO_4	10.0% ± 3.0%
No PFA-fixed cells	Sea salt solution pH 7.7	3.0% ± 1.0%
	Sea salt solution pH 4	0.6% ± 0.1%
	10mM Na_2SO_4	8.6% ± 3%

Figure 11. Percent of precipitation calculated from abiotic precipitation experiments with sulfate solutions consisting of sea salt solution at pH 7.7, sea salt solution at pH 4, and 10mM $NaSO_4$ both with and without PFA-fixed cells. Percents quantified by analysis of media plates using ImageJ.

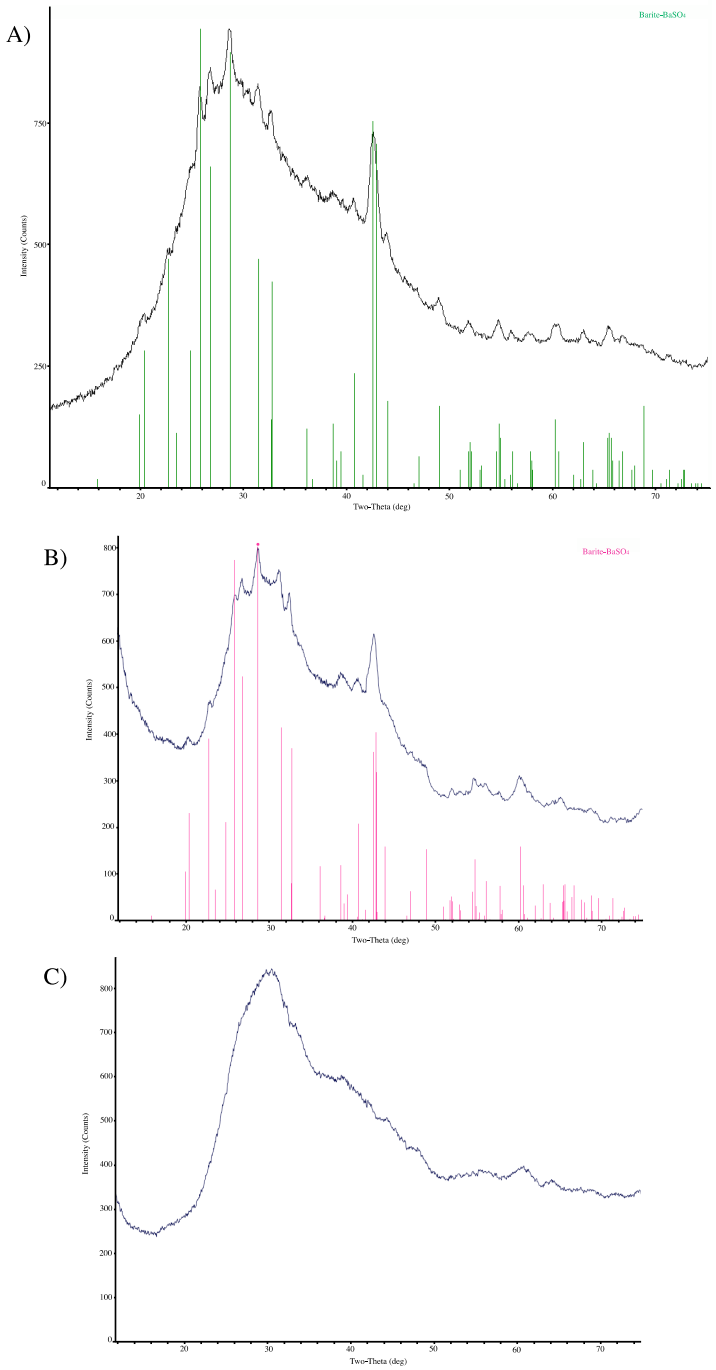

Figure 12. X-ray diffraction diagram of precipitates formed in culture experiments; barite peaks are indicated with green (A) and pink bars (B). A: Analysis of mineral precipitates associated with *Roseobacter* sp. strain LH4. All assayed bacterial experiments produced similar precipitates. B: Analysis of mineral precipitates from abiotic experiments of 2mM Ba^{2+} with 10 mM $S_2O_3^{2-}$ and no PFA-fixed cells. All assayed abiotic experiments produced similar precipitates. C: Control of 2mM Ba^{2+} with 10 mM $S_2O_3^{2-}$, plate shows high intensity region from 2θ 24° - 46°.

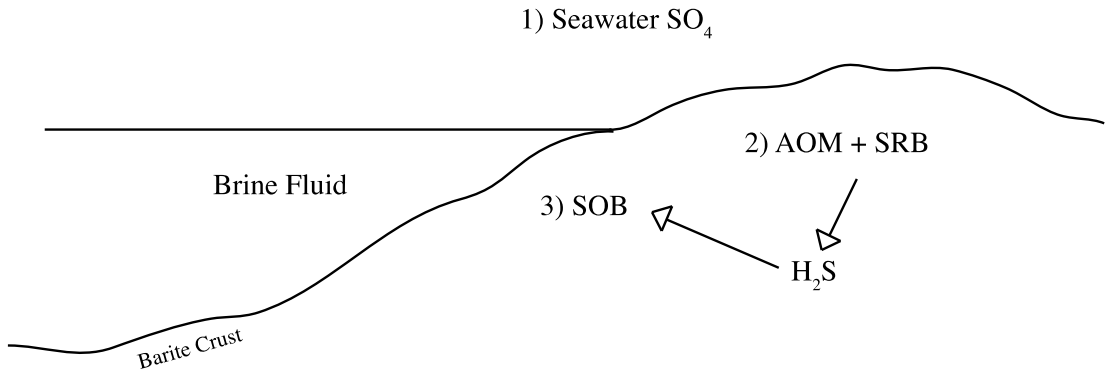

Figure 13. Diagram showing three potential sulfate sources involved in the formation of Gulf of Mexico barite crusts: 1) Seawater sulfate; 2) a sulfate pool with an isotopic composition influenced by bacterial sulfate reduction and the anaerobic oxidation of methane (AOM); and 3) sulfate produced by sulfur-oxidizing bacteria (SOB).