Microbial communities at the borehole observatory on the Costa Rica Rift flank (Ocean Drilling Program Hole 896A)

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INTRODUCTION

The deep-sea subsurface is characterized by relatively low organic carbon input, elevated pressures, geographically variable temperatures, and sparse nutrient availability. Together, these conditions create unique and challenging habitats for microorganisms. Sub-seafloor sediments are estimated to constitute a large proportion of Earth’s biomass (Whitman et al., 1998; Parkes et al., 2000), although local cell densities and microbial activities are low compared to surficial sediments (D’Hondt et al., 2004, 2009; Jørgensen and Boetius, 2007). The microbiology of subsurface, hydrothermally influenced basaltic crust flanking mid-ocean ridges has remained understudied, due to the difficulty in accessing the subsurface environment. The instrumented boreholes resulting from scientific ocean drilling offer access to samples of the formation fluids circulating through oceanic crust. We analyzed the phylogenetic diversity of bacterial communities of fluid and microbial mat samples collected in situ from the observatory at Ocean Drilling Program Hole 896A, drilled into ~6.5 million-year-old basaltic crust on the flank of the Costa Rica Rift in the equatorial Pacific Ocean. Bacterial 16S rRNA gene sequences recovered from borehole fluid and from a microbial mat coating the outer surface of the fluid port revealed both unique and shared phylotypes. The dominant bacterial clones from both samples were related to the autotrophic, sulfur-oxidizing genus Thiomicrospira. Both samples yielded diverse gamma- and alphaproteobacterial phylotypes, as well as members of the Bacteroidetes, Planctomycetes, and Verrucomicrobia. Analysis of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) genes (cbbL and cbbM) from the sampling port mat and from the borehole fluid demonstrated autotrophic carbon assimilation potential for in situ microbial communities; most cbbL genes were related to those of the sulfur-oxidizing genera Thioalkalivibrio and Thiomicrospira, and cbbM genes were affiliated with uncultured phylotypes from hydrothermal vent plumes and marine sediments. Several 16S rRNA gene phylotypes from the 896A observatory grouped with phylotypes recovered from seawater-exposed basalts and sulfide deposits at inactive hydrothermal vents, but there is little overlap with hydrothermally influenced basaltic boreholes 1026B and U1301A on the Juan de Fuca Ridge flank, suggesting that site-specific characteristics of Hole 896A (i.e., seawater mixing into borehole fluids) affect the microbial community composition.

Keywords: basalt, chemolithoautotrophic bacteria, CORKs, Costa Rica rift, formation fluids, ocean drilling program, subsurface, thiomicrospira

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The microbiology of subsurface, hydrothermally influenced basaltic crust flanking mid-ocean ridges has remained understudied, due to the difficulty in accessing the subsurface environment. The instrumented boreholes resulting from scientific ocean drilling offer access to samples of the formation fluids circulating through oceanic crust. We analyzed the phylogenetic diversity of bacterial communities of fluid and microbial mat samples collected in situ from the observatory at Ocean Drilling Program Hole 896A, drilled into ~6.5 million-year-old basaltic crust on the flank of the Costa Rica Rift in the equatorial Pacific Ocean.
Analysis of borehole fluids and mineral crusts from these sites (Orcutt et al., 2011). Investigations of other subsurface crusts, it nonetheless harbors a unique subsurface crustal biosphere with mineralogical and geochemical characteristics with the other habitats are necessary to evaluate this hypothesis. Such borehole observatories, known as Circulation Obviation Retrofit Kits or “CORKs” (Davis et al., 1992; Becker and Davis, 2005), have been used successfully in the past to study hydrogeologic properties of crustal aquifers (Fisher et al., 2003) and to examine the biogeochemical composition of fluids circulating within ocean crust (Wheat et al., 2010).

More recently, the composition of microbial communities inhabiting deep basaltic crust has been investigated through the collection of observatory fluids (Cowen et al., 2003), mineral crusts formed on the surface on observatories (Nakagawa et al., 2006), and mineral chip colonization experiments conducted within the observatories (Orcutt et al., 2011). Those experiments have focused on a series of CORK observatories, mostly at ODP Hole 1026B and IODP Hole U1301A (Fisher et al., 2003), installed on the eastern flank of the Juan de Fuca Ridge in the northeastern Pacific Ocean. Here, reduced, anoxic, sulfate-rich hydrothermal (~64°C) fluids flow within 3.5 million-year-old basaltic crust. Analysis of borehole fluids and mineral crusts from these sites revealed diverse microbial communities containing novel Firmicutes bacteria, some of which are distantly related to *Ammonifex degensii*, a chemolithoautotrophic, thermophilic bacterium that oxidizes hydrogen, formate or pyruvate with nitrate, sulfate or elemental sulfur (Cowen et al., 2003; Nakagawa et al., 2006; Orcutt et al., 2011). Furthermore, enrichment experiments conducted on mineral crusts from the Hole 1026B observatory yielded anaerobic thermophiles, including methanogens, fermenters, and sulfate reducers (Nakagawa et al., 2006; Steinbu et al., 2010). Archaean communities in these observatories appear to be less diverse and align more closely with cultivated members of thermophilic, hydrogenotrophic methanogens of the genus *Methanobacterium*. Such communities have been observed in many environments, such as hydrothermal vents, geothermal areas, and deep-sea hydrothermal systems.

In this study, samples were collected from an observatory placed at Hole 896A in 2001 and revisited in 2002 (Becker et al., 2004) to conduct comparative phylogenetic analysis for evaluating the in situ microbial communities.

**MATERIALS AND METHODS**

**STUDY SITE**

ODP Hole 896A on the Costa Rica Rift (1°15′ N, 83°45′ W; Figure 1) is 3,463 m below sea level and drilled to a depth of 469 mbsf (Figure 1). The lower 290 m of the hole consists of altered basaltic oceanic crust. During drilling in 1993, the upper 196 m section of Hole 896A was cased, thus sealing out sediment pore water and allowing the influx and accumulation of hydrothermal subsurface fluids from the basaltic crust. In 2001, roughly 8 years after the borehole was originally drilled, a wireline packer seal apparatus similar to a CORK observatory was deployed with the intent to plug the hole, record the pressure and temperature in the sealed zones, sample borehole formation fluids, and monitor the return to in situ hydrogeological conditions (Becker et al., 2004). The wireline CORK apparatus was constructed primarily from mild steel, and it included one packer in the cased section and a second packer intended to be set about 50 m into the open-hole section, as well as steel tubing umbilicals to bring formation fluids to sampling ports at the wellhead. However, on deployment, the lower packer became stuck in the hole about 20 m above the intended setting depth. In the attempt to deal with this, it is likely that the tubing umbilicals were damaged and the packers could not be inflated to seal the hole.
**Table 1** | Comparative chemistry of fluids from Costa Rica Ridge flank Holes 896A and 504B with Juan de Fuca Ridge flank fluids, with seawater for comparison.

| Component | Units | Hole 896A | Hole 504B | Hole U1301A | Baby Bare | Seawater |
|-----------|-------|-----------|-----------|--------------|-----------|----------|
| Temperature | °C | -60 | 58 | 64 | 64 | 2 |
| Cl\(^{-}\) | mmol/kg | 547 | 546 | 553 | 554 | 541 |
| SO\(_4^{2-}\) | mmol/kg | 18.5 | 17 | 17.6 | 17.8 | 279 |
| Alkalinity | meq/kg | 0.6 | 0.1 | 0.4 | 0.43 | 2.44 |
| Na\(^{+}\) | mmol/kg | 460 | 456 | 463 | 473 | 463.5 |
| K\(^{+}\) | mmol/kg | 6.8 | 7 | 6.9 | 6.88 | 10.1 |
| Ca\(^{2+}\) | mmol/kg | 50.5 | 58 | 55.8 | 55.2 | 10.2 |
| Mg\(^{2+}\) | mmol/kg | 8.5 | 8 | 1.9 | 0.98 | 52.6 |

a) Data extrapolated from basal sediment pore water samples collected from ODP Holes 501, 504, 677, and 678 (Holes 678 became Hole 896A; Mottl, 1989).
b) Data from Hole 504B borehole fluids collected in situ 1,233 days after drilling (Mottl and Gieskes, 1990; Wheat and Mottl, 2000).
c) Near steady-state formation fluid from Hole U1301A CORK OsmoSamplers deployed in basement borehole for ~4 years (Wheat et al., 2010).
d) Fluids sampled from the Baby Bare basalt outcrop of the Juan de Fuca Ridge flank (Wheat and Mottl, 2000).
e) Bottom seawater from the Costa Rica Ridge flank (Mottl, 1989).

**SAMPLE COLLECTION**

Roughly 15 months after the installation of the observatory, fluid and microbial mat samples were collected on November 18, 2002 during submersible operations with DSV Alvin (Dive 3840; Woods Hole Oceanographic Institution). Because the wireline CORK packers had not been inflated, it is likely that the uphole flow of upper basement fluids revealed by the 2001 temperature log was continuing up around the wellhead. The microbial mat sample, referred to as "mat," consisted of a flocculent grey crust growing on the seawater exposed exterior of Port L that was swabbed with a fresh green plastic sponge, which was exposed to seawater and therefore not sterile (Figure 2A). The sponge was stored on the submersible in a closed plastic biobox filled with site bottom water before return to the ship. The fluid sample, referred to as "bore," consisted of fluids collected at a wellhead sampling port about 10 min after the valve (Port L; Figure 2A) was opened, into an ethanol-sterilized titanium bottle using a flexible sampling hose (Figure 2B). The sampling port was connected to the tubing leading from just below the lower packer. However, because of the possibility of damage to the umbilical and lack of packer seal, it is likely that a mix of bottom water and true formation fluids was being sampled. No warm water venting and efflux of borehole water was observed at Port L. After return to the ship, approximately 0.75 l of borehole fluid was filtered, using a handpump, through a 90-mm diameter 0.22 μm mesh nylon filter to collect particulate material. Both samples were returned to the ship (RV Atlantis, Woods Hole Oceanographic Institution) within a few hours and immediately frozen at −80°C for preservation of DNA. Samples were then transported frozen to a shore-based laboratory.

**DNA EXTRACTION, AMPLIFICATION, AND SEQUENCING**

Environmental DNA was extracted from samples following a protocol modified from two published procedures for extracting DNA from deep subsurface sediments (Juniper et al., 2001; Kormas...
Near full-length 16S rRNA genes were amplified by polymerase chain reaction (PCR) using bacterial primers BAC-8F (5′-AGRRTTTCCTACGTGCTAG-3′) and BAC-1492R (5′-CGGCTACCTTGGTATCACT-3′; Lane, 1991). The PCR mixture for the phenol/chloroform/isoamyl alcohol extracted samples consisted of 1 μl of the environmental DNA, 2 μl of each primer (0.5 mM), 0.5 μl of enzyme included in the Failsafe™ PCR System kit (EpiCentre Biotechnologies), 25 μl of Failsafe™ Premix B and a balance of water for a total reaction volume of 50 μl. The PCR cycle conditions involved an initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 3 min. These 30 cycles were followed by a final extension at 72°C for 10 min. Samples extracted with the Mobio Powersoil DNA Isolation Kit were amplified with Speedstar HS DNA Polymerase (Takara) using 2 μl of DNA sample and the manufacturer’s recommendations concentrations of buffer, dNTPs, and polymerase in a final volume of 25 μl. Thermal cycling was performed as previously described above with the exception that cycling times were 95°C for 10 s, 52°C for 15 s, and 72°C for 20 s over 28 cycles.

To examine the potential for autotrophy using the Calvin cycle, cbbL and cbbM genes involved in the formation of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) from the mat sample environmental DNA were PCR amplified as described previously (Elsworth and Nagamuna, 2001). An initial round of PCR, cloning and sequencing yielded only one cbbL clone and three cbbM clones (mat clones A06, E03, H09, and F03). Additional DNA was amplified from the Powersoil Isolation Kit extractions with the GoTaq Flexi DNA Polymerase (Promega) using a final concentration of 3 mM MgCl₂, 1 μl of each primer, and the manufacturer’s recommendation of dNTP, buffer, and polymerase concentration. Primers for the cbbL gene included cbbL5F172 (5′-ACNTGGACNACNGTNTGG-3′) and cbbL1AR1382 (5′-TCR AARCTGATTTCTGTCTC-3′). Primers for the cbbM gene included cbbM337F (5′-AACCARGY A TGGGYGA Y-3′) and cbbM1126R (5′-TATCCVRCCVCGADAT-3′). In the bore sample, non-specific priming was extensive in one cbbL PCR sample and an additional primer, cbbL1AR1142 (5′-CCGCKTTDGCACANCR GRAT-3′), was used with primer cbbL5F172 in a nested PCR using 0.2 μl of the bore PCR sample and the same mixture and cycling conditions described above, with the exception that the number of cycles was reduced to 20. We note that the cbbL primers did not fully target the total diversity of the “red-like” IC form of Rubisco (Badger and Bel, 2008) due to the need to limit the number of degeneracies, some diversity may have been missed.

The PCR products were subjected to 1.5% agarose gel electrophoresis, stained with 0.5 μg/ml of ethidium bromide or 1× GelRed (Biotium), and visualized by UV excitation for bands indicating successful DNA amplification. PCR products were either excised from the agarose gel or directly purified using the Wizard® PCR preparation kit (Promega) or the Quagen-MineSelect Gel Extraction kit, and then cloned using TOPO XL PCR cloning kit (Invitrogen). From each clone library, clones were selected randomly for sequencing at the Josephine Bay Field Center of the Marine Biological Laboratory in Woods Hole, MA, USA or Genewiz (Research Triangle Park, NC, USA).
Results

16S rRNA gene phylogeny

A total of 66 and 60 nearly full-length 16S rRNA gene clones were successfully sequenced from the bore and mat sample clone libraries, respectively. Phylogenetic analysis of the clone libraries revealed 41 phylotypes from several bacterial phylum-level groups including Bacteroidetes, aerobic and anaerobic heterotrophs, widespread in soil and water (Kirchman, 2002), Cyanobacteria, Actinobacteria (OM1 group; Rappé et al., 1997), oxygenic marine phototrophs, the phylum-level lineages Versicococcus and Planctomycetes, often detected in oxygen-depleted marine habitats (Kirkpatrick et al., 2006) and with relatively few cultured chemosynthetic/archaeans isolates (Wagner and Horn, 2006); and diverse Alphaproteobacteria, Gammaproteobacteria and Planctomycetes (Figures 3 and 4). The seven most abundant phylotypes (highlighted in purple in Figures 3 and 4) were found in both the borehole fluids and the microbial mat.

Despite using bacteria-specific 16S rRNA gene primers, one archaeal clone was also recovered from the mat sample. This clone clone, mat1, was related to Thermodesulfobacterium moelkeri, an extremely thermophilic crenarchaeate isolated from acidic hot spring areas in Japan (Itoh et al., 1998). The presence of an archaeal clone in a bacterial 16S rRNA gene clone library was unexpected, given that the bacterial and archaeal versions of the forward primers had five nucleotide mismatches and thus strong PCR bias against archaeal gene amplification.

A number of sequences grouped near known sulfur cycling microorganisms. Phytophore mcz2 was related to the free-living, autotrophic, oxygen- and nitrate-respiring, sulfur-oxidizing genus Thiosphaera (the Gammaproteobacteria; this phylotype comprised nearly half of the sequences of both the bore and mat clone libraries (Figure 3). This phylotype has also been observed in clone libraries from seawater-exposed massive sulfides (Sylvan et al., 2012) and in fluids sampled from the ODP Hole 1026B observatory on the Juan de Fuca Ridge flank (Huber et al., 2006). One bore phylotype affiliated with the sulfur-oxidizing chemolithotrophic Epsilonproteobacteria Sulfurimonas denitrificans and S. autotrophica. Generally, hydrogen- and sulfur-oxidizing, chemolithoautotrophic Epsilonproteobacteria have a wide environmental distribution at hydrothermal vents and in marine surficial sediments (Campbell et al., 2006; Nakagawa and Takai, 2008) and employ an alternative pathway of autotrophic CO2 fixation, the reverse tricarboxylic acid (TCA) cycle (Hügler et al., 2005). The whitish mats deposited on the walls of the observatory and recorded in video logs of the Hole 896A borehole (Becker et al., 2004) bear a conspicuous resemblance to the sulfur precipitates and flocs produced by sulfur-oxidizing bacteria (Kuenen and Velkamp, 1972; Taylor and Wirsen, 1997; Wirsen et al., 2002).

Several other bore and mat sequences also grouped most closely with uncultivated environmental sequences recovered from seawater-exposed basalts and inactive sulfides from the East Pacific Rise and Hawaii (Santelli et al., 2009; Sylvan et al., 2012), from hydrothermal fluids from a basaltic outcrop (Huber et al., 2006) and from cold seep and hydrothermally influenced deep marine sediments. These phylotypes are highlighted with colored circles in Figures 3 and 4. Nine of the forty observed bacterial
phyotypes, marked with black circles, grouped closely with phylotypes from inactive massive sulfide samples collected from the East Pacific Rise (Sylvan et al., 2012), whereas eight phylotypes, marked with green circles, were shared with clone libraries from seafloor-exposed basalts (Santelli et al., 2009). Three phylotypes, marked with white and yellow circles, were similar to clones from samples from other deep basalt observatories. Of these, one alphaproteobacterial sequence (Figure 4) grouped closely with a sequence from a biofilm formed on pyrite incubated in the subsurface for 4 years (Ovask et al., 2013); the Thiomicrospira-related clone mix 2 and Prochlororaphidophyte clone mat 3 are close to clones from venting basalts at Baby Bare on the Juan de Fuca ridge (Huber et al., 2006). Two phylotypes were most closely related (99% identity) to sequences from the marine cyanobacterial genus Prochlorococcus, which is found at varying depths in the water column in oceans worldwide (West et al., 2001). The presence of these phylotypes is unexpected in samples of oceanic basement formation fluids, and indicates seawater entrainment and contamination during sampling of the hole and mat samples at the observatory platform, or seawater entrainment into the mixed borehole fluids itself. Moreover, the identification of shared phylotypes is robust, shared diversity estimates between the sample sets were tested, but they were skewed due to the difference in clone library sizes between the Hole 896A samples and the comparison studies, and the possibility of seawater entrainment in the Hole 896A samples.

Several sequences grouped most closely to environmental sequences commonly found in seawater (Figures 3 and 4). For example, the bore18 phylotype grouped near Palagibacter ubique within the SAR 11 cluster, a cosmopolitan clade of marine oligotrophic bacteria (Rappe et al., 2002). Several alphaproteobacterial phylotypes from each sample grouped within the Rhodobacteraceae or marine Roseobacter group, a cosmopolitan group of marine bacteria that often metabolize and oxidize organosulfur compounds (Buchan et al., 2005; Brinkhoff et al., 2008). Two phylotypes were most closely related (99% identity) to sequences from the marine cyanobacterial genus Prochlorococcus, which is found at varying depths in the water column in oceans worldwide (West et al., 2001). The presence of these phylotypes is unexpected in samples of oceanic basement formation fluids, and indicates seawater entrainment and contamination during sampling of the bore and mat samples at the observatory platform, or seawater entrainment into the mixed borehole fluids itself. Moreover, the identification of shared phylotypes is robust, shared diversity estimates between the sample sets were tested, but they were skewed due to the difference in clone library sizes between the Hole 896A samples and the comparison studies, and the possibility of seawater entrainment in the Hole 896A samples.
occasional close phylogenetic association of some phylotypes with known aerobic chemooorganotrophic isolates, such as Flaviramus basaltis and Mariovoga tractuosus (Figure 3), suggests chronic oxic seawater mingling with basement fluids at the Hole 896A observatory.

**RuBisCO PHYLOGENY**

Amplification of the *cbbM* and *cbbL* genes of RuBisCO yielded sequences of sufficient length and quality for phylogenetic analysis from the mat sample and from the borehole fluid sample. BLAST search based on amino-acid sequence, and subsequent phylogenetic analysis (Figure 5) revealed that most *cbbL* sequences (145 clones) as well as the borehole sample (16 clones) were most closely related to the obligately chemolithoautotrophic, sulfur-oxidizing gammaproteobacterial genera Thioalkalivibrio, isolated from Siberian and East African soda lakes (Boroskin et al., 2001; Thioalkalivibrio thio-cyanoxidans, GenBank accession ZP_08930733, 93% identity), to sequences from hypersaline soda lake sediment in Kulunda Steppe (Russia; Kolosiva et al., 2011; GenBank AIN96357, 97% identity), and to the facultatively phototrophic sulfur oxidizer *Thio-

capsa* (Guyonaud et al., 1998), capable of chemolithoautotrophic growth with reduced sulfur compounds under microoxic conditions (Caumette, 1986). Other clones (mix clone ODP 4_1_9; five bore and one mat clone) were most closely related to Thiomicrospira (*Thiomicrospira crunogena*, GenBank YP_391108, 94% identity) isolated from marine sediments and hydrothermal vents (Jannasch et al., 1985; Scott et al., 2006) and the obligately autotrophic hydrogen oxidizer *Hydrogenovibrio marinus*, phylogenetically a lineage within the genus *Thiomicrospira* (GenBank BAD15312, 93% identity; Nishihara et al., 1998). Two other borehole sequences were most closely related to those from a pogonophoran bacterial endosymbiont from a cold methane seep in the Japan Trench (Nagamuna et al., 2007).

Only five *cbbM* sequences from the mat and bore sample were obtained in total despite attempts to amplify the gene with published (Elhaied and Nagamuna, 2001) and newly designed primers (this study). The *cbbM* sequences recovered from the Hole 896A samples were related to *cbbM* sequences of uncultivated bacteria from the hydrothermally active Suiyo Seamount, a submarine black smoker volcano in the Izu-Bonin trench off Japan (Clone Suiyo Suiyo II-5, nucleotide accession number AB174731, protein ID BAI13340; Elhaied et al., 2007) and to *cbbM* sequences obtained from reducing sediments near the deepest known chemosynthetic microbial community, in deep-sea sediments of the Japan Trench at 7,434 m depth (Clone JT-Sed(II)-5, nucleotide accession number AB040517, protein ID BAD94441; Elhaied and Nagamuna, 2001; Figure 5).

**DISCUSSION**

The phylogenetic data presented here from Hole 896A on the Costa Rica Rift flank represents the second dataset from a basaltic crust borehole observatory, providing the first comparison to the available data from the Holes 1026B and U1301A CORK observatories.
Veldkamp, 1972). The known to prevail in Holes 1026B and U1301A (Wheat et al., 2010). Fluids (despite the similarity in major ion concentrations of the formation ences as well, may be different redox regimes within the boreholes, of the microbial communities, with suspected metabolic differ-
culent crusts in Hole 896A (Becker et al., 2004) suggests microoxic produce extracellular sulfur in large amounts (Taylor and Wirsen, 1997). The formation of extensive white flocs is also known from biore-
ment fluid chemistry, and temperature (Table 1). Holes 1026B and U1301A on the Juan de Fuca ridge flank reveal surface microbial communities characterized by an abundance of Firmicutes bacteria (Cowen et al., 2003; Nakagawa et al., 2006; Orcutt et al., 2011). The predominant bacterial phylotypes in the Hole 1026B clone libraries were distantly related to thermophilic, nitrate-, and sulfate-reducing bacteria, such as the hydrogen-oxidizing nitrate-reducing ammonia producer Ammonifex denitrius, and the gram-positive, spore-forming sulfate reducing genus Desulfotomaculum. In contrast, the Hole 896A observational libraries were dominated by sequences grouping with Gammaproteobacteria related to the chemolithoautotrophic, sulfur-oxidizing genus Thiomicrospira that is predominantly iso-
ated from sulfidic marine sediments and hydrothermal vents, and hydrothermal plumes; these bacteria were not detected in other borehole surveys (Cowen et al., 2003; Nakagawa et al., 2006; Orcutt et al., 2011).

One explanation for these differences in the dominant members of the microbial communities, with suspected metabolic differences as well, may be different redox regimes within the boreholes, despite the similarity in major ion concentrations of the formation fluids (Table 1). Namely, the extensive accumulation of white flocculent crusts in Holes 896A (Becker et al., 2004) suggests microoxic or nitrate-reducing conditions, while an anaerobic environment is known to prevail in Holes 1026B and U1301A (Wheat et al., 2010). The formation of extensive white flocs is also known from bioreactor experiments, where microaerobic sulfur-oxidizing bacteria produce extracellular sulfur in large amounts (Taylor and Wirsen, 1997); sulfur precipitation is also a characteristic by-product of aerobic, sulfur-oxidizing Thiomicrospira spp. growing in laboratory culture (for an instructive example, see Figure 2 in Kuenen and Veldkamp, 1973). The in situ observation of the flocculent mat-like material within the Hole 896A borehole (Becker et al., 2004) indicates in situ production of biomass and flocculent mats by sulfur-oxidizing bacteria within the borehole. Seawater influence at the Hole 896A CORK observatory is consistent with this interpretation. If seawater were entrained or mixed with the borehole fluids, which are presumably rich in reduced substrates such as sulfur and iron, this might create an ideal niche for the enrichment of the sulfide-oxidizing and biofilm-forming phylotypes observed. The sulfur-oxidizing bacteria that dominate the borehole could eventually be derived from bottom water mixed with highly dilute hydrothermal plumes and microbial populations (Huber et al., 2006, 2007). The observation of seawater-related phylotypes such as SAR11, Roseobacter, Prochlorothrix, and OM1 Actinobacteria in the bore hole sample clone libraries also supports the argument for seawater entrainment. In consequence, the in situ enrichment of bacteria growing under likely conditions of seawater entrainment in the borehole and within the CORK distorts the assessment of potential indigenous microbial diversity in basaltic basement fluids (Cowen, 2004).

**Comparison of Hole 896A microbial communities to those from other habitats**

Based on 16S rRNA gene clone libraries, the microbial communities observed in the Hole 896A samples (Figures 3 and 4) bore little resemblance to the communities described in formation fluids and mineral crusts from Holes 1026B and U1301A, despite the previously reported similarities in mineralogy, base-

**Autotrophic potential**

Based on theoretical models of the energy available from sulfur and iron oxidation in basaltic crust, significant levels of primary production should occur in the subsurface (Bach and Edwards, 2003), a prediction testable by RubisCO genes analysis. RubisCO catalyzes the assimilation of carbon dioxide to organic carbon via the Calvin–Benson–Bassham cycle. Of its currently four known forms, form I is oxygen tolerant and found predominantly in cyanobacteria, chloroplasts, and aerobic chemolithoautotrophic bacteria, while form II is adapted to high CO2 conditions and found predominantly in microaerobic or anaerobic bacteria (Deliwiche and Palmer, 1996; Badger and Bek, 2008). We observed both forms of RubisCO in the Hole 896A mat and borehole samples. (Figure 5). RubisCO sequences were phylogenetically related to *Thiomicrospira*, the closest cultured relative of the most frequently recovered 16S rRNA sequences at the 896A CORK. The form I RubisCO sequences obtained from the bore hole and from the mat sample were most closely related to the sulfur-oxidizing chemolithoautotrophic genera *Thiobacillus* and *Thioploca*, and *Thiomicrospira*, within the form I LA “green-type” clade that is associated with proteobacteria and cyanobacteria (Badger and Bek, 2008). This pattern is consistent with the 16S rRNA sequencing results, and with the interpretation that the borehole fluid and the mat sample contain autotrophic, sulfur-oxidizing bacteria related to these gammaproteobacterial genera. The presence of additional autotrophic bacteria (or of bacteria that contain form II in addi-
tion to one of the form I sequences found here) is indicated by the form II sequences in the mat; these sequences were related to RubisCO of uncultured marine bacteria, not from the open water column but from methane seep sediments and hydrothermal plumes (Figure 5).

As a note of caution, RubisCO has a high rate of horizontal gene transfer events (Deliwiche and Palmer, 1996). The gammapro-
tebacterial form I types are also found in cyanobacteria, for example, the common marine cyanobacterium *Prochlorococcus* which most likely acquired its RubisCO genes by horizontal gene transfer (Hess et al., 2001). Thus, the RubisCO sequence do not rule out seawater contamination, as indicated by the two *Prochlorococcus* 16S rRNA gene sequences found in the borehole.
fluid (Figure 3). A certain degree of seawater contamination is obvious, but it does not invalidate the abundance of phyto- lysts most closely related to sulfur-oxidizing bacteria (Thiomicrospira, Sulfitoloma) and to basalt-associated bacterial phyotypes. With this caveat, the 16S rRNA gene and RuBisCO sequence data are most closely related to sulfur-oxidizing bacteria (Becker et al., 2004). This interpretation is fully consistent with in situ borehole observations of microbial mat growth within the borehole (Becker et al., 2004).

RECOMMENDATIONS FOR FUTURE OBSERVATORY STUDIES

The Hole 896A observatory was installed for primarily geophysical experiments (Becker et al., 2005) and was not designed for high-quality sampling for microbiological analysis of the subsurface crustal biosphere. Nevertheless, samples were collected opportunistically and analyzed despite potential contamination pitfalls, as they represented a unique chance to evaluate the crustal biosphere. Our analysis indicates that fluids from the crustal subsurface mixed with seawater can support microbial communities that appear to form biofilms, and that some of these biofilm-forming species are related to known sulfide oxidizing microbial groups like Thiomicrospira. Given the stark differences in the microbial communities observed between subsurface observatories placed in similar crustal settings (i.e., between the Hole 896A observatory and the Juan de Fuca Ridge Flank Holes 1026B and U1301A), enhanced sample characterization may shed light on the underlying environmental conditions that could explain these differences. For example, analysis of borehole fluid oxygen, nitrate and redox conditions may help resolve whether the presence of these electron acceptors may have influenced the Hole 896A community. A time series analysis of the Hole 896A observatory would provide background information on whether the observed community was representative of “steady state” formation fluids or if it instead represented a transitory evolution of the community following post-observatory installation, as has been observed elsewhere for microbial communities in other boreholes (Curcuit et al., 2011). Most importantly, improvements in the Hole 896A observatory infrastructure to allow cleaner microbiological sampling would reduce the confounding influence of seawater contamination.

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