

ORIGINAL ARTICLE

Prokaryotic diversity, distribution, and insights into their role in biogeochemical cycling in marine basalts

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We used molecular techniques to analyze basalts of varying ages that were collected from the East Pacific Rise, 9° N, from the rift axis of the Juan de Fuca Ridge and from neighboring seamounts. Cluster analysis of 16S rDNA terminal restriction fragment polymorphism data revealed that basalt endoliths are distinct from seawater and that communities clustered, to some degree, based on the age of the host rock. This age-based clustering suggests that alteration processes may affect community structure. Cloning and sequencing of bacterial and archaeal 16S rRNA genes revealed 12 different phyla and subphyla associated with basalts. These include the Gemmatimonadetes, Nitrospirae, the candidate phylum SBR1093 in the bacteria, and in the Archaea Marine Benthic Group B, none of which have been previously reported in basalts. We delineated novel ocean crust clades in the γ -Proteobacteria, Planctomycetes and Actinobacteria that are composed entirely of basalt-associated microflora, and may represent basalt ecotypes. Finally, microarray analysis of functional genes in basalt revealed that genes coding for previously unreported processes such as carbon fixation, methane oxidation, methanogenesis and nitrogen fixation are present, suggesting that basalts harbor previously unrecognized metabolic diversity. These novel processes could exert a profound influence on ocean chemistry.

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Introduction

Oceanic basalts are one of the most abundant rock types on Earth, covering upward of 60% of the Earth's surface. These rocks typically have high permeabilities, which enables infiltration and circulation of large quantities of seawater (Fisher, 1998; Fisher and Becker, 2000). The rock–seawater interaction results in a significant flux of energy and solutes between basalt crust and the overlying seawater (Fisher, 1998). Recent quantitative analyses revealed that basalts harbor 6×10^5 – 4×10^6 and 3×10^6 – 1×10^9 cells per g rock (Einen *et al.*, 2008; Santelli *et al.*, 2008). In fact, Einen *et al.* (2008) suggested that the total number of microorganisms

present in ocean crust exceeds the number present in seawater. These observations raise intriguing questions about the role that microorganisms play in biogeochemical cycling in basalts. Biological alteration of basalt by microorganisms has been the focus of numerous studies, with compelling evidence suggesting that they do play a part in this process (Thorseth *et al.*, 1995; Giovannoni *et al.*, 1996; Fisk *et al.*, 1998, 2003; Torsvik *et al.*, 1998; Furnes and Staudigel, 1999; Furnes *et al.*, 2001, 2004; Banerjee and Muehlenbachs, 2003).

Alteration, whether abiotic or biotic, intrinsically changes the chemistry and mineralogy of the rock. For example, alteration of reactive primary minerals to secondary minerals, changing rock permeabilities, and changes in the oxidation state of the rocks alter the chemical milieu in which endolithic microorganisms reside. These changes may result in shifts in the microbial community. Analysis of prokaryotic communities associated with marine basalts revealed that several clades appear to be

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cosmopolitan in their distribution, as they are associated with globally distributed basalts (Mason *et al.*, 2007; Santelli *et al.*, 2008), regardless of rock age and degree of alteration. The ubiquity of certain clades, such as the α -Marine Group I ocean crust clade IX delineated by Mason *et al.* (2007), regardless of the age of the host rock, suggests that overall basalt microflora do not change on a temporal scale. However, Lysnes *et al.* (2004) reported that basalts of varying ages support different microbial phyla and subphyla. For example, the Actinobacteria were associated with older basalts, but were absent in recently erupted material. Therefore, certain clades may, in fact, respond to alteration processes, which could, for example, affect the available electron donors and acceptors.

Certain microbial taxa may be associated with rocks of varying ages, as suggested by Lysnes *et al.* (2004); however, it is unclear what factors contribute to this habitat specificity. Fresh basalts are ~8% wt FeO and 2% wt Fe₂O₃. The increasing oxidation of reduced iron with time could lead to a shift in the microbial community from oxidizers to reducers. In fact, Edwards *et al.* (2003b) demonstrated that chemolithoautotrophic, iron-oxidizing α - and γ -Proteobacteria isolated from sulfides and metalliferous sediments are able to grow on basalt glass. These isolates are capable of using oxygen and nitrate as electron acceptors. The ability to use multiple electron acceptors would be a requisite as basalt alteration progresses and the *in situ* redox conditions change.

Alternately, the reduced iron available to iron-oxidizing prokaryotes may become hydrated during fluid-rock interactions. This reaction can evolve hydrogen (Janecky and Seyfried Jr, 1986; Berndt *et al.*, 1996), which can serve as an electron donor for numerous microorganisms including methanogens and sulfate reducers. In fact, Bach and Edwards (2003) estimated that autotrophic sulfate reduction and methanogenesis in marine basalts could result in substantial prokaryotic biomass ($9 \pm 7 \times 10^{10}$ and $3 \pm 2 \times 10^{10}$ g C per year, respectively).

Although the geological characteristics of basalts, such as the availability of FeO for microbial iron oxidation, discussed above, do provide some insight into the potential metabolic functions in this environment, examination of the *in situ* metabolic diversity of prokaryotes by cultivation efforts is limited to one study. Templeton *et al.* (2005) isolated Mn-oxidizing, heterotrophic bacteria from Loihi Seamount. Thus, there is a need to circumvent the lack of cultured microorganisms using a molecular approach to determine metabolic diversity in basalts. GeoChip is a molecular tool that does not rely on cultivation-based methods to assay for functional diversity. Specifically, it is a functional gene microarray that has 24 243 oligonucleotide probes covering >10 000 genes in >150 functional groups involved in nitrogen, carbon, sulfur and phosphorus cycling (He *et al.*, 2007). GeoChip can provide

significant insight into metabolic potential in a given environment, such as marine basalts.

In this study, we used terminal restriction fragment length polymorphism (T-RFLP), cloning and sequencing, and microarray analysis of functional genes to (1) assess successional changes in the microbial communities associated with basalts of varying ages and from different geographical locations, (2) examine species composition and distribution and (3) determine potential metabolic function in basalts by examining functional genes.

Our analyses revealed that rock age, or degree of alteration, may, to some degree, play a role in community succession. In addition, we report previously unrecognized phyla in basalts and several novel ocean crust clades of microorganisms that may represent basalt specialists. Finally, the examination of functional genes in basalt revealed the genetic potential for several novel metabolic processes. This analysis provides insight into biogeochemical cycling in this ocean crust environment.

Materials and methods

Sample collection

Glassy pillow basalts were collected from areas of low (or no) sediment accumulation using the DSV Alvin on two separate cruises to East Pacific Rise (9° N) and to the Co-axial segment of the Juan de Fuca Ridge (JdF) and neighboring seamounts (Table 1). Basalt samples were collected and placed inside a collection box, or 'biobox', which was designed to prevent sample exposure to ambient seawater during the ascent to the surface. Before the dive, the box was filled with either 0.2- μ m filtered seawater or sterile Millipore water. During Alvin's descent, residual airspace was replaced with seawater that passed through 0.2 μ m filters embedded in the lid. The biobox volume (16 l) allowed for several liters of ambient deep seawater to be collected with the basalts. Once on deck, the samples were removed from the biobox using sterile (flamed) tongs and placed into separate freezer bags. Samples were immediately frozen at -80 °C and remained frozen until shore-based analyses. To control for deep-sea planktonic organisms that may have found their way into fractures and pores in the basalt samples, the biobox water was filtered and the filters were frozen and analyzed along with the basalts (see below).

Nucleic acid extraction from the basalt samples

For molecular analyses, all rock sample-handling and all extraction steps were performed in a sterile laminar flow hood. Ceramic tumbling vessels, chisels, mortar and pestles were baked at 220 °C for at least 24 h. The outer rock surface was removed by tumbling the rock several times for 20 min, replacing with sterile grit each time. The glassy rind

Table 1 Basalt samples collected from the East Pacific Rise and the Juan de Fuca Ridge

Alvin dive ^a	Date	Latitude	Longitude	Depth (m)	Dive feature ^b	Age ^c	Molecular analyses
<i>East Pacific Rise (EPR) R/V Atlantis Voyage 7 Leg 3</i>							
D3713C	10/19/01	09° 50.80' N	104° 17.63' W	2493	Base of Q vent	1991 eruption	T-RFLP
D3716A	10/22/01	09° 50.30' N	104° 17.51' W	2499	Axial caldera	1991 eruption	T-RFLP
D3718B	10/24/01	09° 50.78' N	104° 17.58' W	2493	North of Q vent	1991 eruption	T-RFLP, cloning and sequencing ^d
D3719D	10/25/01	09° 50.78' N	104° 17.58' W	2496	Near M vent	1991 eruption	T-RFLP
D3720R	10/26/01	09° 50.78' N	104° 17.58' W	2498	Near TY vent	1991 eruption	T-RFLP
D3721D	10/27/01	09° 50.79' N	104° 17.59' W	2495	Near Q vent	1991 eruption	T-RFLP
D3721E	10/27/01	09° 50.79' N	104° 17.59' W	2496	Near Q vent	1991 eruption	T-RFLP
<i>Juan de Fuca Ridge (JdF) R/V Atlantis Voyage 7 Leg 19</i>							
D3815F	8/05/02	45° 59.50' N	129° 56.59' W	2135	Helium Basin	<100 Ka	T-RFLP, cloning and sequencing ^e
D3816F-1,2	8/06/02	46° 31.34' N	129° 29.94' W	2653	Co-Axial Rift	10–170 Ka	T-RFLP
D3823M	8/19/02	46° 41.95' N	130° 55.94' W	1909	Cobb Seamount	3.3 Myr	T-RFLP, cloning and sequencing ^d
D3826U	8/23/02	46° 31.16' N	129° 34.92' W	2409	Lava flow	1993 eruption	T-RFLP

Abbreviation: T-RFLP, terminal restriction fragment length polymorphism.

^aThe Alvin dive number and suffix is the sample identifier.

^bThe 9° N samples were collected from the area of the 1991 eruption (Haymon *et al.*, 1993) and were 11 years old at the time of collection. The region of EPR vents is described in Fornari and Embley (1995).

^cThe ages of Juan de Fuca samples from Helium Basin and Co-Axial Rift were inferred from seafloor-spreading rate and distance from the ridge axis. Age of the Cobb Seamount sample from Desonie and Duncan (1990). D3826U was collected from the 1993 lava flow (Embley *et al.*, 2000).

^dD3718B and D3823M clones were analyzed and presented in Mason *et al.* (2007) and are only included in dendrograms in this study if they are part of novel clades delineated here or are closely related to clones from D3815F.

^eD3815F clones are presented in this study.

was panned away with a chisel and/or sterile rock splitter. Approximately 1 cm³ was powdered with a tungsten mortar and pestle, and 2 ml of the powder was used in each extraction. Two control DNA extractions, to which either 2 ml of the grit from the last tumbling step or no rock or grit material was added, were used to assess contamination introduced by the tumbling steps or by the DNA-extraction reagents, respectively.

The DNA-extraction protocol was optimized for extracting DNA from basalts. Specifically, each extraction tube contained 2 ml of rock powder and final concentrations of the following: 4.5 mM Tris·HCl, pH 7.4; 185 mM EDTA, pH 8.0; 4.5% Chelex-100 (w v⁻¹); 0.7% sodium dodecyl sulfate (w v⁻¹); and 2 mg proteinase K (Qiagen Inc., Valencia, CA, USA). Tubes were placed in a 37 °C rotator with gentle agitation (180 r.p.m.) overnight. The rock powder was separated from the supernatant by low-speed centrifugation and mixed with final concentrations of the following: 923 mM NaCl and 1.3% cetyltrimethylammonium bromide (w v⁻¹). Samples were incubated at 65 °C for 30 min, extracted once with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) (pH 7.4) and then extracted twice with an equal volume of chloroform/isoamyl alcohol (24:1). Nucleic acids were precipitated by adding one volume of isopropanol and incubating the samples overnight at 4 °C. Samples were spun at 24 000 g for 1 h at 4 °C in a TL-100 ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA, USA) to pellet the precipitated nucleic acids. Pellets were washed with 70% ethanol (v v⁻¹), dried in a laminar

flow hood at room temperature and suspended in sterile water. Replicate extracts were combined (40 µl final volume) and stored at –80 °C.

DNA extraction from the filtered biobox water samples
At least 12 l of biobox water from each dive was filtered through a 142-mm 0.2-µm Supor filter (Pall Gelman Laboratory, Ann Arbor, MI, USA) in a polycarbonate filter holder (Geotech Environmental Equipment Inc., Denver, CO, USA) that was connected to a peristaltic pump. Filters were immediately preserved in 5 ml of sucrose lysis buffer (20 mM EDTA, 400 mM NaCl, 0.75 M sucrose, 50 mM Tris·HCl (pH 9.0)) and stored at –80 °C. Total community nucleic acids were extracted from the filters according to Giovannoni *et al.* (1990).

Terminal restriction fragment length polymorphism analysis

Terminal restriction fragment length polymorphism analysis was used to compare the archaeal and bacterial communities from several rocks and corresponding biobox seawater samples according to Moeseneder *et al.* (1999) with few modifications. The archaeal 16S rRNA genes were amplified using the primers Arch20F (DeLong *et al.*, 1999) and Arch915R (Stahl and Amann, 1991), with the forward primer 5'-end-labeled with phosphoramidite fluorochrome 5-carboxyfluorescein (6-FAM) and the reverse primer labeled with 5-hexachloro-fluorescein. Fifty PCR cycles were necessary to

amplify archaeal DNA, whereas a semi-nested approach was required to amplify bacterial 16S rRNA genes from nearly all of the basalts, with primers 27F-B (5'-AGRGTTYGATYMTGGCTCAG) and 1492RY (5'-GGYTACCTTGTACGACTT) modified from Lane (1991) used in the initial PCR (30 cycles) and primers 27F-B-[FAM] and 1391R (Lane, 1991) used in the second reaction (20 cycles). Only the forward strand of this PCR product was labeled for the T-RFLP analysis. For both archaeal and bacterial amplifications, three replicate PCRs (50 μ l) for each DNA sample contained final concentrations of the following: 1 μ l of DNA extract; 1% (v v⁻¹) PCR buffer (+ NH₄SO₄; MBI Fermentas, Hanover, MD, USA); 0.2 mM each deoxynucleotide triphosphate; 0.2 μ M each primer; 2 mM MgCl₂ (MBI Fermentas); 1.2 mg ml⁻¹ bovine serum albumin (non-acetylated; Sigma, St Louis, MO, USA); 1% (w v⁻¹) PVP (polyvinylpyrrolidone); and 2.5 U Taq polymerase (MBI Fermentas). PCR cycling consisted of denaturation at 94 °C for 1.5 min, annealing at 55 °C for 1.5 min and extension at 72 °C for 1.5 min. The filtered biobox seawater samples from each dive were also analyzed using the same cycling conditions as the basalts, except that the number of cycles was reduced to 30.

PCR products (50 ng) were digested with 10 U of enzyme for 6 h at 37 °C with each of three separate restriction enzymes: *AluI*, *BsuRI* (*HaeIII*), and *Hin6I* (*HhaI*) (MBI Fermentas). Samples were run on an ABI 3100 (Applied Biosystems Inc. (ABI), Foster City, CA, USA). The fingerprint patterns for the rock and seawater communities were compared according to Moeseneder *et al.* (1999); however, only peaks longer than 70 bp in length were included in the analysis. Data were standardized by inclusion of peaks that represented > 1% of the total peak height for each fingerprint and were then converted to binary matrices. Binary data were analyzed by the unweighted pair group with mathematical averages method in Phylogenetic Analysis Using Parsimony* (and other methods) version 4.0 b10 (Swofford, 1998) using the site distance-matrix method of Nei and Li (1979) according to Moeseneder *et al.* (1999).

PCR amplification and cloning of prokaryotic 16S rRNA genes

Data from the unweighted pair group with mathematical averages analysis were used to select three basalt samples that differed in age and community structure (D3718B, 9° N EPR, D3815F and D3823M JdF) for cloning and sequencing of archaeal and bacterial 16S rRNA genes. The archaeal communities were amplified according to the PCR conditions described above for T-RFLP analysis, except that the primers were not fluorescently labeled. To amplify archaeal 16S rDNA from D3815F, a semi-nested approach was employed using the primers Arch20F and 1492RY in the initial reaction and Arch20F and Arch915R in the semi-nested reaction.

The bacterial community from D3718B was amplified using the semi-nested approach described above. Amplification of bacterial 16S rDNA from D3823M did not require a semi-nested approach. As with the T-RFLP analysis, the corresponding seawater samples from each dive were also cloned for comparison. PCRs (50 μ l volume) were cloned into the pGEM-T Easy vector (Promega Corp., Madison, WI, USA). Clone libraries were constructed and screened according to the methods of Vergin *et al.* (2001). Briefly, clones were assigned to clone families based upon shared patterns for two separate restriction digests. Digested PCR products were resolved on a 3% agarose gel. One clone from each unique RFLP pattern was sequenced using an ABI 3730 capillary sequencer. Full-length sequences were obtained for clones representing each phylo-type. Clones with restriction patterns that appeared only once in the library were designated 'unique'. Percent coverage was calculated based upon the number of unique clones versus total clones according to the method of Good (1953). Chimeric sequences were identified with the CHECK_CHIMERA program (Maidak *et al.*, 1997, 1999) and Mallard (Ashelford *et al.*, 2006).

Phylogenetic analysis

The phylogenies of microorganisms from D3718B and D3823M were extensively reviewed in Mason *et al.* (2007). Clones from these libraries are presented here in phylogenetic dendrograms only if they are part of novel ocean crust clades delineated here or if they are highly similar to clones from D3815F. However, clones from all libraries were analyzed during phylogenetic reconstruction. Phylogenetic analyses and clade delineations were carried out according to Mason *et al.* (2007), using the Greengenes database (DeSantis *et al.*, 2006). Briefly, neighbor-joining, maximum parsimony and maximum-likelihood trees of near-full-length sequences were generated in ARB (Ludwig *et al.*, 2004). Maximum-likelihood trees were generated using Tree-Puzzle (Schmidt *et al.*, 2002), with the Hasegawa–Kishino–Yano model (Hasegawa *et al.*, 1985). Quartet-puzzling (QP) reliability values are not shown at bifurcations if they are below 50%. In determining clades, QP values from 90% to 100% are strongly supported; however, QP values less than 70% can also be trusted (Schmidt *et al.*, 2002). Clades delineated here with QP values lower than 70% were analyzed relative to QP support values of the other branches in the tree (Schmidt *et al.*, 2002).

Nucleotide sequence accession numbers

The 16S rRNA gene sequences for the archaeal and bacterial clones were submitted to the GenBank database and have been assigned the following accession numbers: DQ070750–DQ070835 (D3718B and D3823M) and FJ024305–FJ024341 (D3815F).

Functional genes

Basalt sample D3815F was selected for functional gene analysis because it had several clades that have not been previously reported from this environment, particularly the Marine Benthic Group B. We hypothesized that this diversity of species would be mirrored in the diversity of functional genes. Second, thin sections of this sample showed textures that suggest bioalteration; therefore, analysis of functional genes in this sample would provide insight into the biological processes that may result in these textural features. Functional genes were assayed for using the GeoChip 2.0 (He *et al.*, 2007) microarray following previously described methods (Wu *et al.*, 2006; He *et al.*, 2007). Briefly, DNA from D3815F was amplified in triplicate using a TempliPhi 500 amplification kit (Amersham Biosciences, Piscataway, NJ, USA) following the manufacturer's protocol. To facilitate amplification, spermidine (0.1 µg µl⁻¹) and single-strand binding protein (0.04 mM) were added to the reaction mixture. Amplified DNA was fluorescently labeled with Cy5. Hybridizations were performed using a HS4800Pro Hybridization Station (TECAN US, Durham, NC, USA) overnight at 42 °C. Microarrays were scanned using a ProScanArray (PerkinElmer, Waltham, MA, USA). Images were then analyzed using ImaGene 6.0 (BioDiscovery, El Segundo, CA, USA) to designate the identity of each spot and to determine spot quality. Data were processed as described by Wu *et al.* (2006). Briefly, raw data from ImaGene were analyzed using a GeoChip data

analysis pipeline. A signal-to-noise ratio of ≥3 was considered a positive signal. A positive signal in at least one-third of the probes for a particular gene (minimum of two probes) was required for a gene to be considered positive. Each gene had one, two or three probes per array based on the number of probes available, meeting the criteria described by He *et al.* (2007).

Results and discussion

T-RFLP

Unweighted pair group with mathematical averages cluster analysis of T-RFLP data revealed that the archaeal and bacterial communities were distinct from deep seawater communities (Figure 1). Further, there was striking congruency in the unweighted pair group with mathematical averages clustering patterns for the four oldest JdF samples. These old samples, ranging from a few thousand to about 3 Myr in age, clustered together, whereas the younger basalts from 9° N (from an eruption in 1991) clustered with one JdF sample of a similar age (D3826U, from the 1993 lava flow). This clustering is evidence that there are differences in microbial communities present in recently erupted basalts compared with older, more weathered rocks. The observed clustering is supported, to some degree, by phylogeny. For example, the Planctomycetes ocean crust clade XIV members (see below) are from recently erupted to medium-aged basalts. Overall,

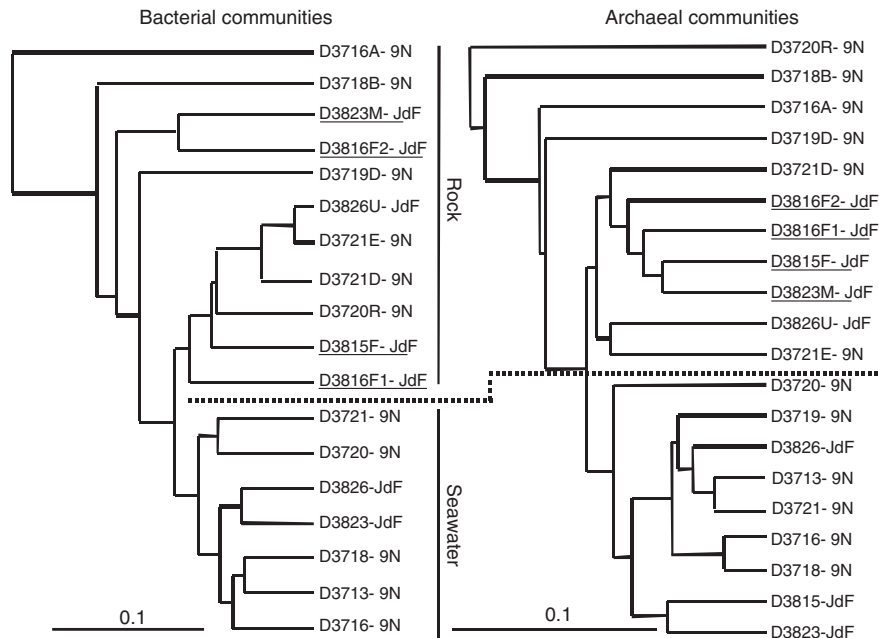


Figure 1 UPGMA analysis of T-RFLP fingerprint patterns for the bacterial (left) and archaeal (right) communities recovered from basalts (above the dashed line) and background seawater from 9° N and JdF (below the dashed line). Older basalts (> 20 years) from JdF are underlined. All 9° N samples are less than 20 years. Sample numbers indicate Alvin dive number and location: 9N is 9° N on the East Pacific Rise and JdF is Juan de Fuca Ridge and Cobb Seamount. The scale bar represents similarity. T-RFLP, terminal restriction fragment length polymorphism; UPGMA, unweighted pair group with mathematical averages.

however, there is a distinct overlap in the microbial communities regardless of rock age. For example, the basalt-specific ocean crust clade presented here, such as the γ -Proteobacteria ocean crust clade XII, is composed of microorganisms from young, fresh basalts to 3.3-Myr-old basalts. This pattern suggests that basalt microflora are largely associated with rocks of varying ages, but that a minority may reside in, for example, younger, less-altered rocks to the exclusion of older, more-weathered rocks. This finding is consistent with that of Lysnes *et al.* (2004), who reported that specific bacterial species are found only in rocks of a certain age.

Phylogenetic analysis

A total of 547 bacterial and archaeal 16S rDNA clones were analyzed and 173 unique clones were sequenced (Table 2). This analysis revealed that Gemmatimonadetes, Nitrospirae, SBR1093 and in the Archaea, the Marine Benthic Group B (Figures 2–4), were present in basalt samples. None of these clades have been previously reported in marine basalts. In addition, microorganisms in the α -, δ - and γ -Proteobacteria, Acidobacteria, Actinobacteria, Bacteroidetes and Planctomycetes in the bacterial domain are reported (Figures 2 and 3). The most prevalent microorganisms were Proteobacteria (56%), the majority of which were γ - (25%), α - (15%) and δ - (13%), followed by Bacteroidetes (10%), Actinobacteria (9%), Planctomycetes (7%), Acidobacteria (6%) and Gemmatimonadetes (3%). The remaining clades were observed in a single rock sample.

Our observations are consistent with those reported by Santelli *et al.* (2008), who analyzed basalts from the East Pacific Rise and from Hawaii and found 68%/66% (EPR%/Hawaii%) Proteobacteria, 8%/5% Planctomycetes, 7%/8% Actinobacteria, 4%/1% Bacteroidetes and 3%/4% Acidobacteria. The similarity in bacterial communities associated with basalts from a broad geographic distribution suggests cosmopolitan distributions of these clades, which is in agreement with the findings presented by Mason *et al.* (2007) and Santelli *et al.* (2008).

Phylogenetic reconstruction revealed three novel ocean crust clades composed entirely of microorgan-

isms associated with basalt. These new clades are the γ -Proteobacteria ocean crust clade XII (Figure 2), Actinobacteria ocean crust clade XIII (Figure 3) and Planctomycetes ocean crust clade XIV (Figure 3). These clades are comprised of bacteria sampled from JdF (this study), East Pacific Rise, 9° N (this study and Santelli *et al.*, 2008), and Hawaiian (Santelli *et al.*, 2008) basalts. These cosmopolitan basalt clades may represent ecotypes of bacteria that are specifically adapted to this environment.

Cloning and sequencing of archaeal 16S rDNA revealed that Marine Benthic Group B were present in basalts (Figure 4). This is the first report of this clade in this environment, as previous studies that examined the archaeal communities in basalts revealed only Marine Group I Crenarchaeota (Thorseth *et al.*, 2001; Fisk *et al.*, 2003; Lysnes *et al.*, 2004; Mason *et al.*, 2007). Recently, quantitative analyses of the microbial communities in basalts revealed that Archaea comprise 4–12% and 0.02% or less of the prokaryotic communities (Santelli *et al.*, 2008; Einen *et al.*, 2008), respectively. Although these estimates are disparate, they do reveal that Archaea are a minor component in the overall microbial communities that reside in basalt. Although Archaea are less prevalent, they are ubiquitous in basalts and have been reported in all studies that assayed for their presence (Thorseth *et al.*, 2001; Fisk *et al.*, 2003; Lysnes *et al.*, 2004; Mason *et al.*, 2007; Einen *et al.*, 2008; Santelli *et al.*, 2008). Further, as discussed previously, a clade of Marine Group I Archaea appears to be endemic to basalt (Mason *et al.*, 2007). This habitat specificity and global distribution indicate that some Archaea, although less abundant than bacteria, are particularly adapted to life in basalt and likely play a role in biogeochemical cycling.

Functional genes

GeoChip (He *et al.*, 2007) microarray analysis of functional genes in basalt sample D3815F revealed the presence of genes coding for metabolic processes previously unrecognized in this environment. In this analysis, a total of 604 probes of the 24 243 total probes present on GeoChip were positive.

Table 2 Clone libraries of archaeal and bacterial 16S rRNA genes from basalts and seawater

Sample ID	Sample site	Sample type	Prokaryote domain	Clone families	Unique/total	Good's coverage (%)
D3718B	9° N	Basalt	Archaea	20	11/94	88.3
D3718F	9° N	Seawater	Archaea	22	13/96	86.5
D3815F	JdF	Basalt	Archaea	33	24/95	74.7
D3823M	JdF	Basalt	Archaea	20	13/95	86.3
D3718B	9° N	Basalt	Bacteria	10	64/93	31.2
D3718F	9° N	Seawater	Bacteria	10	44/89	50.5
D3815F	JdF	Basalt	Bacteria	43	25/96	73.4
D3823M	JdF	Basalt	Bacteria	15	38/74	48.6

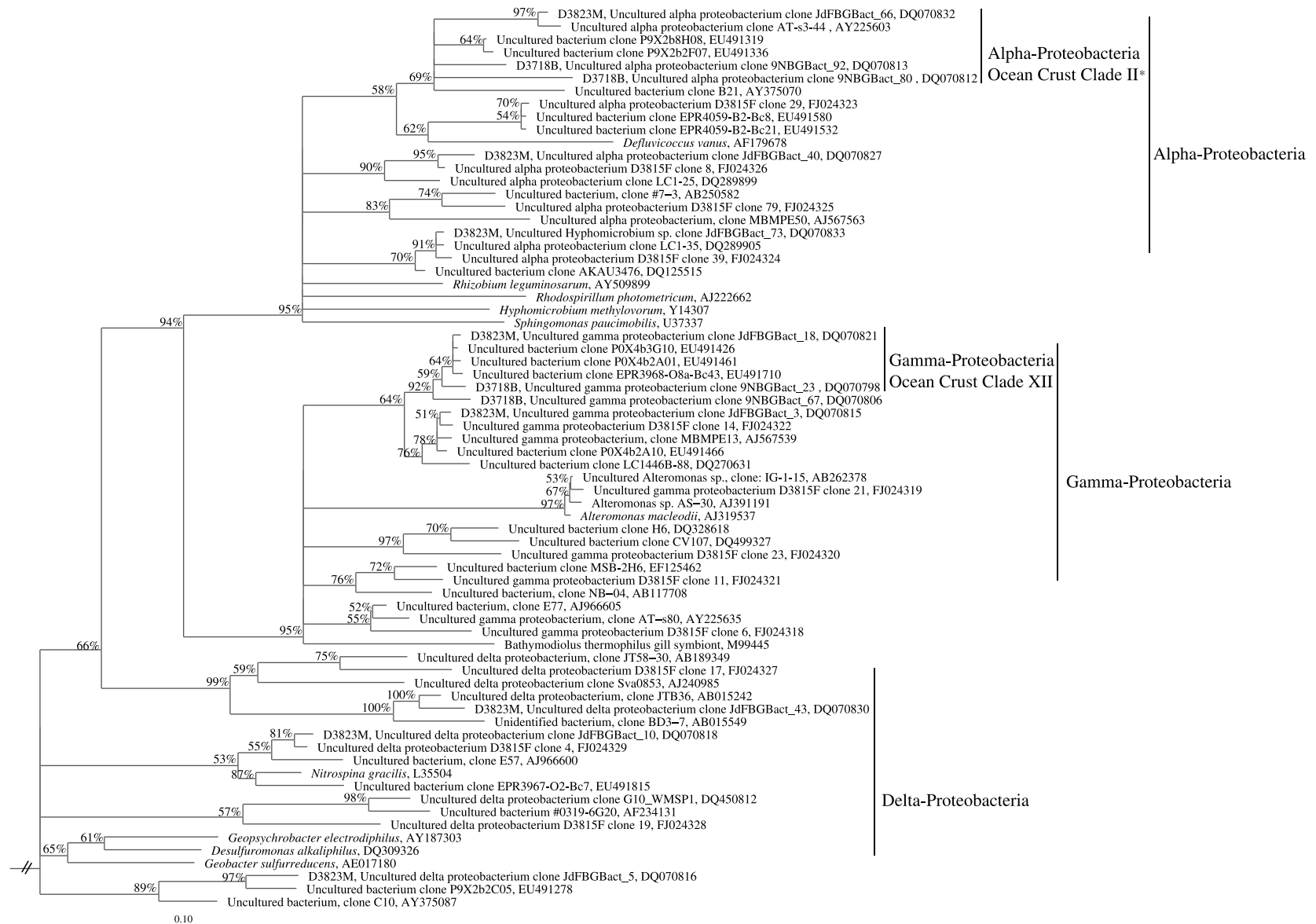


Figure 2 Maximum-likelihood phylogenetic tree of proteobacterial 16S rRNA gene sequences from basalt samples. The Proteobacteria tree was constructed with 25 000 puzzling steps. A general Bacteria filter was used. The 16S rDNA sequence of *Aquifex pyrophilus* (M83548) was used as the outgroup (not shown). The α -Proteobacteria ocean crust clade II, designated by an (*), was delineated by Mason *et al.* (2007) and is included here because new basalt sequences extend this clade. The scale bar indicates 0.1 nucleotide substitutions per site.

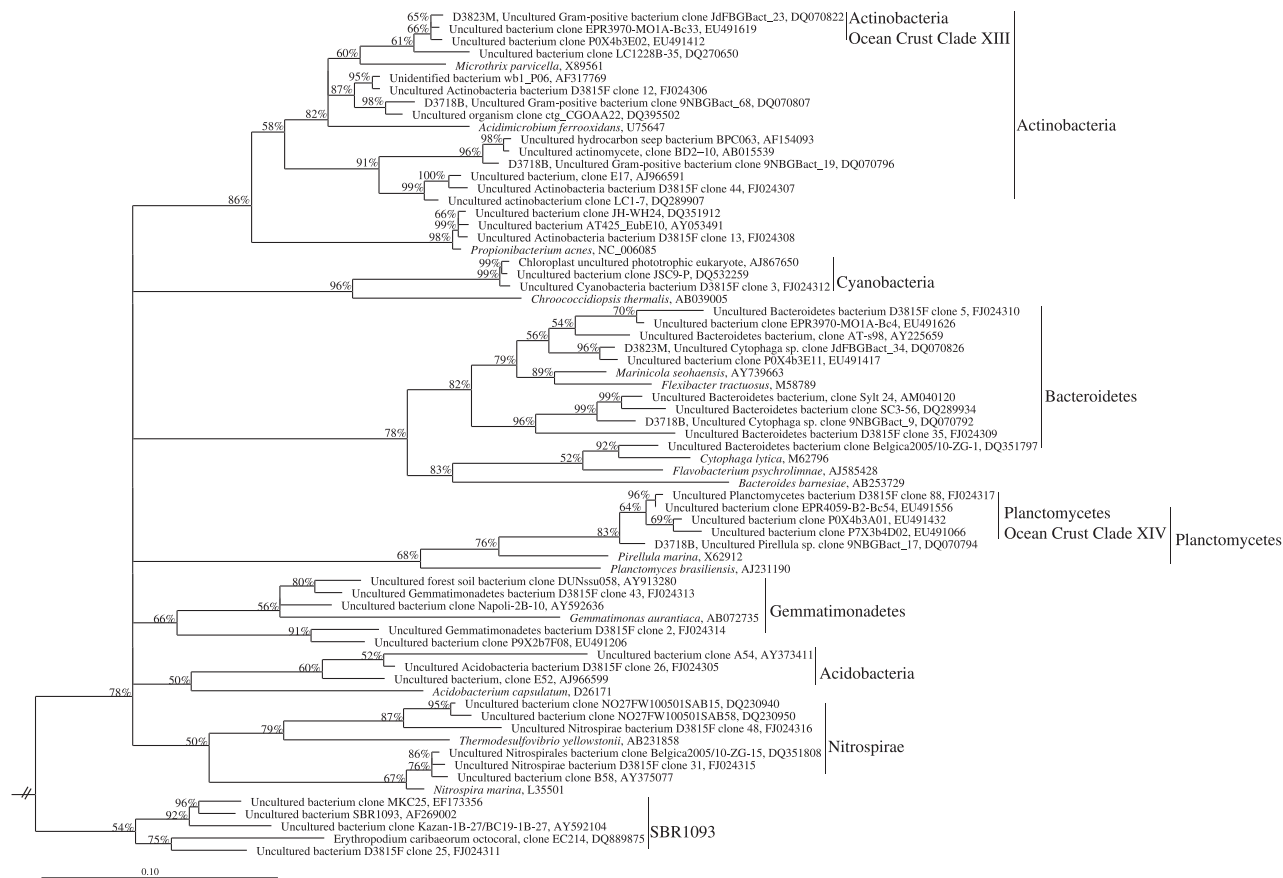


Figure 3 Maximum-likelihood phylogenetic tree of Actinobacteria, Cyanobacteria, Bacteroidetes, Planctomycetes, Gemmatimonadetes, Acidobacteria, Nitrospirae and SBR1093 16S rRNA gene sequences from basalt samples. The bacterial tree was constructed with 25 000 puzzling steps. A general Bacteria filter was used. The 16S rDNA sequence of *Aquifex pyrophilus* (M83548) was used as the outgroup (not shown). The scale bar indicates 0.1 nucleotide substitutions per site.

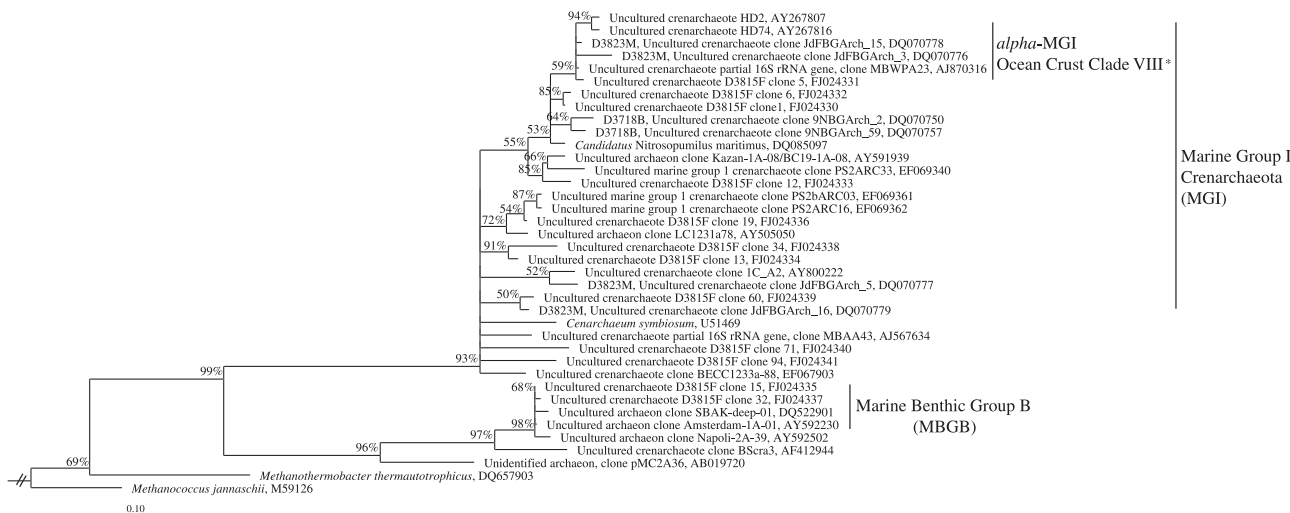


Figure 4 Maximum-likelihood phylogenetic tree of archaeal 16S rRNA gene sequences from basalt samples. The archaeal tree was constructed with 25 000 puzzling steps. A general Archaea filter was used. The 16S rDNA sequence of *Aquifex pyrophilus* (M83548) was used as the outgroup (not shown). The α -MGI ocean crust clade VIII, designated by an (*), was delineated by Mason et al. (2007) and is included here because new basalt sequences extend this clade. The scale bar indicates 0.1 nucleotide substitutions per site. MGI, Marine Group I Crenarchaeota.

Specifically, we found genes coding for carbon fixation, methane production and oxidation, nitrogen fixation, ammonium oxidation, nitrate

and nitrite reduction, dissimilatory sulfate reduction and iron reduction (see Supplementary Table 1 for a complete list).

Here we report genes coding for carbon fixation. Basalts lacking a sediment layer are considered to be an oligotrophic, low-carbon environment (Edwards *et al.*, 2003a); thus carbon cycling in this habitat is particularly significant. The oligotrophic nature of this environment suggests that carbon fixation would be paramount in this habitat. In fact, chemolithoautotrophic processes in marine subsurface ridge flank hydrothermal environments have been theoretically shown to provide energy that could result in significant microbial biomass ($\sim 1 \times 10^{12}$ g C per year) (Bach and Edwards, 2003). Therefore, chemolithoautotrophic processes occurring *in situ* could serve to underpin a basalt-hosted biosphere. One such process is methanogenesis, where hydrogen can serve as the electron donor to reduce carbon dioxide, evolving methane. During fluid–rock interactions when the basalt minerals olivine and pyroxene react with water, hydrogen may be evolved (Janecky and Seyfried Jr, 1986; Berndt *et al.*, 1996). Thus, the requisite electron donor may be present as a result of this abiotic reaction.

Here, we report that genes coding for methanogenesis are present in basalt. Methanogens have not been reported in molecular analyses of Archaea in basalts conducted to date (Thorseth *et al.*, 2001; Fisk *et al.*, 2003; Lysnes *et al.*, 2004; Mason *et al.*, 2007). However, Lysnes *et al.* (2004) reported that methane was evolved in enrichment cultures inoculated with marine basalts. Although the Marine Benthic Group B clade currently lacks a cultured representative (Knittel *et al.*, 2005), they are frequently associated with environments dominated by methane, methanogens and methanotrophs (Knittel *et al.*, 2005; Kendall and Boone, 2006; Kendall *et al.*, 2007). The role of this clade in the environment is unknown, but it is plausible that they are involved in methane biogeochemical cycling. Although no known methanogens were observed in our study, the diversity of *mcr* genes (Supplementary Table 1) in conjunction with a clade typically observed in methane-rich environments suggests that this metabolic process may be occurring in basalts. Methane resulting from biological processes could serve as a carbon and energy source for heterotrophic processes. In fact, we found genes coding for methane oxidation. Methane cycling in marine basalts would have a direct impact on the overlying hydrosphere.

As discussed above, basalts are not carbon replete. Similarly, they are composed of only a small amount of nitrogen, averaging approximately 2 p.p.m. (Marty *et al.*, 1995). Therefore, the detection of genes coding for nitrogen fixation is intriguing. Cowen *et al.* (2003), and more recently Huber *et al.* (2006), investigated ocean crust fluids and reported elevated levels of ammonium compared with seawater. Cowen *et al.* (2003) suggested that nitrogen fixation may serve as the source of this excess ammonium. Mehta *et al.* (2005) attributed nitrogen fixation in crustal fluids and in deep seawater to non-methano-

genic Archaea, which are the only known archaeal nitrogen fixers. In that study, *nifH* genes were detected in crustal fluids. Nitrogen fixation may also be taking place in the host rocks themselves, given the presence of *nifH* genes in our basalt sample.

Nitrogen fixation could augment the low nitrogen concentrations in basalts and may ultimately support ammonium-oxidizing microorganisms. This hypothesis is supported by the presence of genes that code for ammonium oxidation in our basalt sample. As reported by Mason *et al.* (2007) (see Figures 3 and 4), basalt sequences similar to *Nitrosococcus oceani* (89% similar) and *Nitrosospira multiformis* (96% similar), both of which are known ammonium-oxidizing microorganisms (Watson, 1965; Watson *et al.*, 1971), were derived from basalts from JdF and 9° N (this study), and Mohs Ridge (Einen *et al.*, 2006). Thus, phylogenetic and functional gene analyses both suggest that ammonium oxidation may be occurring in basalts.

Further, basalt clones closely related to the nitrite-oxidizing *Nitrospina gracilis* (91–94% similar) and *Nitrospira marina* (95–96% similar) (Watson and Waterbury, 1971; Tal *et al.*, 2003) were found (Figures 2 and 3), suggesting that nitrite oxidation, the second step in nitrification, may be occurring in basalts. This observation could not be confirmed using GeoChip, however, because genes coding for nitrite oxidation are not present on the gene chip.

Nitrification could provide the substrate for both denitrification and anaerobic ammonium oxidation (anammox), both of which lead to loss of nitrogen (Lam *et al.*, 2007). In fact, we found numerous genes coding for nitrate and nitrite reduction; therefore, the genetic potential for denitrification is present in this environment. Recently, Edwards *et al.* (2003b) demonstrated that chemolithoautotrophic iron-oxidizing bacteria are able to grow on basalt glass using nitrate as the electron acceptor. Whether anaerobic ammonium oxidation is occurring in basalts remains unclear. Although Planctomycetes have been reported in basalts, including the novel ocean crust clade Planctomycetes XIV delineated here, microorganisms closely related to known anammox bacteria, such as *Kuenenia stuttgartiensis* (77% similar to basalt-associated microorganisms), have not been detected. Therefore, it is unclear if this process is important in considering nitrogen loss from the basalt layer. Our data do suggest, however, that nitrogen could be lost from marine crust by denitrification processes.

In addition to genes coding for denitrification processes, we also detected genes coding for iron reduction and dissimilatory sulfate reduction in basalt. Together, these genes suggest that anaerobic respiration may be occurring in basalt. The presence of genes that code for aerobic respiration (for example, ammonium oxidation) in the same sample indicates that aerobic and anaerobic processes may occur simultaneously on a small spatial scale,

suggesting, perhaps that microniches are occupied by prokaryotes in basalt. Consistent with our findings, it was reported that in upper basaltic crust, redox conditions are such that aerobic and anaerobic processes are likely supported (Bach and Edwards, 2003).

Conclusion

Basalts from JdF, neighboring seamounts and 9° N, EPR harbor cosmopolitan microorganisms that are distinct from seawater prokaryotes. Several novel ocean crust clades, composed only of microorganisms from basalts, suggest that some bacteria are specifically adapted to this ocean crust environment. Our analysis of geochemically important functional genes revealed the potential for several metabolic processes not known to be occurring in basalts, particularly carbon fixation, methanogenesis, methane oxidation, nitrogen fixation and denitrification. Our data suggest that basalts not only harbor a diversity of broadly distributed microbial species, but also unexpected metabolic diversity. Future studies should utilize culture-dependent and culture-independent methods to analyze biogeochemical cycling in basalts to better understand the biological processes in this vast subsurface environment and how these processes ultimately affect ocean chemistry.

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