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

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Abstract

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 twelve different phyla and sub-phyla 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 gamma-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.

Keywords: Archaea/Bacteria/biogeochemical cycling/functional genes/microbial ecology/prokaryotic basalt alteration.

Introduction

Oceanic basalts are one of the most abundant rock types on Earth, covering upwards 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 to 4×10^6 and 3×10^6 to 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; Torsvik et al., 1998; Furnes and Staudigel, 1999; Furnes et al., 2001; Banerjee and Muehlenbachs, 2003; Fisk et al., 2003; Furnes et al., 2004).

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 alters 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 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 alpha-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 sub-phyla. 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 Fe_2O_3. 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 chemolithoautrophic, iron-oxidizing alpha- and gamma-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 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 ($9 \pm 7 \times 10^{10}$ g C/yr and $3 \pm 2 \times 10^{10}$ g C/yr, respectively).

While the geological characteristics of basalts, such as the availability of FeO for microbial iron-oxidation, discussed above, do provide some insight into potential metabolic

function 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 in marine basalts.

In this study we used terminal restriction fragment 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. Additionally, we report previously unrecognized phyla in basalts and several novel ocean crust clades of microorganisms that may represent basalt specialists. Finally, 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.

Material 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 CoAxial 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. Prior to the dive, the box was filled with either 0.2 µm filtered seawater or sterile Millipore water. During Alvin's descent, residual airspace was replaced with seawater that passed through 0.2 µm filters embedded in the lid. The biobox volume (16 liters) 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 ºC 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 hours. The outer rock surface was removed by tumbling the rock several times for 20 minutes, replacing with sterile grit each time. The glassy rind was pared away with a chisel and/or sterile rock splitter. Approximately 1 cm^3 was powdered with a tungsten mortar and pestle, and 2 ml of 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 were added,

were used to assess contamination introduced by the tumbling steps or from 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); 0.7% sodium dodecyl sulfate (w/v); 2 mg proteinase K (Qiagen Inc., Valencia, CA). Tubes were placed in a 37 °C rotator with gentle agitation (180 rpm) overnight. The rock powder was separated from the supernatant through low speed centrifugation and mixed with final concentrations of the following: 923 mM NaCl and 1.3% CTAB (cetyltrimethylammonium bromide; w/v). 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 hr at 4°C in a TL-100 ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA) to pellet the precipitated nucleic acids. Pellets were washed with 70% ethanol (v/v), dried in a laminar flow hood at room temperature, and suspended in sterile water. Replicate extracts were combined (40 µl final vol.) 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) in a polycarbonate filter holder (Geotech Environmental Equipment, Inc. Denver, CO) 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 (T-RFLP) analysis

T-RFLP analysis was used to compare the archaeal and bacterial communities from several rock 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-carboxy-fluorescein (6-FAM) and the reverse primer labeled with 5-hexachlorofluorescein (5-HEX). Fifty PCR cycles were necessary to amplify archaeal DNA, while 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'-GGYTACCTTGTTACGACTT) modified from (Lane, 1991) used in the initial PCR reaction (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 PCR reactions (50 µl) for each DNA sample contained final concentrations of the following: 1µl of DNA extract; 1% (v/v) PCR buffer (+ NH_4SO_4; MBI Fermentas, Hanover, MD); 0.2 mM each deoxynucleotide triphosphate; 0.2 µM each primer; 2 mM $MgCl_2$ (MBI Fermentas); 1.2 mg ml^{-1} bovine serum albumin (non-acetylated, SIGMA); 1% (wt/v) PVP (polyvinylpyrrolidone); 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 the number of cycles was reduced to 30.

PCR products (50 ng) were digested with 10 units of enzyme for 6 hrs at 37 ºC 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). 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 (UPGMA) method in PAUP* (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 UPGMA analysis was used to select three basalt samples that differed in age and community structure (D3718B, 9 °N EPR, D3815F and D3823M Juan de Fuca) 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 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. PCR reactions (50 µl vol) were cloned into the pGEM®-T Easy vector (Promega Corp., Madison, WI). 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 phylotype. Clones with restriction patterns that only appeared 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; Maidak et al., 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 to 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) following the manufacturer's protocol. To facilitate amplification spermidine (0.1 $\mu g\ \mu l^{-1}$) 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) overnight at 42 °C. Microarrays were scanned using a ProScanArray (PerkinElmer, Waltham, MA). Images were then analyzed using ImaGene 6.0 (BioDiscovery, El Segundo, CA) to designate the identity of each spot and to determine spot quality. Data was processed as described by Wu et al (2006). Briefly, raw data from Imagene was 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 1/3 of the probes for a particular gene (minimum of 2 probes) was required for a gene to be considered positive. Each gene had 1, 2, or 3 probes per array based on the number of probes available meeting the criteria described by He et al. (2007)

Results and Discussion

T-RFLP

UPGMA 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 UPGMA clustering patterns for the four oldest JdF samples. These old samples, ranging from a few thousand to about three million years in age, clustered together, while 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 to 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, however, there is distinct overlap in the microbial communities regardless of rock age. For example, the basalt specific ocean crust clade presented here, such as the gamma-Proteobacteria ocean crust clade XII, is composed of microorganisms from young, fresh basalts to 3.3 Ma year 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, 3, and 4) were present in basalt samples. None of these clades have been previously reported in marine basalts. Additionally, microorganisms in the alpha-, delta-, and gamma-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 gamma- (25%), alpha- (15%), and delta- (13%), followed by the 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

311 3%/4% Acidobacteria. The similarity in bacterial communities associated with basalts from a
312 broad geographic distribution suggests cosmopolitan distributions of these clades, which is in
313 agreement with findings presented by Mason et al. (2007) and Santelli et al. (2008).
314 Phylogenetic reconstruction revealed three novel ocean crust clades composed entirely of
315 microorganisms associated with basalt. These new clades are the gamma-Proteobacteria ocean
316 crust clade XII (Figure 2), Actinobacteria ocean crust clade XIII (Figure 3), and Planctomycetes
317 ocean crust clade XIV (Figure 3). These clades are comprised of Bacteria sampled from Juan de
318 Fuca (this study), East Pacific Rise, 9 °N (this study and Santelli et al., 2008) and Hawaiian
319 (Santelli et al., 2008) basalts. These cosmopolitan basalt clades may represent ecotypes of
320 Bacteria that are specifically adapted to this environment.
321 Cloning and sequencing of Archaeal 16S rDNA revealed that Marine Benthic Group B
322 (MBGB) were present in basalts (Figure 4). This is the first report of this clade in this
323 environment, as previous studies that examined the archaeal communities in basalts revealed
324 only Marine Group I Crenarchaeota (MGI) (Thorseth et al., 2001; Fisk et al., 2003; Lysnes et al.,
325 2004; Mason et al., 2007). Recently, quantitative analyses of the microbial communities in
326 basalts revealed that Archaea comprise 4-12% and 0.02% or less of the prokaryotic communities
327 (Einen et al., 2008; Santelli et al., 2008), respectively. While these estimates are disparate they
328 do reveal that Archaea are a minor component in the overall microbial communities that reside in
329 basalt. Although Archaea are less prevalent they are ubiquitous in basalts and have been
330 reported in all studies that assayed for their presence (Thorseth et al., 2001; Fisk et al., 2003;
331 Lysnes et al., 2004; Mason et al., 2007; Einen et al., 2008; Santelli et al., 2008). Further, as
332 discussed previously a clade of Marine Group I Archaea appear to be endemic to basalt (Mason
333 et al., 2007). This habitat specificity and global distribution indicates that some Archaea, while

334 less abundant than Bacteria, are particularly adapted to life in basalt and likely play a role in
335 biogeochemical cycling.
336
337 **Functional genes**
338 GeoChip (He et al., 2007) microarray analysis of functional genes in basalt sample
339 D3815F revealed the presence of genes coding for metabolic processes previously unrecognized
340 in this environment. In this analysis a total of 604 probes of the 24 243 total probes present on
341 GeoChip were positive. Specifically, we found genes coding for carbon fixation, methane
342 production and oxidation, nitrogen fixation, ammonium-oxidation, nitrate and nitrite reduction,
343 dissimilatory sulfate reduction, and iron reduction (see Supplementary Table 1 for a complete
344 list).
345 Here we report genes coding for carbon fixation. Basalts lacking a sediment layer are
346 considered to be an oligotrophic, low carbon environment (Edwards et al., 2003a), thus carbon
347 cycling in this habitat is particularly significant. The oligotrophic nature of this environment
348 suggests that carbon fixation would be paramount in this habitat. In fact, chemolithoautotrophic
349 processes in marine subsurface ridge flank hydrothermal environments have been theoretically
350 shown to provide energy that could result in significant microbial biomass ($\sim 1 \times 10^{12}$ g C yr^{-1})
351 (Bach and Edwards, 2003). Therefore, chemolithoautrophic processes occurring *in situ* could
352 serve to underpin a basalt hosted biosphere. One such process is methanogenesis, where
353 hydrogen can serve as the electron donor to reduce carbon dioxide, evolving methane. During
354 fluid-rock interactions when the basalt minerals olivine and pyroxene react with water, hydrogen
355 may be evolved (Janecky and Seyfried Jr, 1986; Berndt et al., 1996). Thus the requisite electron
356 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 ppm (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 to 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-methanogenic 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 Juan de Fuca and 9 °N (this study), and Mohns 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, that 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 does 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 (e.g. 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 Juan de Fuca, 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 suggests that basalts not only harbor a diversity of broadly distributed microbial species, but also unexpected metabolic diversity. Future studies should utilize culture-dependent and -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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References

Ashelford, K.E., Chuzhanova, N.A., Fry, J.C., Jones, A.J., and Weightman, A.J. (2006) New Screening Software Shows that Most Recent Large 16S rRNA Gene Clone Libraries Contain Chimeras. *Appl Environ Microbiol* **72**: 5734-5741.

Bach, W., and Edwards, K.J. (2003) Iron and sulfide oxidation within the basaltic ocean crust: implications for chemolithoautotrophic microbial biomass production. *Geochim Cosmochim Ac* **67**: 3871-3887.

Banerjee, N.R., and Muehlenbachs, K. (2003) Tuff life: Bioalteration in volcaniclastic rocks from the Ontong Java Plateau. *Geochem Geophy Geosy* **4**: 10.1029/2002GC000470.

Berndt, M.E., Allen, D.E., and Seyfried, W.E. (1996) Reduction of CO2 during serpentinization of olivine at 300 °C and 500 bar. *Geology* **24**: 351-354.

Cowen, J.P., Giovannoni, S.J., Kenig, F., Johnson, H.P., Butterfield, D., Rappe, M.S. et al. (2003) Fluids from Aging Ocean Crust That Support Microbial Life. *Science* **299**: 120-123.

DeLong, E.F., Taylor, L.T., Marsh, T.L., and Preston, C.M. (1999) Visualization and Enumeration of Marine Planktonic Archaea and Bacteria by Using Polyribonucleotide Probes and Fluorescent In Situ Hybridization. *Appl Environ Microbiol* **65**: 5554-5563.

DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K. et al. (2006) Greengenes, a Chimera-Checked 16S rRNA Gene Database and Workbench Compatible with ARB. *Appl Environ Microbiol* **72**: 5069-5072.

Desonie, D.L., and Duncan, R.A. (1990) The Cobb-Eickelberg seamount chain: hotspot volcanism with mid-ocean ridge basalt affinity. *J Geophys Res* **95**: 697-692, 711.

Edwards, K.J., Bach, W., and Rogers, D.R. (2003a) Geomicrobiology of the Ocean Crust: A Role for Chemoautotrophic Fe-Bacteria. *Biol Bull* **204**: 180-185.

Edwards, K.J., Rogers, D.R., Wirsen, C.O., and McCollom, T.M. (2003b) Isolation and Characterization of Novel Psychrophilic, Neutrophilic, Fe-Oxidizing, Chemolithoautotrophic alpha- and gamma-Proteobacteria from the Deep Sea. *Appl Environ Microbiol* **69**: 2906-2913.

480 Einen, J., Thorseth, I.H., and Ovreas, L. (2008) Enumeration of Archaea and Bacteria in seafloor
481 basalt using real-time quantitative PCR and fluorescence microscopy. *FEMS Microbiol Lett* **282**:
482 182-187.
483

484 Einen, J., Kruber, C., Øvreas, L., Thorseth, I.H., and Torsvik, T. (2006) Microbial colonization
485 and alteration of basaltic glass. *Biogeosciences Discussions* **3**: 273-307.
486

487 Embley, R.W., Chadwick, W.W., Perfit, M.R., Smith, M.C., and Delaney, J.R. (2000) Recent
488 eruptions on the co-axial segment of the Juan de Fuca Ridge: Implications for mid-ocean ridge
489 accretion processes. *J Geophys Res* **105**: 501-516,525.
490

491 Fisher, A.T. (1998) Permeability within basaltic ocean crust. *Rev Geophys* **36**: 143–182.
492

493 Fisher, A.T., and Becker, K. (2000) Channelized fluid flow in oceanic crust reconciles heat-flow
494 and permeability data. *Nature* **403**: 71-74.
495

496 Fisk, M.R., Giovannoni, S.J., and Thorseth, I.H. (1998) Alteration of Oceanic Volcanic Glass:
497 Textural Evidence of Microbial Activity. *Science* **281**: 978-980.
498

499 Fisk, M.R., Storrie-Lombardi, M.C., Douglas, S., Popa, R., McDonald, G., and Di Meo-Savoie,
500 C.A. (2003) Evidence of biological activity in Hawaiian subsurface basalts. *Geochem Geophy*
501 *Geosy* **4**: 10.1029/2002GC000387.
502

503 Fornari, D.J., and Embley, R.W. (1995) Tectonic and volcanic controls on hydrothermal
504 processes at the mid-ocean ridge: An overview based on near-bottom and submersible studies. In
505 *Seafloor hydrothermal systems* Humphris, S.E., Zierenberg, R.A., Mullineaux L.S., and al., e.
506 (eds), pp. 1-46.
507

508 Furnes, H., and Staudigel, H. (1999) Biological mediation in ocean crust alteration: how deep is
509 the deep biosphere? *Earth Planet Sc Lett* **166**: 97-103.
510

511 Furnes, H., Banerjee, N.R., Muehlenbachs, K., Staudigel, H., and de Wit, M. (2004) Early Life
512 Recorded in Archean Pillow Lavas. *Science* **304**: 578-581.
513

514 Furnes, H., Staudigel, H., Thorseth, I.H., Torsvik, T., Muehlenbachs, K., and Tumyr, O. (2001)
515 Bioalteration of basaltic glass in the oceanic crust. *Geochem Geophy Geosy* **2**.
516

Giovannoni, S.J., DeLong, E.F., Schmidt, T.M., and Pace, N.R. (1990) Tangential flow filtration and preliminary phylogenetic analysis of marine picoplankton. *Appl Environ Microbiol* **56**: 2572-2575.

Giovannoni, S.J., Fisk, M.R., Mullins, T.D., and Furnes, H. (1996) Genetic evidence for endolithic microbial life colonizing basaltic glass-seawater interfaces. *Proc ODP, Sci Results* **148**: 207-214.

Good, I.J. (1953) The Population Frequencies of Species and the Estimation of Population Parameters. *Biometrika* **40**: 237-264.

Hasegawa, M., Kishino, H., and Yano, T. (1985) Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *J Mol Evol* **22**: 160-174.

Haymon, R.M., Fornari, D.J., Von Damm, K.L., Lilley, M.D., Perfit, M.R., Edmond, J.M. et al. (1993) Volcanic eruption of the mid-ocean ridge along the East Pacific Rise crest at 9°45-52'N: direct submersible observations of seafloor phenomena associated with an eruption event in April, 1991. *Earth Planet Sc Lett* **119**: 85-101.

He, Z., Gentry, T.J., Schadt, C.W., Wu, L., Liebich, J., Chong, S.C. et al. (2007) GeoChip: a comprehensive microarray for investigating biogeochemical, ecological and environmental processes. *ISME J* **1**: 67-77.

Huber, J.A., Johnson, H.P., Butterfield, D.A., and Baross, J.A. (2006) Microbial life in ridge flank crustal fluids. *Environ Microbiol* **8**: 88-99.

Janecky, D.R., and Seyfried Jr, W.E. (1986) Hydrothermal serpentinization of peridotite within the oceanic crust: Experimental investigations of mineralogy and major element chemistry. *Geochim Cosmochim Ac* **50**: 1357-1378.

Kendall, M.M., and Boone, D. (2006) Cultivation of methanogens from shallow marine sediments at Hydrate Ridge, Oregon. *Archaea* **1**: 31-38.

Kendall, M.M., Wardlaw, G.D., Tang, C.F., Bonin, A.S., Liu, Y., and Valentine, D.L. (2007) Diversity of Archaea in Marine Sediments from Skan Bay, Alaska, Including Cultivated Methanogens, and Description of Methanogenium boonei sp. nov. *Appl Environ Microbiol* **73**: 407-414.

555 Knittel, K., Losekann, T., Boetius, A., Kort, R., and Amann, R. (2005) Diversity and Distribution
556 of Methanotrophic Archaea at Cold Seeps. *Appl Environ Microbiol* **71**: 467-479.
557

558 Lam, P., Jensen, M.M., Lavik, G., McGinnis, D.F., Müller, B., Schubert, C.J. et al. (2007)
559 Linking crenarchaeal and bacterial nitrification to anammox in the Black Sea. *Proc Natl Acad*
560 *Sci* **104**: 7104–7109.
561

562 Lane, D.J. (1991) 16S/23S rRNA sequencing. In *Nucleic acid techniques in bacterial*
563 *systematics*. Stackenbrandt, E., and Goodfellow, M. (eds). New York: John Wiley, pp. 115-148.
564

565 Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar et al. (2004) ARB: a
566 software environment for sequence data. *Nucl Acids Res* **32**: 1363-1371.
567

568 Lysnes, K., Thorseth, I.H., Steinsbu, B.O., Øvreas, L., Torsvik, T., and Pedersen, R.B. (2004)
569 Microbial community diversity in seafloor basalt from the Arctic spreading ridges. *FEMS*
570 *Microbiol Ecol* **50**: 213-230.
571

572 Maidak, B.L., Olsen, G.J., Larsen, N., Overbeek, R., McCaughey, M.J., and Woese, C.R. (1997)
573 The RDP (Ribosomal Database Project). *Nucl Acids Res* **25**: 109-111.
574

575 Maidak, B.L., Cole, J.R., Parker, C.T., Jr., Garrity, G.M., Larsen, N., Li, B. et al. (1999) A new
576 version of the RDP (Ribosomal Database Project). *Nucl Acids Res* **27**: 171-173.
577

578 Marty, B., Lenoble, M., and Vassard, N. (1995) Nitrogen, helium and argon in basalt: A static
579 mass spectrometry study. *Chem Geol* **120**: 183-195.
580

581 Mason, O.U., Stingl, U., Wilhelm, L.J., Moeseneder, M.M., Di Meo-Savoie, C.A., Fisk, M.R.,
582 and Giovannoni, S.J. (2007) The phylogeny of endolithic microbes associated with marine
583 basalts. *Environ Microbiol* **9**: 2539-2550.
584

585 Mehta, M.P., Huber, J.A., and Baross, J.A. (2005) Incidence of novel and potentially archaeal
586 nitrogenase genes in the deep Northeast Pacific Ocean. *Environ Microbiol* **7**: 1525-1534.
587

588 Moeseneder, M.M., Arrieta, J.M., Muyzer, G., Winter, C., and Herndl, G.J. (1999) Optimization
589 of Terminal-Restriction Fragment Length Polymorphism Analysis for Complex Marine
590 Bacterioplankton Communities and Comparison with Denaturing Gradient Gel Electrophoresis.
591 *Appl Environ Microbiol* **65**: 3518-3525.
592

Nei, M., and Li, W.-H. (1979) Mathematical Model for Studying Genetic Variation in Terms of Restriction Endonucleases. *Proc Natl Acad Sci* **76**: 5269-5273.

Santelli, C.M., Orcutt, B.N., Banning, E., Bach, W., Moyer, C.L., Sogin, M.L. et al. (2008) Abundance and diversity of microbial life in ocean crust. *Nature* **453**: 653-656.

Schmidt, H.A., Strimmer, K., Vingron, M., and von Haeseler, A. (2002) TREE-PUZZLE: maximum likelihood phylogenetic analysis using quartets and parallel computing. *Bioinformatics* **18**: 502-504.

Stahl, D.A., and Amann, R. (1991) Development and application of nucleic acid probes. In *Nucleic acid techniques in bacterial systematics*. Stackebrandt, E., and Goodfellow, M. (eds). Chichester, England: John Wiley and Sons, pp. 205-248.

Swofford, D.L. (1998) PAUP*. Phylogenetic analysis using parsimony (*and other methods). *Sinauer Associates*.

Tal, Y., Watts, J.E.M., Schreier, S.B., Sowers, K.R., and Schreier, H.J. (2003) Characterization of the microbial community and nitrogen transformation processes associated with moving bed bioreactors in a closed recirculated mariculture system. *Aquaculture* **215**: 187–202.

Templeton, A., Staudigel, H., and Tebo, B. (2005) Diverse Mn(II)-Oxidizing Bacteria Isolated from Submarine Basalts at Loihi Seamount. *Geomicrobiol J* **22**: 127-139.

Thorseth, I.H., Torsvik, T., Furnes, H., and Muehlenbachs, K. (1995) Microbes play an important role in the alteration of oceanic crust. *Chem Geol* **126**: 137-146.

Thorseth, I.H., Torsvik, T., Torsvik, V., Daae, F.L., Pedersen, R.B., and Party, K.-S. (2001) Diversity of life in ocean floor basalt. *Earth Planet Sc Lett* **194**: 31-37.

Torsvik, T., Furnes, H., Muehlenbachs, K., Thorseth, I.H., and Tumyr, O. (1998) Evidence for microbial activity at the glass-alteration interface in oceanic basalts. *Earth Planet Sc Lett* **162**: 165-176.

Vergin, K.L., Rappé, M.S., and Giovannoni, S.J. (2001) Streamlined Method to Analyze 16S rRNA Gene Clone Libraries. *BioTechniques* **30**: 938-943.

Watson, S.W. (1965) Characteristics of a marine nitrifying bacterium, Nitrosocystis oceanus sp. n. *Limnol Oceanogr* **10(Suppl.):R274-R289**.

Watson, S.W., and Waterbury, J.B. (1971) Characteristics of two marine nitrite oxidizing bacteria, Nitrospina gracilis nov. gen. nov. sp. and Nitrococcus mobilis nov. gen. nov. sp. *Arch Microbiol* **77**: 203-230.

Watson, S.W., Graham, L.B., Remsen, C.C., and Valois, F.W. (1971) A lobular, ammonia-oxidizing bacterium, Nitrosolobus multiformis Nov. Gen. Nov. sp. *Arch Microbiol* **76**: 183-203.

Wu, L., Liu, X., Schadt, C.W., and Zhou, J. (2006) Microarray-Based Analysis of Subnanogram Quantities of Microbial Community DNAs by Using Whole-Community Genome Amplification. *Appl Environ Microbiol* **72**: 4931-4941.

Figure legends

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.

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 alpha-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 Bacteria 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 Archaea 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 alpha-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.

Tables.

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
East Pacific Rise (EPR) R/V Atlantis Voyage 7 Leg 3							
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	"	T-RFLP
D3718B	10/24/01	09° 50.78' N	104° 17.58' W	2493	north of Q vent	"	T-RFLP, cloning & sequencing[d]
D3719D	10/25/01	09° 50.78' N	104° 17.58' W	2496	near M vent	"	T-RFLP
D3720R	10/26/01	09° 50.78' N	104° 17.58' W	2498	near TY vent	"	T-RFLP
D3721D	10/27/01	09° 50.79' N	104° 17.59' W	2495	near Q vent	"	T-RFLP
D3721E	10/27/01	09° 50.79' N	104° 17.59' W	2496	near Q vent	"	T-RFLP
Juan de Fuca Ridge (JdF) R/V Atlantis Voyage 7 Leg 19							
D3815F	8/05/02	45° 59.50' N	129° 56.59 W	2135	Helium Basin	<100 Ka	T-RFLP, cloning & 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 Ma	T-RFLP, cloning & sequencing[d]
D3826U	8/23/02	46° 31.16' N	129° 34.92 W	2409	lava flow	1993 eruption	T-RFLP

[a]The Alvin dive number and suffix is the sample identifier.

[b]The 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).

[c]The 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).

[d]D3718B 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.

[e]D3815F clones are presented in this study.

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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

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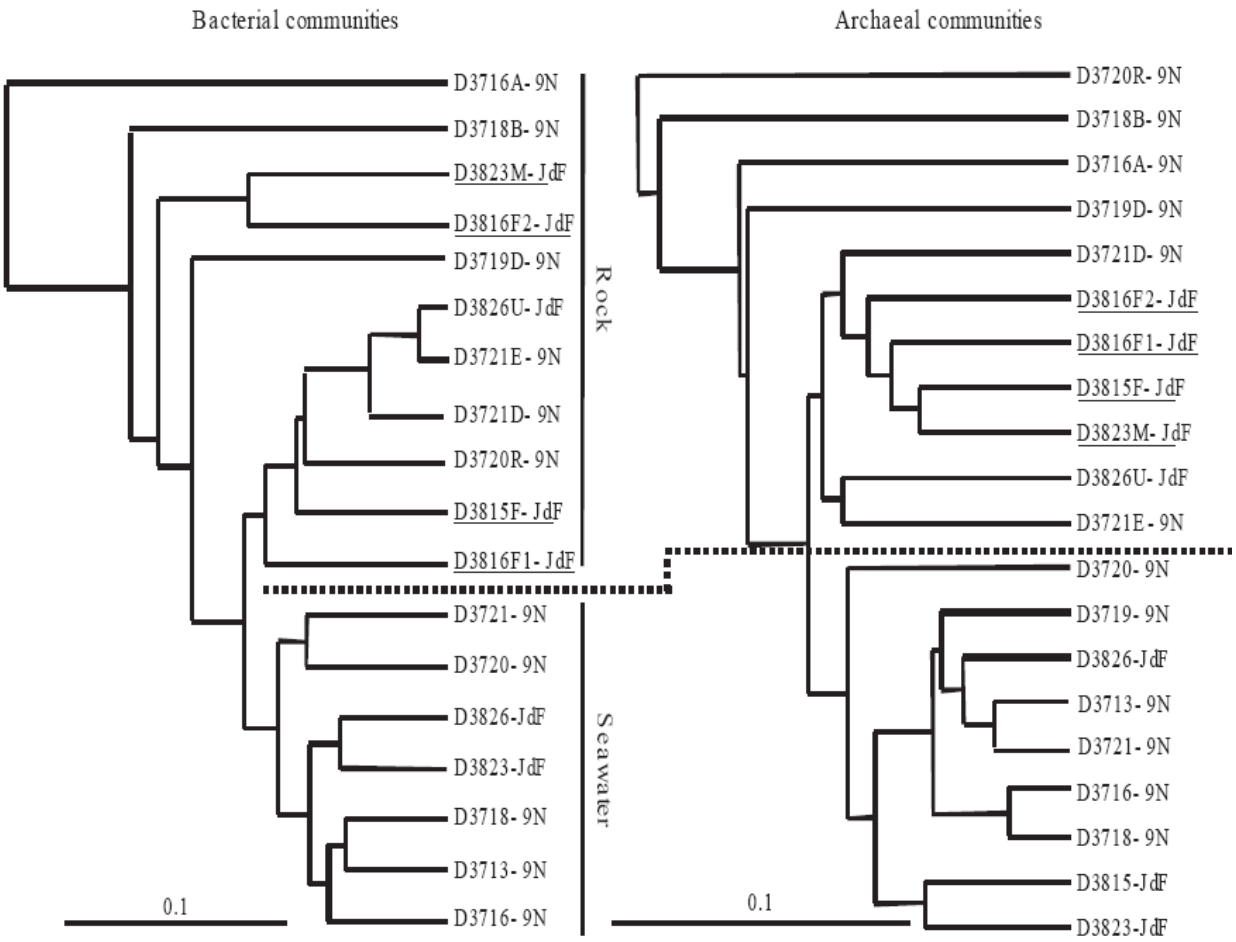
697 **Figures.**



698 **Figure 1.**

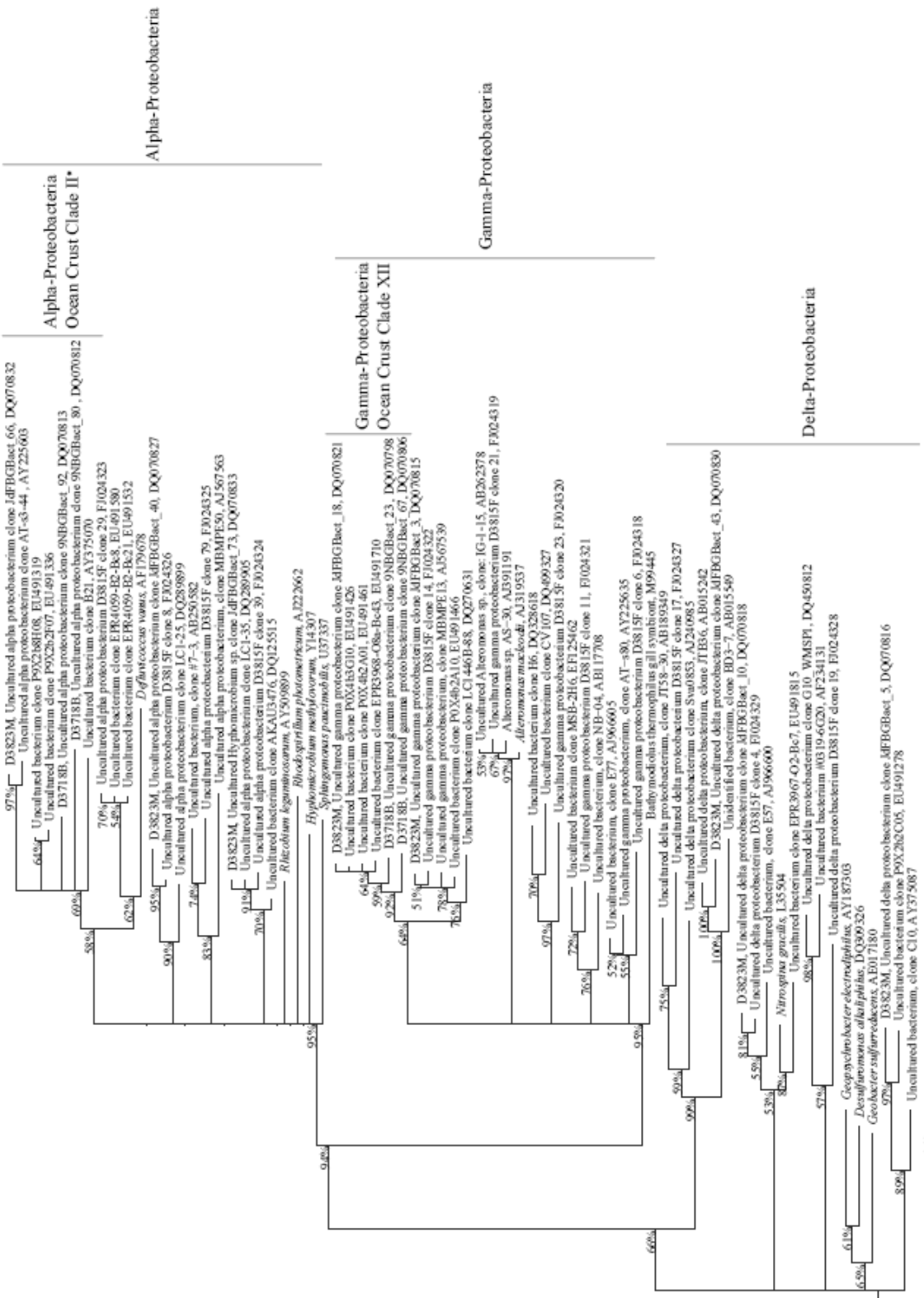

Alpha-Proteobacteria
Alpha-Proteobacteria Ocean Crust Clade II*
Gamma-Proteobacteria Ocean Crust Clade XII
Gamma-Proteobacteria
Delta-Proteobacteria
D3823M, Uncultured alpha proteobacterium clone JdFBGBact_66, DQ070832
Uncultured alpha proteobacterium clone AT-s3-44 , AY225603
Uncultured bacterium clone P9X2b8H08, EU491319
Uncultured bacterium clone P9X2b2F07, EU491336
D3718B, Uncultured alpha proteobacterium clone 9NBGBact_92, DQ070813
D3718B, Uncultured alpha proteobacterium clone 9NBGBact_80 , DQ070812
Uncultured bacterium clone B21, AY375070
Uncultured alpha proteobacterium D3815F clone 29, FJ024323
Uncultured bacterium clone EPR4099-B2-B8, EU491580
Uncultured bacterium clone EPR4099-B2-B21, EU491532
Dyadobacter vanus, AF179678
D3823M, Uncultured alpha proteobacterium clone JdFBGBact_40, DQ070827
Uncultured alpha proteobacterium D3815F clone 8, FJ024326
Uncultured alpha proteobacterium clone LC1-25, DQ289899
Uncultured bacterium, clone #7--3, AB250582
Uncultured alpha proteobacterium D3815F clone 79, FJ024325
Uncultured alpha proteobacterium, clone MBMPE50, AJ567563
D3823M, Uncultured Hyphomicrobium sp. clone JdFBGBact_73, DQ070833
Uncultured alpha proteobacterium clone LC1-35, DQ289905
Uncultured bacterium D3815F clone 39, FJ024324
Uncultured bacterium clone AKAU3476, DQ125515
Rhizobium leguminosarum, AY509899
Rhodospirillum photometricum, AJ222662
Hyphomicrobium methylovorum, Y14307
Sphingomonas paucimobilis, U37337
D3823M, Uncultured gamma proteobacterium clone JdFBGBact_18, DQ070821
Uncultured bacterium clone P0X4h3G10, EU491426
Uncultured bacterium clone P0X4h2A01, EU491461
Uncultured bacterium clone EPR3968-O8a-Bc43, EU491710
D3718B, Uncultured gamma proteobacterium clone 9NBGBact_23 , DQ070798
D3718B, Uncultured gamma proteobacterium clone 9NBGBact_67, DQ070806
D3823M, Uncultured gamma proteobacterium clone JdFBGBact_3, DQ070815
Uncultured gamma proteobacterium D3815F clone 14, FJ024325
Uncultured gamma proteobacterium, clone MBMPE13, AJ567539
Uncultured bacterium clone P0X4h2A10, EU491466
Uncultured bacterium clone LC1-446B-83, DQ270631
Uncultured Alteromonas sp., clone: IG-1-15, AB262378
Uncultured gamma proteobacterium D3815F clone 21, FJ024319
Alteromonas sp. AS--30, AJ391191
Alteromonas macleodii, AJ319537
Uncultured bacterium clone H6, DQ328618
Uncultured bacterium clone CV107, DQ499327
Uncultured gamma proteobacterium D3815F clone 23, FJ024320
Uncultured bacterium clone MSB-2H6, EF125462
Uncultured gamma proteobacterium D3815F clone 11, FJ024321
Uncultured bacterium, clone NB--04, AB117708
Uncultured bacterium, clone E77, AJ966605
Uncultured gamma proteobacterium, clone AT--s80, AY225635
Uncultured gamma proteobacterium D3815F clone 6, FJ024318
Butyrivibrio thermophilus gill symbiont, M99445
Uncultured delta proteobacterium, clone JTS8--30, AB189349
Uncultured delta proteobacterium D3815F clone 17, FJ024327
Uncultured delta proteobacterium clone Sva0853, AJ240985
Uncultured delta proteobacterium, clone JTB36, AB015242
D3823M, Uncultured delta proteobacterium clone JdFBGBact_43, DQ070830
Unidentified bacterium, clone BD3--7, AB015549
D3823M, Uncultured delta proteobacterium clone JdFBGBact_10, DQ070818
Uncultured delta proteobacterium D3815F clone 4, FJ024329
Uncultured bacterium, clone E57, AJ966600
Nitrospina gracilis, L35504
Uncultured bacterium clone EPR3967-O2-Bc7, EU491815
Uncultured delta proteobacterium clone G10_WMSP1, DQ450812
Uncultured bacterium #0319-6G20, AF234131
Uncultured delta proteobacterium D3815F clone 19, FJ024328
Geopsychrobacter electrodiphilus, AY187303
Desulfuromonas alkaliphilus, DQ300326
Geobacter sulfurreducens, AE017180
D3823M, Uncultured delta proteobacterium clone JdFBGBact_5, DQ070816
Uncultured bacterium clone P9X2b2C05, EU491278
Uncultured bacterium, clone C10, AY375087
0.10

Figure 2.

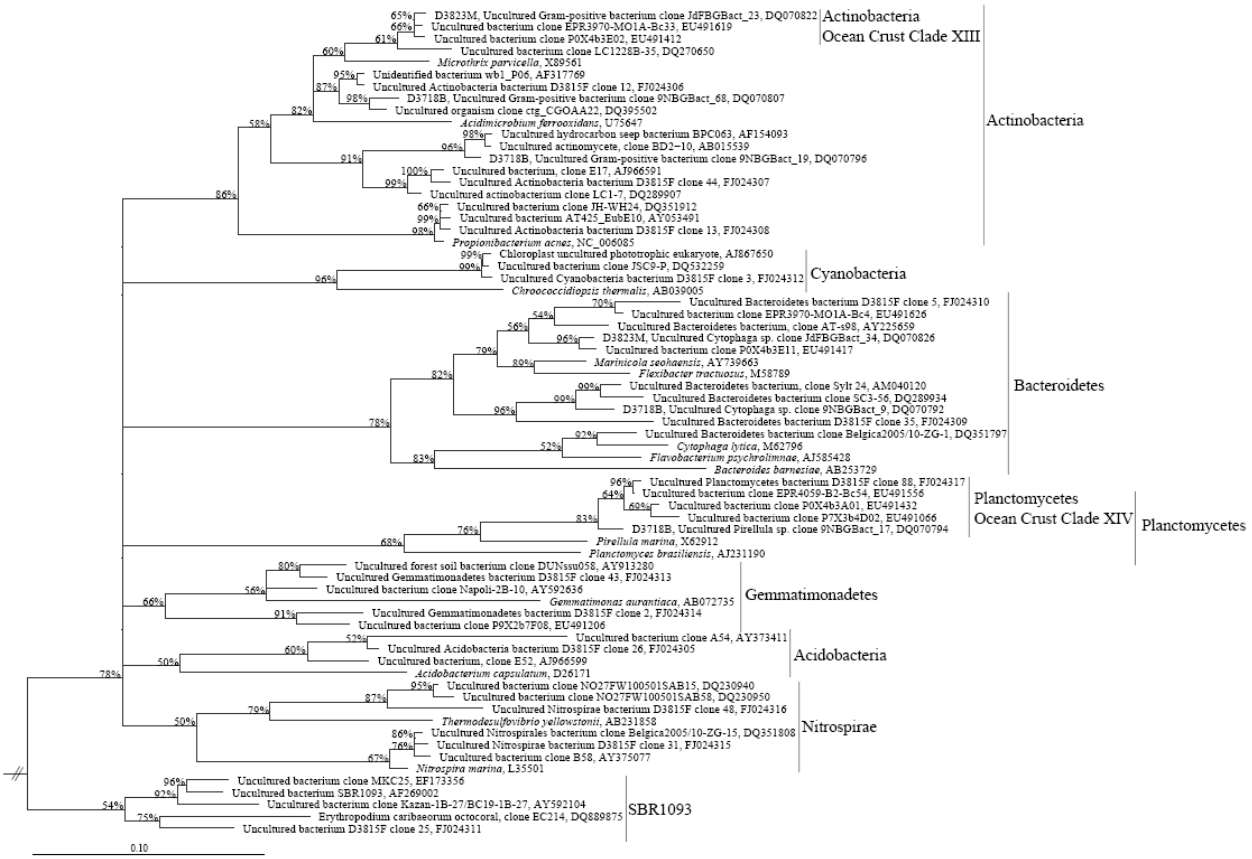


Figure 3.

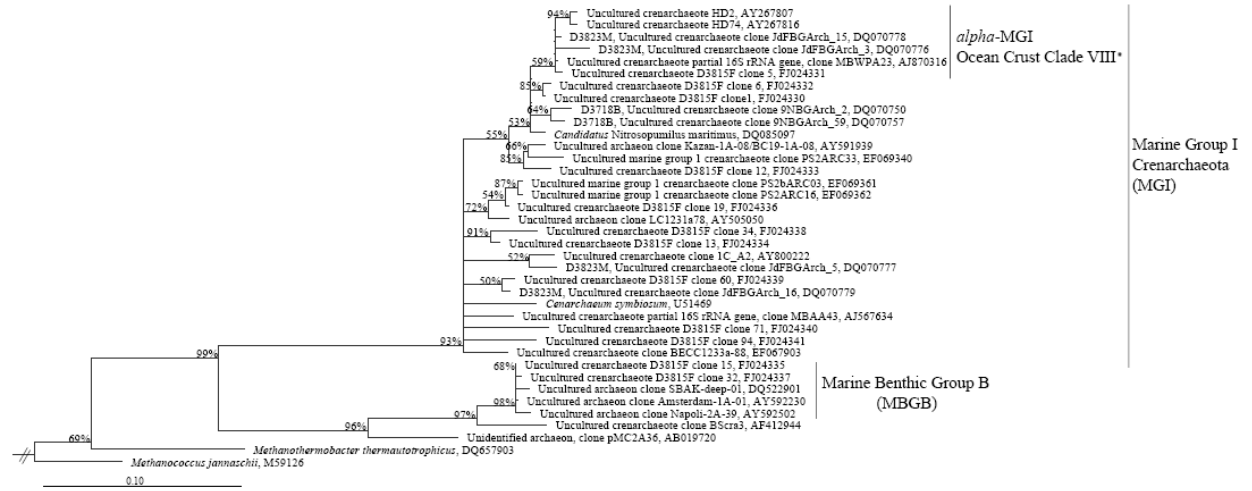


Figure 4.

Supplementary Table 1. Functional genes present in basalt sample D3815F determined by microarray analysis.

Genbank ID	Gene short description	Gene category	Organism
23012702	0	Carbon fixation	Magnetospirillum magnetotacticum
12407235	aclB	Carbon fixation	Chlorobium limicola
30721807	FTHFS	Carbon fixation	Methylobacterium extorquens
32307749	rbcL	Carbon fixation	uncultured bacterium
505126	rbcL	Carbon fixation	Hydrogenophilus thermoluteolus
37791353	rbcL	Carbon fixation	uncultured proteobacterium
7592878	rbcL	Carbon fixation	uncultured deep-sea autotrophic bacterium ORII-2
7592885	rbcL	Carbon fixation	uncultured deep-sea autotrophic bacterium ORII-5
37791373	rbcL	Carbon fixation	uncultured proteobacterium
21217703	rbcL	Carbon fixation	uncultured bacterium
7229162	rbcL	Carbon fixation	uncultured deep-sea autotrophic bacterium SBI-5
132036	rbcL	Carbon fixation	Rhodospirillum rubrum
7592852	rbcL	Carbon fixation	uncultured deep-sea autotrophic bacterium SBII-4
6778693	dsrA	Sulfate reduction	uncultured sulfate-reducer HMS-25
40253098	dsrA	Sulfate reduction	uncultured sulfate-reducing bacterium
FW015084A	dsrA	Sulfate reduction	lab clone
34017136	dsrA	Sulfate reduction	uncultured bacterium
34017154	dsrA	Sulfate reduction	uncultured bacterium
13898425	dsrA	Sulfate reduction	uncultured phenanthrene mineralizing bacterium
20501981	dsrA	Sulfate reduction	uncultured sulfate-reducing bacterium
FW010274A	dsrA	Sulfate reduction	lab clone
13898437	dsrA	Sulfate reduction	uncultured phenanthrene mineralizing bacterium
12667570	dsrA	Sulfate reduction	uncultured sulfate-reducing bacterium UMTRAdsr626-27
7262420	dsrA	Sulfate reduction	uncultured sulfate-reducer HMS-54
20501993	dsrA	Sulfate reduction	uncultured sulfate-reducing bacterium
34017094	dsrA	Sulfate reduction	uncultured bacterium
7262428	dsrA	Sulfate reduction	uncultured sulfate-reducer HMS-24
20502017	dsrA	Sulfate reduction	uncultured sulfate-reducing bacterium
14090290	dsrA	Sulfate reduction	Desulfomicrobium escambiense
14389123	dsrA	Sulfate reduction	uncultured sulfate-reducing bacterium
25990790	dsrA	Sulfate reduction	uncultured bacterium
40253034	dsrA	Sulfate reduction	uncultured sulfate-reducing bacterium
14389119	dsrA	Sulfate reduction	uncultured sulfate-reducing bacterium
15055587	dsrA	Sulfate reduction	Desulfococcus multivorans
22900884	dsrA	Sulfate reduction	uncultured bacterium
22999275	dsrA	Sulfate reduction	Magnetotactic cocci
14276799	dsrA	Sulfate reduction	Desulfotomaculum geothermicum

6778709	dsrA	Sulfate reduction	uncultured sulfate-reducer HMS-4
12667674	dsrA	Sulfate reduction	uncultured sulfate-reducing bacterium UMTRAdsr826-16
TPB16340A	dsrA	Sulfate reduction	lab clone
20501983	dsrA	Sulfate reduction	uncultured sulfate-reducing bacterium
FW300226A	dsrA	Sulfate reduction	lab clone
6179922	dsrB	Sulfate reduction	Solar Lake Mat Clone9065
18034323	dsrB	Sulfate reduction	Desulfacinum infernum
FW005271B	dsrB	Sulfate reduction	lab clone
13249527	dsrB	Sulfate reduction	uncultured sulfate-reducing bacterium
13561055	dsrB	Sulfate reduction	Desulfosarcina variabilis
TPB16051B	dsrB	Sulfate reduction	lab clone
15076856	dsrB	Sulfate reduction	Desulfosporosinus orientis
FW003272B	dsrB	Sulfate reduction	lab clone
14389217	dsrB	Sulfate reduction	uncultured sulfate-reducing bacterium
FW003264B	dsrB	Sulfate reduction	lab clone
28974756	dsrB	Sulfate reduction	uncultured bacterium
3892198	dsrB	Sulfate reduction	Archaeoglobus profundus
15077475	dsrB	Sulfate reduction	Desulfovibrio desulfuricans subsp. desulfuricans
FW015318B	dsrB	Sulfate reduction	lab clone
13249551	dsrB	Sulfate reduction	uncultured sulfate-reducing bacterium
FW010117B	dsrB	Sulfate reduction	lab clone
10716971	dsrB	Sulfate reduction	unidentified sulfate-reducing bacterium
TPB16055B	dsrB	Sulfate reduction	lab clone
28974734	dsrB	Sulfate reduction	uncultured bacterium
21673682	dsrB	Sulfate reduction	Chlorobium tepidum TLS
FW003269B	dsrB	Sulfate reduction	lab clone
TPB16070B	dsrB	Sulfate reduction	lab clone
13249539	dsrB	Sulfate reduction	uncultured sulfate-reducing bacterium
34017190	dsrB	Sulfate reduction	uncultured bacterium
40253070	dsrB	Sulfate reduction	uncultured sulfate-reducing bacterium
39998311	cytochrome	Metal resistance/reduction	Geobacter sulfurreducens PCA
24372200	cytochrome	Metal resistance/reduction	Shewanella oneidensis MR-1
39995722	cytochrome	Metal resistance/reduction	Geobacter sulfurreducens PCA
39996424	cytochrome	Metal resistance/reduction	Geobacter sulfurreducens PCA
24373346	cytochrome	Metal resistance/reduction	Shewanella oneidensis MR-1
39998423	cytochrome	Metal resistance/reduction	Geobacter sulfurreducens PCA
39935825	cytochrome	Metal resistance/reduction	Rhodopseudomonas palustris CGA009
2865528	cytochrome	Metal resistance/reduction	Shewanella putrefaciens
39998372	cytochrome	Metal resistance/reduction	Geobacter sulfurreducens PCA
39998004	cytochrome	Metal	Geobacter sulfurreducens PCA

		resistance/reduction	
39997392	cytochrome	Metal resistance/reduction	Geobacter sulfurreducens PCA
23475584	cytochrome	Metal resistance/reduction	Desulfovibrio desulfuricans G20
39996164	cytochrome	Metal resistance/reduction	Geobacter sulfurreducens PCA
39937295	cytochrome	Metal resistance/reduction	Rhodopseudomonas palustris CGA009
145083	cytochrome	Metal resistance/reduction	Desulfovibrio vulgaris
15022433	mcr	Methane generation	Treponema medium
12802200	mcrA	Methane generation	uncultured archaeon 85A
38570220	mcrA	Methane generation	uncultured euryarchaeote
13259189	mcrA	Methane generation	uncultured methanogen RS-MCR04
38570178	mcrA	Methane generation	uncultured euryarchaeote
38570176	mcrA	Methane generation	uncultured euryarchaeote
799197	mcrA	Methane generation	Methanolobus oregonensis
34305116	mcrA	Methane generation	uncultured archaeon
13259303	mcrA	Methane generation	uncultured methanogen RS-ME32
7445687	mcrA	Methane generation	Methanothermobacter thermautotrophicus
34305109	mcrG	Methane generation	uncultured archaeon
20094092	mcrG	Methane generation	Methanopyrus kandleri AV19
6002398	mmo	Methane oxidation	Methylomonas sp. KSPIII
141050	mmo	Methane oxidation	Methylococcus capsulatus
6002402	mmo	Methane oxidation	Methylomonas sp. KSPIII
6002409	mmo	Methane oxidation	Methylomonas sp. KSWIII
2098700	mmo	Methane oxidation	Methylocystis sp. M
37813016	mmoA	Methane oxidation	uncultured bacterium
34915630	mmoA	Methane oxidation	uncultured methanotrophic proteobacterium
21685061	mmoA	Methane oxidation	Methylocella palustris
37813004	mmoA	Methane oxidation	uncultured bacterium
2098698	mmoA	Methane oxidation	Methylocystis sp. M
11038435	mmoA	Methane oxidation	uncultured putative methanotroph
37813020	mmoA	Methane oxidation	uncultured bacterium
7188932	pmo	Methane oxidation	Methylosinus trichosporium
7188937	pmo	Methane oxidation	Methylocystis sp. M
34733039	pmoA	Methane oxidation	uncultured bacterium
37496853	pmoA	Methane oxidation	uncultured bacterium
6424923	pmoA	Methane oxidation	uncultured eubacterium pAMC512
7578614	pmoA	Methane oxidation	uncultured bacterium FW-47
11493646	nifD	Nitrogen fixation	Azoarcus sp. BH72
29293348	nifH	Nitrogen fixation	uncultured bacterium
12001854	nifH	Nitrogen fixation	uncultured bacterium NR1611
13173333	nifH	Nitrogen fixation	uncultured bacterium
780721	nifH	Nitrogen fixation	unidentified marine eubacterium
780717	nifH	Nitrogen fixation	unidentified marine eubacterium
22449921	nifH	Nitrogen fixation	uncultured bacterium
12659182	nifH	Nitrogen fixation	Spirochaeta zuelzerae
3157500	nifH	Nitrogen fixation	unidentified nitrogen-fixing bacteria

10863131	nifH	Nitrogen fixation	marine stromatolite eubacterium HB(0898) Z02
1236929	nifH	Nitrogen fixation	Anabaena variabilis
10863141	nifH	Nitrogen fixation	marine stromatolite eubacterium HB(0697) A100
12659198	nifH	Nitrogen fixation	Treponema azotonutricium
3157624	nifH	Nitrogen fixation	unidentified nitrogen-fixing bacteria
13173301	nifH	Nitrogen fixation	uncultured bacterium
3157594	nifH	Nitrogen fixation	unidentified nitrogen-fixing bacteria
1255464	nifH	Nitrogen fixation	unidentified bacterium
22449901	nifH	Nitrogen fixation	uncultured bacterium
19070843	nifH	Nitrogen fixation	unidentified nitrogen-fixing bacteria
13173319	nifH	Nitrogen fixation	uncultured bacterium
29649383	nifH	Nitrogen fixation	uncultured nitrogen-fixing bacterium
13173335	nifH	Nitrogen fixation	uncultured bacterium
33385573	nifH	Nitrogen fixation	uncultured bacterium
780713	nifH	Nitrogen fixation	unidentified marine eubacterium
1572591	nifH	Nitrogen fixation	Desulfovibrio gigas
29293188	nifH	Nitrogen fixation	uncultured bacterium
13173305	nifH	Nitrogen fixation	uncultured bacterium
5701924	nifH	Nitrogen fixation	Paenibacillus polymyxa
3157704	nifH	Nitrogen fixation	unidentified nitrogen-fixing bacteria
12001832	nifH	Nitrogen fixation	uncultured bacterium NR1600
12001842	nifH	Nitrogen fixation	uncultured bacterium NR1605
3157506	nifH	Nitrogen fixation	unidentified nitrogen-fixing bacteria
20804123	nifH	Nitrogen fixation	Mesorhizobium loti
22450003	nifH	Nitrogen fixation	uncultured bacterium
22988609	0	Nitrification	Rhodobacter sphaeroides
7595786	amoA	Nitrification	unidentified bacterium
7544069	amoA	Nitrification	Nitrosomonas halophila
7578632	amoA	Nitrification	uncultured bacterium WC306-54
27529221	amoA/pmoA	Nitrification	uncultured bacterium
26278794	narG	Denitrification	uncultured bacterium
29652478	narG	Denitrification	uncultured bacterium
26278922	narG	Denitrification	uncultured bacterium
26278882	narG	Denitrification	uncultured bacterium
29652532	narG	Denitrification	uncultured bacterium
38427014	narG	Denitrification	uncultured bacterium
26278770	narG	Denitrification	uncultured bacterium
32308011	narG	Denitrification	uncultured bacterium
32307889	narG	Denitrification	uncultured bacterium
38427060	narG	Denitrification	uncultured bacterium
32307981	narG	Denitrification	uncultured bacterium
26278784	narG	Denitrification	uncultured bacterium
29652590	narG	Denitrification	uncultured bacterium
29652508	narG	Denitrification	uncultured bacterium
38427022	narG	Denitrification	uncultured bacterium
29652428	narG	Denitrification	uncultured bacterium
26278684	narG	Denitrification	uncultured bacterium
32307917	narG	Denitrification	uncultured bacterium

26278870	narG	Denitrification	uncultured bacterium
17385544	narG	Denitrification	uncultured bacterium
26278676	narG	Denitrification	uncultured bacterium
30269569	nasA	Denitrification	uncultured bacterium
30269577	nasA	Denitrification	uncultured bacterium
12597209	nirK	Denitrification	Alcaligenes sp. STC1
3758830	nirK	Denitrification	Hyphomicrobium zavarzinii
NBPd1-B05	nirK	Denitrification	lab clone
27125563	nirK	Denitrification	uncultured bacterium
37999212	nirK	Denitrification	uncultured bacterium
ORA-NIRK-C01	nirK	Denitrification	lab clone
1488172	nirK	Denitrification	Rhizobium sullae
3758901	nirK	Denitrification	Rhodobacter sphaeroides f. sp. denitrificans
28542653	nirS	Denitrification	uncultured bacterium
24421455	nirS	Denitrification	uncultured organism
24421507	nirS	Denitrification	uncultured organism
37999198	nirS	Denitrification	uncultured bacterium
24421269	nirS	Denitrification	uncultured organism
24528368	nirS	Denitrification	uncultured bacterium
28542627	nirS	Denitrification	uncultured bacterium
38455926	nirS	Denitrification	uncultured bacterium
24421271	nirS	Denitrification	uncultured organism
22252866	nirS	Denitrification	uncultured bacterium
34391466	norB	Denitrification	Nitrosomonas europaea
29466090	norB	Denitrification	uncultured bacterium
29466092	norB	Denitrification	uncultured bacterium
38373207	nosZ	Denitrification	uncultured bacterium
3057083	nosZ	Denitrification	Paracoccus pantotrophus
29125972	nosZ	Denitrification	uncultured soil bacterium
13959038	nosZ	Denitrification	Azospirillum lipoferum
4633572	nosZ	Denitrification	uncultured bacterium ProR
14994626	nosZ	Denitrification	uncultured bacterium

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