LETTERS

Abundance and diversity of microbial life in ocean crust

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Oceanic lithosphere exposed at the sea floor undergoes seawater–rock alteration reactions involving the oxidation and hydration of glassy basalt. Basalt alteration reactions are theoretically capable of supplying sufficient energy for chemolithoautotrophic growth[1]. Such reactions have been shown to generate microbial biomass in the laboratory[2], but field-based support for the existence of microbes that are supported by basalt alteration is lacking. Here, using quantitative polymerase chain reaction, *in situ* hybridization and microscopy, we demonstrate that prokaryotic cell abundances on seafloor-exposed basalts are 3–4 orders of magnitude greater than in overlying deep sea water. Phylogenetic analyses of basaltic lavas from the East Pacific Rise (9° N) and around Hawaii reveal that the basalt-hosted biosphere harbours high bacterial community richness and that community membership is shared between these sites. We hypothesize that alteration reactions fuel chemolithoautotrophic microorganisms, which constitute a trophic base of the basalt habitat, with important implications for deep-sea carbon cycling and chemical exchange between basalt and sea water.

We assessed the abundance, species richness and phylogenetic diversity of endolithic and epilithic microbial communities inhabiting young, unsedimented ocean crust at the sea floor. Basaltic seafloor lavas from the East Pacific Rise (EPR), 9° N (Fig. 1a; Supplementary Fig. 1a, b), were sampled to represent various ages (up to 20 thousand years) and alteration states (Fig. 1b–e; Supplementary Table 1) for analysis. Quantitative polymerase chain reaction (qPCR) measurements (Supplementary Table 2) of the glassy rinds of lava flows indicate total bacterial and archaeal cell densities range from $\sim 3 \times 10^6$ to $\sim 1 \times 10^9$ cells per g rock (10^7 to 3×10^9 gene copies per g rock). Bacteria dominated (88–96%) all samples examined. Catalysed reporter deposition–fluorescent *in situ* hybridization (CARD–FISH) analyses revealed dense populations of Bacteria on basalt surfaces, which were significantly more abundant than Archaea (Fig. 2a–c). In contrast, the total microbial cell number of deep ocean waters is only 8×10^3 cells per ml sea water to 9×10^4 cells per ml sea water[3,4] in the deep ocean (>1,000 m), half of which are Archaea.

The bacterial community composition was evaluated using full-length 16S ribosomal RNA gene clone libraries that were constructed from basalt samples and surrounding sea water. Statistical approaches were applied to evaluate species richness, the number of operational taxonomic units (OTUs) of these communities, as compared to other environments. We define an OTU at 97% sequence similarity—a commonly recognized level for comparative analysis. Rarefaction was used to compare bacterial richness of the

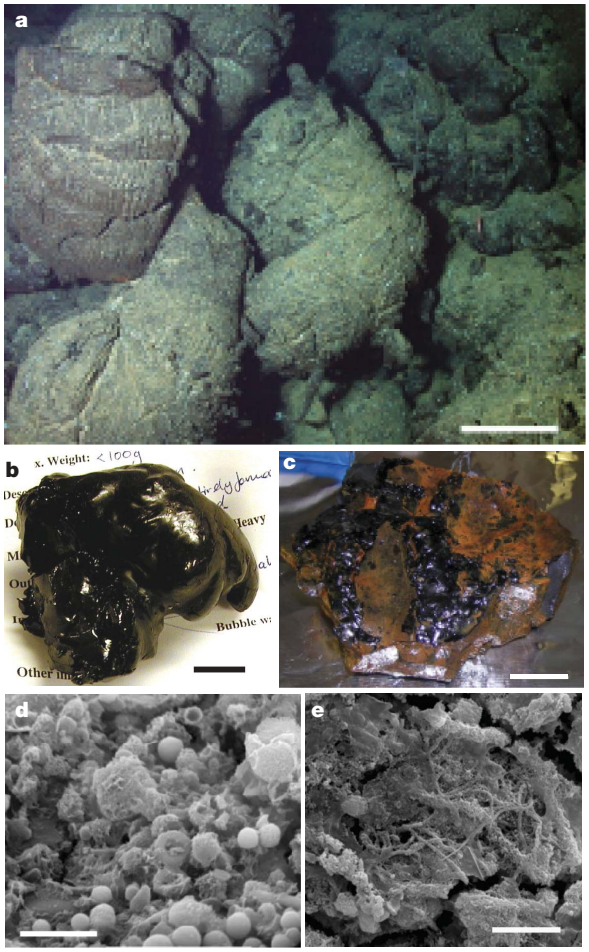

Figure 1 | A highly diverse and abundant epilithic and endolithic microbial community exists on basaltic lavas from the East Pacific Rise. a, The EPR at 9° N is characterized by lava flows, such as pillow basalts that outcrop at the sea floor. Scale bar, 40 cm. **b**, **c**, Photographs showing the range of volcanic samples used in this study from fresh and glassy (**b**) to more altered and oxide-coated (**c**). Scale bars, 2 cm (**b**) and 4 cm (**c**). **d**, **e**, Scanning electron microscopy (SEM) images of different presumed cellular morphologies, such as coccoidal (**d**) and filamentous (**e**) structures that were observed on ferromanganese-oxide-encrusted and iron-oxide-coated samples. Scale bars, 5 μm.

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collective EPR basalts with that of other oceanic habitats (Fig. 3a) and other known high-diversity environments (Fig. 3b). Although richness varies among the samples (Fig. 3c), cumulative treatment is warranted here for comparison with the selective studies, which similarly sum multiple samples from a single habitat (see Supplementary Information). The comparative oceanic habitats included a hydrothermal white smoker from the EPR[5], the upper water column of the Sargasso Sea[6], hydrothermal vent fluids from the Mid-Atlantic Ridge (MAR) captured in an *in situ* growth chamber[7], and deep-subsurface sediments from the Nankai Trough[8]. High-diversity environments included for comparison were terrestrial farm soil[9] and a hypersaline microbial mat from the Guerrero Negro[10]. Although the environments were not sampled to saturation, rarefaction analysis revealed that EPR basalts harbour greater bacterial richness than the other marine habitats (Fig. 3a), and are most comparable to richness in mat and soil microbial communities (Fig. 3b).

To test whether the EPR basalt richness is site-specific or common among seafloor-exposed basalts, we also examined 16S rRNA gene clone libraries generated from lavas collected from offshore the big island of Hawaii (hereafter referred to as Hawaii; Supplementary Fig. 1a, c and Supplementary Table 1). The individual (Fig. 3c) and cumulative (Fig. 3b) richness of the Hawaii basalts as estimated by rarefaction analysis are similar to the EPR richness estimates.

Comparative rarefaction results were corroborated by nonparametric-based estimates of total richness and diversity (Supplementary Table 3). The Chao1 total richness estimate for the cumulative EPR basalts is 440 OTUs (lower and upper 95% confidence intervals of 338 and 609 OTUs). The Chao1 estimate for farm soil, the high-diversity end-member, is 1,410 OTUs (95% confidence interval of 1,223–1,658 OTUs), whereas the EPR deep-seawater survey, the low-diversity end-member, is 12 OTUs (95% confidence interval of 11–22 OTUs). Because nonparametric estimators produce widely varying results

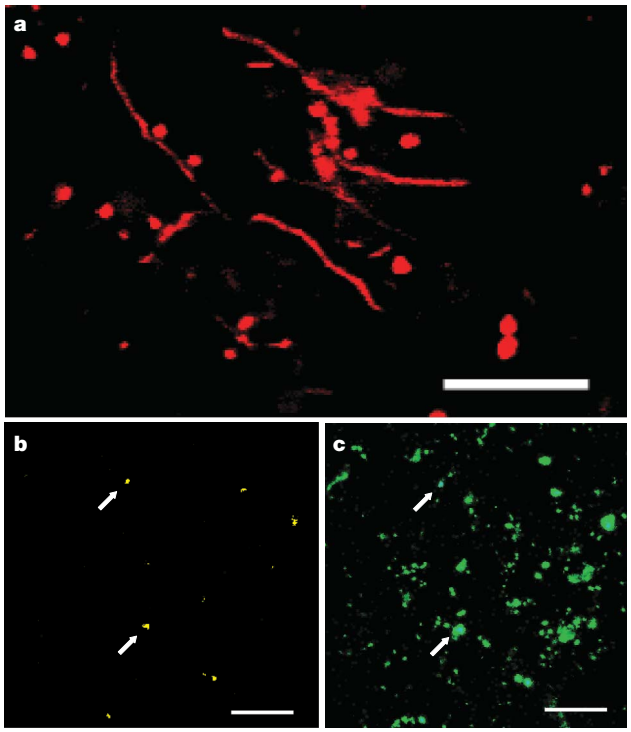

Figure 2 | Confocal laser scanning micrographs of cells hybridized with domain-specific fluorescent probes (via CARD–FISH) on lava surfaces. Microbial cells were hybridized with either probe EUB338(I-III) mix (**a**) or probe ARCH915 (**b**) to target Bacteria or Archaea, respectively. A variety of bacterial cell morphologies such as filaments, cocci and rods were confirmed with hybridizations (**a**). Side-by-side comparisons of Archaea (**b**) versus total cells (**c**) reveals that Archaea account for only a small portion of the total cells. Total cells were identified with the general DNA stain SYBR green. The arrows point to some cells that overlap in each frame. Scale bars, 10 μm (**a**) and 20 μm (**b**, **c**).

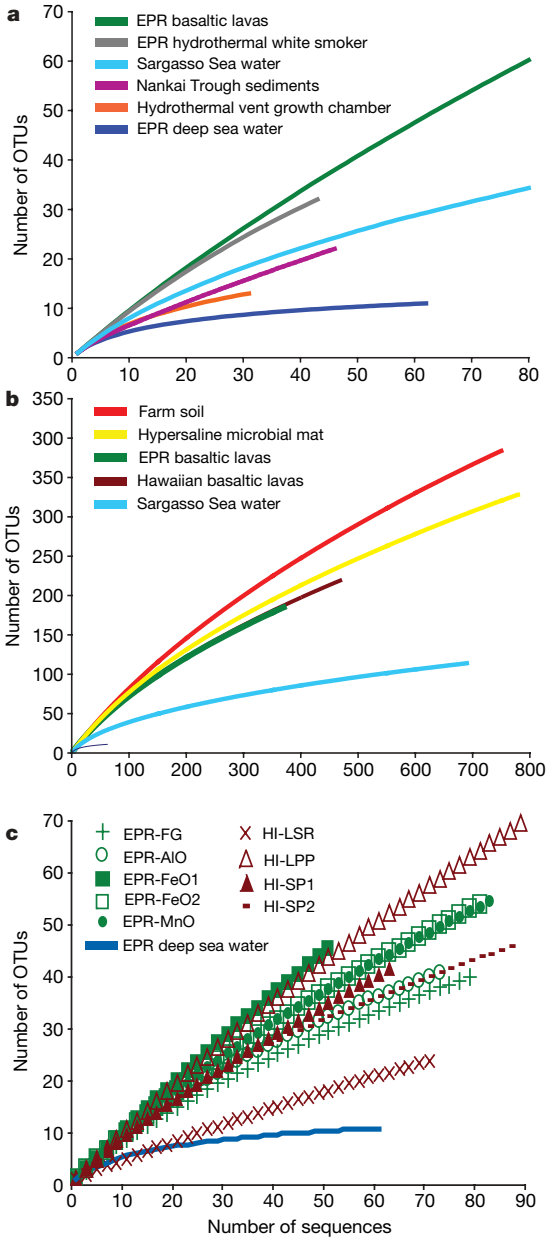

Figure 3 | Relative bacterial richness from several environmental studies shown through rarefaction analyses. **a**, Species richness of Bacteria inhabiting EPR seafloor lavas (cumulative results) is compared with that of other ocean environments, such as the Sargasso Sea[6] (partial curve), a MAR hydrothermal vent *in situ* growth chamber[7], an EPR hydrothermal white smoker spire[5], Nankai Trough deep-sea sediments[8], and EPR deep sea water. The bacterial richness of the EPR basalts is also compared to a basalt-hosted community from Hawaii and other known high-richness environments (**b**), such as a farm soil[9] (partial curve) and a hypersaline microbial mat from the Guerrero Negro[10] (partial curve). Partial rarefaction curves are shown for visualization purposes; however, complete data sets were used in calculating curve projections. **c**, Rarefaction curves for the individual EPR and Hawaii basalt clone libraries. A partial curve is shown for HI-LPP (total clones = 246). Comparative studies in **a** and **b** are based on near full-length 16S rRNA gene sequences, and most studies are the sum of several environmental samples. OTUs are defined at a sequence similarity of ≥ 97%. FG, fresh glass; LSR, Loihi seamount South Rift; LPP, Loihi seamount Pisces Peak; SP1 & SP2, South Point samples.

and underestimate diversity[11,12], we do not interpret these results as accurate predictions of true species richness. Rather, these indicators support our rarefaction observations of the relative differences between environments. This secondary comparison is informative because rarefaction curves can intersect and cross with increased sampling effort, particularly in high-diversity communities in which curves do not plateau[13]. Results showed that the relative estimates of diversity were consistent among the different environments, regardless of the estimator or index used. One caveat of all comparative measurements is that the specific methods used to generate sequences (that is, DNA extraction protocol, PCR amplification, clone library construction) differed slightly in each study, which could influence the observed and predicted phylogenetic diversity. Our conclusions concerning the diversity of basalts relative to other habitats, however, seem unlikely to be altered by these differences.

Examination of the taxonomic groups obtained from various environments (Supplementary Fig. 2) reveals that the phylogenetic diversity of Bacteria in seafloor basalts is distinct from the biotopes of the other habitats that were used for comparison. The 21 taxonomic groups recovered from basalt are dominated by sequences belonging to the Proteobacteria phylum (all subdivisions), which account for 68% and 66% of all sequences in the EPR and Hawaii basalt libraries, respectively (EPR/Hawaii convention is used hereafter). Non-Proteobacteria groups recovered include the Plantomycetes (8%/5%, EPR/Hawaii), Actinobacteria (7%/8%), Bacteroidetes (4%/1%), Acidobacteria (3%/4%) and Verrucomicrobia (2%/2%). The other phyla recovered represent $<2\%$ each of the total clone library, and a small portion (6%/1%) could not be assigned a taxonomic group. Notably, estimates of shared OTU richness for the two geographically separated basalt communities (Supplementary Fig. 3) show considerable overlap in community membership, suggesting that oceanic basalt microbes are widely distributed among this biotope.

The phylogenetic distribution and richness of Bacteria in the other deep-sea environments are lower than deep-sea basalts (Supplementary Fig. 2). The deep seawater library reveals three taxonomic groups, all Proteobacteria: γ-proteobacteria (89% of the total library), α-proteobacteria and β-proteobacteria. Other deep-seawater studies[14,15] have recovered additional taxa belonging to δ-proteobacteria, Bacteroidetes and Planctomycetes. Rarefaction analysis including those taxa (see Supplementary Information and Supplementary Figs 4 and 5) does not, however, significantly increase species richness. To assess the influence of hydrothermal source proximity on phylogenetic diversity, we compare the basalt data with clone library data constructed from several mineralogical layers in a hydrothermal white smoker spire at the EPR[5]. Supplementary Fig. 2 shows that the hydrothermal spire harbours different and fewer taxonomic groups, and in particular prevalence of ε-Proteobacteria, Aquificales and Thermales (microbes typically found in hydrothermal environments[16]) which infrequently occur in the other deep-sea libraries (basalts and sea water).

The differences in phylogenetic diversity, species richness, and total biomass between the basaltic lavas and overlying sea water raise questions about what energy source(s) fuel this biosphere. Potential energy sources capable of sustaining microbial life in basaltic ocean crust include hydrothermal input of both dissolved and particulate manganese and iron phases to the seafloor environment that could provoke redox reactions capable of supporting chemolithoautotrophic growth[1,17]. Reduction reactions with iron or manganese as the electron acceptors could occur if enough hydrogen is present and anaerobic conditions exist. Dissolved organic carbon in sea water or hydrothermal fluids[18] could also provide energy to heterotrophic microorganisms. It is suggested, however, that most dissolved organic carbon in the deep sea is recalcitrant to microbial oxidation[19], but this remains largely unexplored.

Alteration reactions occurring on basalt surfaces could support microbial growth[1]. We inferred that such reactions contribute to biomass production within the basalt-hosted biotope and examined the possibility from a theoretical perspective. Lava surfaces are composed predominantly of volcanic glass, a highly reactive rock component that contains reduced elemental species such as iron, sulphur and manganese. Oxygen and nitrate in deep sea water oxidize these reduced constituents, and chemolithoautotrophic microorganisms could potentially exploit the free energy changes associated with these redox reactions for their metabolic requirements[1].

Support for this hypothesis is provided by laboratory studies that demonstrate that chemolithoautotrophic iron-oxidizing bacteria isolated from the sea floor are able to use rock and minerals, including basalt glass, for metabolism and growth[2]. Furthermore, biomass production calculations[1] suggest that the observed oxidation of iron and sulphur in the upper ocean crust, if entirely mediated by microorganisms, can support the fixation of up to $\sim 5 \times 10^{11}$ g C yr^{-1}. Assuming $\sim 4 \times 10^{15}$ g of fresh basalt yr^{-1} are subject to oxidation reactions[1] and a single cell contains 200 fg C (ref. 20), we estimate that 6×10^{7} cells per g rock may be supported through alteration reactions (if microbial growth efficiency is 0.1 and biomass turnover occurs once per year). These parameters can be adjusted for more conservative estimates preferring a more oligotrophic or slower growing environment in which more energy goes into cell maintenance rather than cell growth, as in deep-sea sediments[21] (for example, 6×10^{9} cells per g basalt could be produced if carbon content is 20 fg C $cell^{-1}$, growth efficiency is 0.01, and biomass turnover is 100 years). Cell densities in volcanic glasses from the EPR established through qPCR measurements, $\sim 10^{6}$ to 10^{9} cells per g basalt, fall within the range of predicted densities of $\sim 10^{7}$ to 10^{9} cells per g basalt based on steady state iron and sulphur oxidation rates.

Our calculations suggest that alteration reactions in the upper ocean crust may fuel microbial ecosystems at the sea floor and contribute to biomass production and diversity in these systems. This hypothesis supports the understanding of the phylogenetically rich and distinct nature of the basalt biotope, otherwise it may be anticipated that the basalt-hosted community would more closely match that of the surrounding environment(s), that is, sea water in the case of unsedimented mid-ocean ridges like the EPR. Enrichment of taxa from diverse metabolic groups may result from the establishment of micro-environments within or on rock cavities and surfaces during alteration, secondary mineral precipitation and biofilm formation. Niche creation would allow for a greater variety of redox reactions and metabolic pathways (for example, heterotrophic, anaerobic, or reductive) within small spatial scales, including those supporting organotrophic and mixotrophic communities. The observed microbial biomass and phylogenetic diversity may consequently be an expression of the range of diverse chemical microenvironments that develop during basalt alteration.

METHODS SUMMARY

Sample collection and analysis. Basaltic seafloor lavas were collected from the EPR at 9° N (Supplementary Fig. 1a, b), during R/V *Atlantis* research cruises AT11-7 and AT11-20 and around Hawaii (Supplementary Fig. 1a, c) during *Ka'imikai-o-Kanaloa* cruise KOK 02-24 using the submersibles *Alvin* and *Pisces V*, respectively. DNA was extracted using the Ultraclean soil DNA kit (MoBio Laboratories) with minor modifications. Near full-length 16S rRNA gene clone libraries were constructed as previously described[22] and sequenced at the Josephine Bay Paul Center (JBPC) at the Marine Biological Laboratories (MBL).

Phylogenetic analysis. Sequences were assembled and edited using an automated pipeline at the JBPC and aligned with the ARB software package (http://www.arb-home.de/). Phylogenetic affiliation was assigned with 'Classifier' available through the Ribosomal Database Project[23] using an 80% confidence threshold. Rarefaction analysis and species richness estimates were performed using the program Distance Based OTU and Richness Determination (DOTUR)[24], and the program Shared OTUs and Similarity (SONS)[25] was used for shared community richness estimates. Sequences from the MAR hydrothermal vent *in situ* growth chamber[7], from the Guerrero Negro hypersaline microbial mat[10] and from the EPR hydrothermal white smoker spire[5] were accessed from GenBank. The full Minnesota farm soil and Nankai Trough deep

subsurface sediment[8] data sets were provided with permissions from the authors[9]. The Sargasso Sea[6] data, previously analysed in ref. 24, were downloaded from the DOTUR website (http://www.plantpath.wisc.edu/fac/joh/DOTUR.html).

CARD–FISH and quantatitive PCR analysis. For CARD–FISH analyses, paraformaldehyde-fixed samples were hybridized with horseradish-peroxidase-linked oligonucleotide probes EUB338-I, EUB338-II and EUB338-III (ref. 26) or ARCH915 (ref. 27) with Cy3-tyramides (Biomers.net). SYBR Green I DNA stain (Invitrogen) was used as a counterstain. Digital images were collected on a LSM510-META laser scanning confocal microscope (Carl Zeiss) at the MBL and processed with the LSM Image Browser version 3.5 and Adobe Photoshop. Environmental DNA from lavas was used for qPCR to quantify bacterial[28] and archaeal[29] 16S rDNA gene copies. Cell densities were approximated using a conversion of 4.1 or 1.6 copies cell for Bacteria or Archaea respectively[30].

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions Phylogenetic analyses were performed by C.M.S. for EPR samples, and B.N.O. and E.B. for Hawaii samples; microscopy was done by C.M.S. Biomass calculations were done by W.B. C.L.M. helped C.M.S. with qPCR analyses. M.L.S. supported Hawaii studies and the pipeline for phylogenetic studies. H.S. provided ship access for Hawaii studies. K.J.E. and W.B. developed and guided this project, and K.J.E., C.M.S. and B.N.O. developed and wrote the paper with input from co-authors.

Author Information Sequences are deposited in the GenBank database under accession numbers EU491521–EU491952 (for EPR basalts) and EU491020–EU491491 (for Hawaii basalts). Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to K.J.E (kje@usc.edu).

METHODS

Sample collection. Basaltic seafloor lavas were collected from the EPR at 9° N (Supplementary Fig. 1a, b) during R/V *Atlantis* research cruises AT11-7 and AT11-20 and from around Hawaii (Supplementary Fig. 1a, c) during the *Ka'imikai-o-Kanaloa* cruise KOK 02-24 using the submersibles *Alvin* and *Pisces V*, respectively. Lavas were placed in bioboxes containing sterile fresh water, allowed to fill with ambient bottom sea water, sealed to minimize contamination, and brought to the surface for processing. Ambient EPR deep sea water was collected at the sea floor with Niskins bottles. Five basaltic lavas from the EPR and four Hawaii basalts (Supplementary Table 1) were used for analyses.

DNA extraction and sequencing. The outer ≤1.5 cm glass portion of each was crushed to millimetre-sized or smaller fractions using asceptic techniques after collection. For the EPR basalts, subsamples were either processed at once or frozen (−80 °C) for less than 24 h until DNA was extracted. Hawaii rock fragments were stored for ~3 yr at −80 °C before DNA extraction. Environmental DNA was extracted from ~1 g crushed basalt using the Ultraclean soil DNA kit (MoBio Laboratories) after a modified manufacturer's protocol. Before bead beating, samples were incubated for 10 min at 70 °C, after which 200 μg of polyadenylic acid (polyA) was added[31]. For the deep-seawater sample, 50 ml of fluid was filtered (0.2-μm-pore-size), and extractions were performed directly on the filter. The 16S rRNA region of environmental DNA was amplified using primer set 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′).

Amplification products were purified using the QIAquick nucleotide removal kit (Qiagen). Clone libraries were constructed from amplification products as described in a previous publication[22]. Plasmid extractions (alkaline lysis) and sequencing (ABI v 3.1 BigDye terminator, Applied Biosystems) were performed at the Keck Facility of the JBPC, MBL, Woods Hole, Massachusetts.

Phylogenetic analysis. Sequence reads were edited and assembled using an automated pipeline, straw, from the JBPC (http://jbpc.mbl.edu/computing-seqinformatics.html). Finalized sequences were aligned using ARB (http://www.arb-home.de/). Chimaeric sequences were identified with Bellerophon[32] (EPR basalts) and Mallard[33] (Hawaiian Island basalts). Putative chimaeras were checked against sequences of several closest relatives (determined by BLAST (Basic Local Alignment Search Tool))[34] using ARB, as well as with the RDP-II chimera detection program[23].

BLAST was used to for comparison against the GenBank 16S rRNA database for phylogenetic assignments. ARB was used to generate distance matrices using the neighbour-joining method, application of a filter for bacteria (positions 1218–42590), and the Jukes–Cantor correction. DOTUR[24] was used for rarefaction analysis and richness estimates using a 97% sequence similarity definition. Shared richness estimates were calculated using SONS[25]. Sequences from the hydrothermal vent *in situ* growth chamber[7] were accessed from GenBank. The farm soil sequences were provided with authors' permission[9]. Sargasso Sea data[6], previously analysed in ref. 24, was downloaded from (http://www.plantpath.wisc.edu/fac/joh/DOTUR/).

Quantatitive PCR. Real-time qPCR using the Taqman approach (Applied Biosystems) was used to quantify bacterial and archaeal 16S rDNA gene copies from environmental DNA. The qPCR assay to estimate Archaea used the primer set Arch349F (5′-GYGCASCAGKCGMGAAW-3′) and Arch806R (5′-GGACTACVSGGGTATCTAAT-3′) with probe Arch516F ((6-FAM)-5′-TGYCAGCCGCCGCGGTAAHACCVGC-3′-(Iowa Black FQ)), previously designed and tested in ref 29. Primers and probe were synthesized by Integrated DNA Technologies. Primers 331F (5′-TCCTACGGGAGGCAGCAGT-3′) and 797R (5′-GGACTACCAGGGTATCTAATCCTGTT-3′) and TaqMan probe (6-FAM)-5′-CGTATTACCGCGGCTGCTGGCAC-3′-(TAMRA) (Thermo Electron Corporation) were used for bacterial DNA quantification[28].

Assays on an ABI Prism 7000 Sequence Detection System in 96-well optical grade plates used the following reagents: 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 1 unit Platinum Taq (Invitrogen), 1× ROX (Invitrogen), 800 nM forward primer, 800 nM reverse primer, 200 nM Taqman probe, template (3 μl), and molecular grade water (total volume of 30 μl). Amplification conditions were 50 °C for 2 min, 95°C for 10 min, and 45 cycles of 95 °C for 15 s and 59°C for 3 min. All reactions (standards, samples, and blanks) were performed in triplicate and included a no template control.

Standard curves for quantifying gene copies were determined using purified PCR products from a mixture of archaeal and bacterial plasmid DNA of known concentration (10 ng μl^{-1} each) isolated from microbial mats at hydrothermal vents along the Mariana Island Arc[35]. For DNA quantification, serial dilutions ($1\times - 10^{-6}\times$ concentration) of PCR products were used to construct standard curves for Bacteria ($E = 94\%$, $r^2 = 0.99$, detection limit = 1.5×10^{-5} ng DNA per reaction) and Archaea ($E = 76\%$, $r^2 = 0.99$, detection limit = 1.5×10^{-5} ng DNA $reaction^{-1}$). DNA concentrations were converted to gene copy number assuming one double-stranded DNA molecule has a mass of 660 g mol^{-1}. Conversion factors of 4.1 (16S rDNA gene copies $cell^{-1}$) and 1.6 were used to convert bacterial and archaeal gene copies to cell numbers[30].

CARD–FISH and microscopy. Lavas were fixed in 4% (w/v) paraformaldehyde, rinsed, and stored in PBS:ethanol (1:1) at −20 °C. Hybridizations were performed as described previously[36,37]. Hybridizations were performed at 35 °C with 55% formamide concentration. Bacteria or Archaea were targeted using horseradish peroxidase-linked oligonucleotide probes EUB338-I, EUB338-II and EUB338-III (ref. 26) or ARCH915 (ref. 27), with Cy3-tyramides synthesized by Biomers.net; SYBR Green I DNA stain (Invitrogen) was used as counterstain. An LSM510-META laser scanning confocal microscope (Carl Zeiss) based on an Axioskop 2FS fixed-stage upright with an Achroplan IR ×63/0.90W (water immersion) objective was used with a HeNe laser (543 nm wavelength) and Ar ion laser (488 nm wavelength) at the MBL Central Microscopy Center. LSM Image Browser v3.5 (Zeiss) was used to create three-dimensional image projections from *z*-stacks, and Adobe Photoshop was used for minimal image processing (cropping and adjusting contrast and/or brightness)

SEM samples were obtained at the MBL Microscopy Center with a JEOL 840 SEM operated at 15 kV.

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