LETTERS

Deep sub-seafloor prokaryotes stimulated at interfaces over geological time

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The sub-seafloor biosphere is the largest prokaryotic habitat on Earth[1] but also a habitat with the lowest metabolic rates[2]. Modelled activity rates are very low, indicating that most prokaryotes may be inactive or have extraordinarily slow metabolism[2]. Here we present results from two Pacific Ocean sites, margin and open ocean, both of which have deep, subsurface stimulation of prokaryotic processes associated with geochemical and/or sedimentary interfaces. At 90 m depth in the margin site, stimulation was such that prokaryote numbers were higher (about 13-fold) and activity rates higher than or similar to near-surface values. Analysis of high-molecular-mass DNA confirmed the presence of viable prokaryotes and showed changes in biodiversity with depth that were coupled to geochemistry, including a marked community change at the 90-m interface. At the open ocean site, increases in numbers of prokaryotes at depth were more restricted but also corresponded to increased activity; however, this time they were associated with repeating layers of diatom-rich sediments (about 9 Myr old). These results show that deep sedimentary prokaryotes can have high activity, have changing diversity associated with interfaces and are active over geological timescales.

Recently, subsurface prokaryotes have been found to be ubiquitous on Earth (for example, in sediments, rocks, aquifers, mines, basalts and crustal fluids, oil reservoirs and ice sheets[3]). In the dark and remote from photosynthetically produced organic matter these environments are among the lowest-energy-flux habitats known[4], with metabolic rates 10^3–10^5 times lower than in near-surface sediments[5]. Their enormous prokaryotic biomass[1] has therefore been questioned, particularly for the largest habitat, namely sub-seafloor sediments[2]. In certain terrestrial habitats higher prokaryotic activity occurs at deep interfaces such as sandstone-shale[6] or sandstone-clay[7], showing active prokaryotic metabolism over geological timescales (for example the Cretaceous period[6]). Although deep stimulation of prokaryotic activity occurs in some sub-seafloor sediments[8,9], the impact of interfaces has not been fully explored, including biodiversity changes expected in dynamic prokaryotic

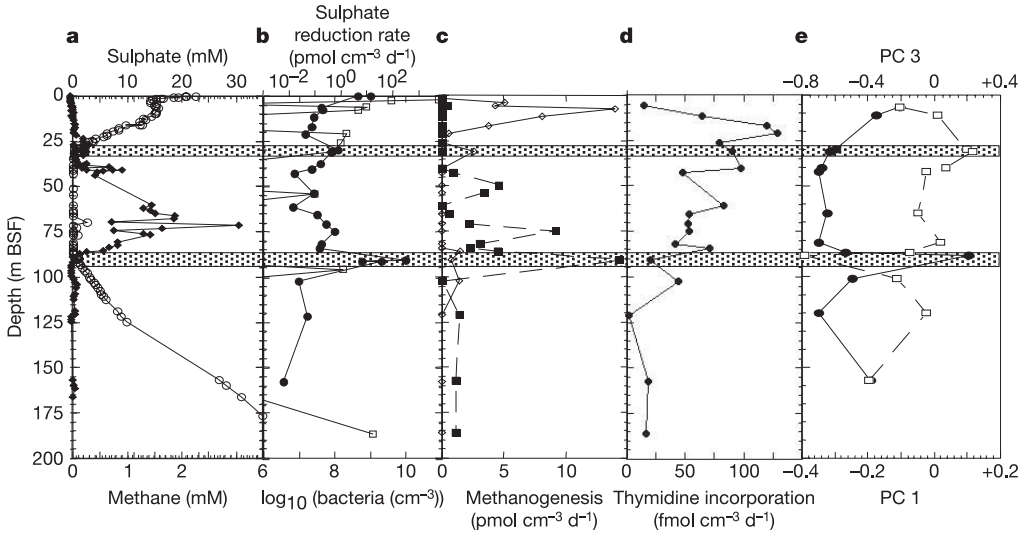

Figure 1 | Biogeochemical process and prokaryotic biodiversity profiles at the Peru margin site (ODP 1229). **a**, Geochemistry[29]: open circles, pore water sulphate; filled diamonds, methane. **b**, Open squares, sulphate reduction rates; filled circles, total population of prokaryotes. **c**, Methanogenic rates: Open diamonds, H_2/CO_2; filled squares, acetate. **d**, Growth rates, measured as thymidine incorporation. **e**, Principal-components profile of diversity of Bacteria from a DGGE analysis of 16S rRNA gene sequences: filled circles, component 1 (56% of variation); open squares, component 3 (9% of variation). Component 2 (24% of variation) had a similar profile to that of component 1. Shaded boxes highlight elevated prokaryotic processes and sulphate/methane interfaces.

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communities adapted to local environments[10]. Prokaryotic stimulation and adaptation has been shown for shallow (4 m) marine layers that are high in organic matter[11] and suggested in deeper (58 m) clays with volcanic ash layers in the absence of geochemical or activity measurements[12]. Here we investigated changes in prokaryotic activity, population size and composition in deep marine sediments (more than 400 m) from the east Pacific Ocean with deep geochemical or lithological interfaces[13].

Sediments were from a continental margin and open ocean site (water depths of 150.5 and 3,297 m, respectively; Ocean Drilling Program (ODP) Leg 201). The margin site was unusual in having a deep brine incursion, so sulphate was present both in the near surface, from sea water, and at depth (Fig. 1a). A high content of organic matter in sediment (2–8% (ref. 14)) greatly stimulated prokaryotic activity, resulting in high populations of prokaryotes (6.5×10^8 cm^{-3}) and high rates of anaerobic reduction of sulphate (about 6,000 pmol cm^{-3} d^{-1}; Fig. 1b) with sulphate reaching zero at about 35 m below sea floor (BSF). Below this depth, biogenic methane increased to about 2,000 μM between 65 and 75 m BSF.

Separation between zones of sulphate and methane is normally interpreted as sulphate-reducing bacteria outcompeting methanogens for limiting substrates[5], and hence the restriction of methanogenesis to deeper, sulphate-free sediments. However, our results show methanogenesis (about 15 pmol cm^{-3} d^{-1}; Fig. 1c) within the sulphate zone, so in high-organic-matter sediments active methanogenesis can coexist with sulphate reduction[15]. The absence of methane in this zone (Fig. 1a) is therefore probably due to methane consumption, presumably by an anaerobic consortium of methane oxidizers and sulphate-reducing bacteria[16]. This process is normally intensified at the sulphate/methane interface[17] and probably accounts for the increase in prokaryotic populations ($P < 0.001$), numbers of dividing and divided cells (data not shown) at about 30 m BSF (6.3-fold and 1.3-fold increase, respectively) and sulphate reduction rates (above and at the interface). There is also an increase in rates of H_2/CO_2 methanogenesis. Prokaryotic stimulation is repeated and intensified at the lower sulphate/methane interface, about 90 m, where total populations and rates of methanogenesis increase markedly (61-fold and 31-fold, respectively; $P < 0.001$). In addition there is a peak of sulphate reduction at the bottom of the interface (about 95 m BSF). At this interface prokaryotic populations are considerably larger (13-fold; $P < 0.001$) and rates of methanogenesis are comparable to those near the surface. Further, acetate becomes the major methanogenic substrate, in contrast to H_2/CO_2 methanogenesis near the surface; there is therefore a shift in carbon flow. Increases in acetate methanogenesis occur at depth at other sites and was related to warming and activation of organic matter with depth[8]. However, increased methane oxidation also occurs[18] and this might result in increased acetate formation, either as a direct intermediate of anaerobic methane oxidation or as a product of recycling dead cells associated with greater prokaryotic biomass.

Prokaryotic growth, measured by thymidine incorporation, is greater in the subsurface than at the surface, with a maximum above the top sulphate/methane interface (Fig. 1d). In the methane zone, thymidine incorporation gradually decreases and reaches low levels that are maintained in the deep sulphate zone. There is no correlation between thymidine incorporation and other prokaryotic measurements, which might reflect the inability of some prokaryotes, including many sulphate-reducing bacteria and methanogens, to incorporate thymidine[19]. Thymidine incorporation is therefore probably reflecting an actively growing subset of the total population.

Gene libraries of 16S rRNA were obtained from high-molecular-mass DNA from all four depths (6.7, 30.2, 42.03 and 86.67 m BSF) although, as expected, extractable DNA yields were low[20,21]. However, diversity in Bacteria was high, with sequences from at least seven major phyla or class-level groups (Fig. 2a, see also Supplementary Information). Gammaproteobacteria were numerous in the shallowest (6.7 m BSF) and dominated the deepest (near the 90-m interface) libraries, whereas green non-sulphur (GNS) bacteria, although abundant throughout, dominated the libraries within the methane zone (30.2 and 42.03 m BSF). There was therefore a distinctly different bacterial community in the near-surface and

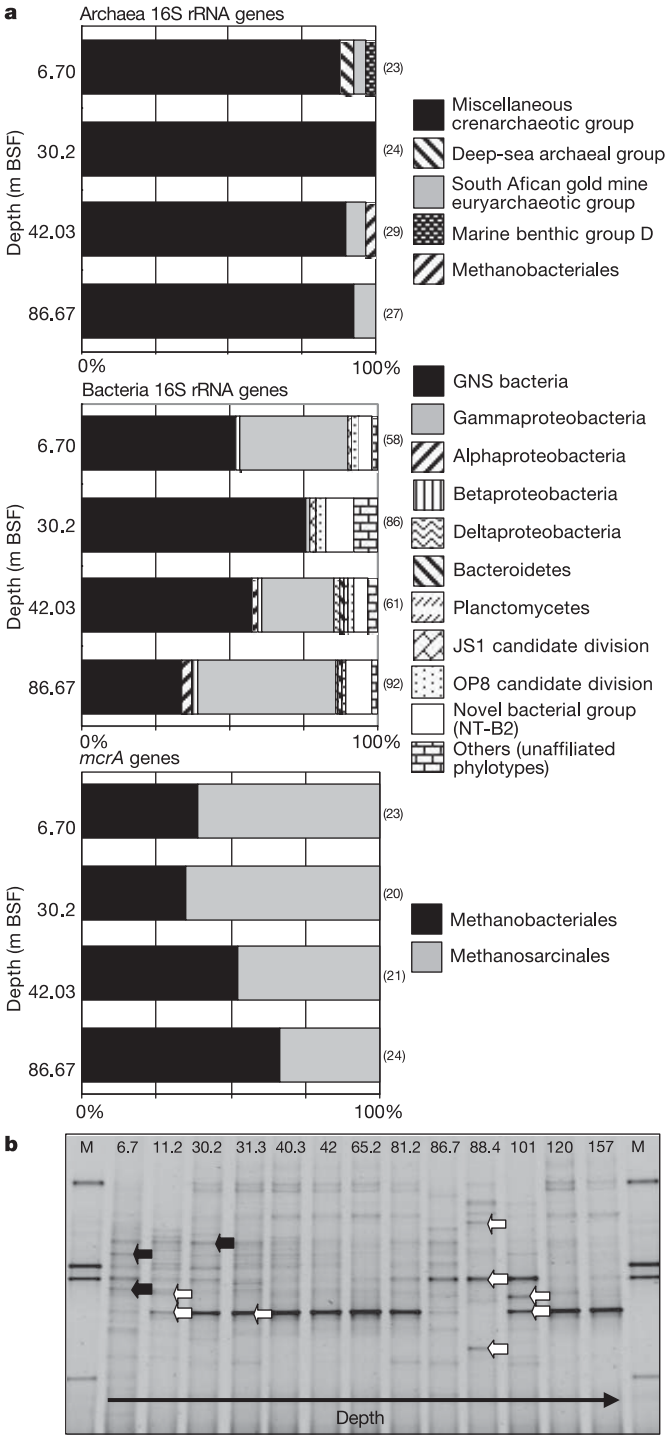

Figure 2 | Prokaryotic biodiversity at the Peru margin site (ODP Leg 201, site 1229). a, Biodiversity based on gene libraries for Archaea, Bacteria (16S rRNA gene) and methanogens (*mcrA* gene) from four depths. Numbers in parentheses are the numbers of sequenced clones in each library. **b**, DGGE profile of Bacteria from 13 depths (depth in metres BSF labelled at the top of individual lanes; M, marker lane[20]). The identities of sequences from representative bands are indicated by arrows: black arrows, GNS bacteria; white arrows, Gammaproteobacteria.

deeper sulphate zones from that in the methane zone, and this corresponds to the zones of different thymidine incorporation (Fig. 1d). A new phylum widely distributed in anoxic sediments, JS1 (ref. 22), was only in the 6.7 and 30.2 m BSF libraries and was a minor component. This is unlike Nankai Trough sediments, in which JS1 dominated and no GNS bacteria were found[21]. However, it is similar to the Sea of Okhotsk deep sediments where Gammaproteobacteria dominated some layers, with GNS and JS1 Bacteria dominating others[12].

Denaturing gradient-gel electrophoresis (DGGE) was used to investigate 16S rRNA gene diversity at nine additional depths; this confirmed high diversity, with 24 distinct bands overall (Fig. 2b). Sequencing representative bands confirmed that GNS and Gammaproteobacteria were most abundant, although the new JS1 phylum was detected at all depths with targeted primers[22]. Principal-component (PC) analysis of DGGE depth profiles (three components comprised 79% of variation) showed distinct changes in biodiversity (Fig. 1e). PC1 and PC2 (70% of variation) had similar depth profiles, with abrupt changes very close to the 90 m interface (88.4 m BSF), whereas PC3 (9% of variation) had a contrasting profile, with positive values in the top 40 m BSF, peaking near the 30 m interface, and then decreasing. However, near the 90 m interface values changed rapidly to a minimum negative value and then increased below. Hence, there are abrupt changes in diversity of Bacteria at the two sulphate/methane interfaces, with populations at each being distinctly different (Fig. 2). The population at the 90 m interface is unique in containing only three bright bands, which were not present together at any other depth. PC1 is strongly positively correlated ($P < 0.05$) with dissolved sulphate and rates of H_2/CO_2 methanogenesis, whereas PC3 is positively correlated with alkalinity and thymidine incorporation but negatively with dissolved sulphate and manganese. This provides a unique insight into the potential characteristics and environmental control of prokaryotic diversity at this site.

We have demonstrated the stimulation of prokaryotic processes at discrete interfaces in deep, sub-seafloor sediments and shown marked changes in biodiversity and biogeochemical processes at subsurface interfaces. These results provide compelling evidence for active and dynamic populations in subsurface marine sediments and are consistent with recent evidence for the viability of microscopically detected cells[23]. Sediments at about 90 m BSF date to the early Pleistocene epoch (about 0.8 Myr ago), whereas the deeper sediments (186 m BSF) are Late Pliocene in age (up to 2 Myr ago[24]). Prokaryotic processes are therefore operating on geological timescales. In addition, this site was studied 18 years ago on ODP Leg 112 (ref. 25) and provided evidence, controversial at the time, for the stimulation of culturable prokaryotes below about 50 m BSF; however, these data now also show the long-term stability of stimulated subsurface prokaryotic populations.

In contrast to Bacteria, the diversity of Archaea was more limited, and as in other subsurface sediments[12,21] all depths were dominated by the diverse miscellaneous crenarchaeotic group (Fig. 2a, see also Supplementary Information). Only one methanogen sequence was detected and this was from the methane zone (42.03 m BSF). However, consistent with the methanogenic rate measurements was the observation that methanogen-specific genes (*mcrA*) were present at all four depths (Fig. 2a). Diversity was limited to Methanobacteriales and Methanosarcinales (taxa using H_2/CO_2 and/or acetate, respectively), which is consistent with methane formation from both these substrates (Fig. 1c). Similar limited methanogenic diversity occurs in other deep sediments[21,26]. Sequences for anaerobic methane oxidizers[16] were absent. However, neither were sulphate-reducing bacteria detected, despite high (near-surface) rates of sulphate reduction, detectable sulphate reduction at depth (Fig. 1) and presumably sulphate reduction coupled to methane oxidation at the two sulphate/methane interfaces[17]. Calculations from the sulphate reduction rates indicate that the maximum proportion of sulphate-reducing bacteria of the total population at the 30 and 90 m interfaces is 0.02% and 0.002%, respectively, and it is most unlikely that these would be in our 16S rRNA gene libraries[27]. Furthermore, low numbers of sulphate-reducing bacteria are consistent with a lack of DNA amplification with a specific gene from sulphate-reducing bacteria (*dsrAB* (ref. 28)). Low numbers could also occur with anaerobic methane-oxidizing prokaryotes, especially when associated with sulphate-reducing bacteria[16]. Hence, prokaryotes directly involved in sulphate reduction and/or anaerobic methane oxidation at this site may be highly active but might represent a small proportion of the total population or be unknown prokaryotes in

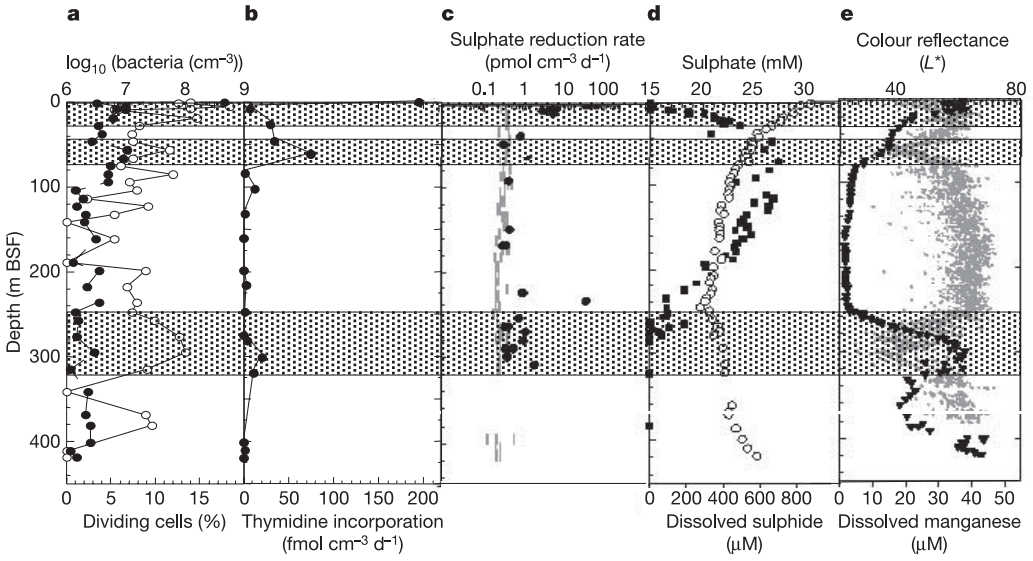

Figure 3 | Biogeochemical processes and prokaryotic populations at the Pacific open ocean site (ODP 1226). **a**, Prokaryotic profiles: filled circles, total prokaryotic population; open circles, percentage of dividing and divided cells. **b**, Rate of prokaryotic growth, measured as thymidine incorporation. **c**, Filled circles, sulphate reduction rates; grey oblongs, minimum detection limits. **d**, Geochemistry[29]: open circles, pore water sulphate; filled squares, hydrogen sulphide. **e**, Colour reflectance as a measure of diatom abundance (low reflectance represents high diatom abundance); filled triangles, pore water manganese concentration. Shaded boxes highlight elevated prokaryotic processes and high diatom layers.

our gene sequences, or their genes might not have been amplified.

Similar stimulation of prokaryotes occurred in the open ocean site but in association with repeated lithological depth changes and their allied high diatom content (Fig. 3). In the three diatom-rich layers between the surface and about 400 m BSF there was a consistent stimulation of prokaryotic activity (sulphate reduction and thymidine incorporation) and total prokaryotic numbers and/or the proportion of dividing and divided cells. Furthermore, in the top and bottom diatom-rich layers there was an increasing concentration of dissolved manganese[29], indicating active manganese reduction by prokaryotes. The mechanism of prokaryotic stimulation in these layers is not clear but it might be that diatomaceous organic matter is considerably less reactive than other sedimentary organic matter and as a consequence can fuel low, but continuing, prokaryotic activity over long periods. The deepest layer (about 250–320 m BSF) is 7–11 Myr old (ref. 29), which markedly extends the timescale of subsurface stimulation of prokaryotic processes at the margin site and any other deep, sub-seafloor sediment[9].

METHODS

Sediment handling, activity and total prokaryotic count measurements. Samples were obtained from Sites 1226 and 1229 on ODP Leg 201 (ref. 29). All sediment was subsampled, aseptically and anaerobically (not molecular genetic samples) at about 4 °C and then rapidly processed for radiotracer or prokaryotic counts or stored for molecular analysis (−80 °C) on board ship. Intact syringe subcores were injected with radiotracer ([^{14}C]bicarbonate or [^{14}C]acetate, [^{35}S]sulphate, or [*methyl*-^{3}H]thymidine) and incubated at close to temperatures *in situ*; activity was then stopped by freezing or the addition of zinc acetate before processing in the laboratory[18,30]. Because incubation conditions were not identical to conditions in the original sediment, measured rates might differ from those *in situ*. Total populations were determined by staining with acridine orange coupled with epifluorescence microscopy (AODC, ref. 25); these were conducted mainly on the ship. Total counts were assessed for significant differences with a one-way analysis of variance, using the Tukey–Kramer method for comparing individual means ($\log_{10}$(minimum significant difference) = 0.59 for $P < 0.05$) and the sum-of-squares simultaneous test procedure for groups of means. Numbers of sulphate-reducing bacteria were estimated from the specific sulphate reduction rate of 0.1 fmol per cell per day (ref. 28) and the measured sulphate reduction rates. This was expressed as a percentage of the AODC total count.

Two types of tracer were used to assess contamination from sea water during drilling: a perfluorocarbon (PFT) and bacteria-sized fluorescent beads (0.5 μm), combined with aseptic handling and subsampling. If present, contamination was concentrated near the core liner. Contamination was low (less than 0.1 μl of sea water per gram of sediment) or below detection in advanced piston cores, all Peru margin samples and to about 300 m BSF at the open ocean site. Contamination was more variable and slightly higher in extended core barrel cores taken below 300 m BSF for the open ocean site (about 0.24 μl of sea water per gram of sediment). A 0.1-μl volume of sea water contains about 50 bacteria, which is 0.000012% of the average prokaryotic population at Site 1229. Because sediment near the core liner was not sampled, and detected contamination was low or absent, subsequent analysis was not subject to seawater contamination.

DNA extraction and polymerase chain reaction (PCR) conditions. DNA was extracted from sediments[20] and then stored at −80 °C. PCR was conducted with 16S rRNA gene primers for Bacteria (27F-907R and 27F-1492R), for Archaea (109F-958R), for candidate division JS1 (63F-665R) and primers for *mcrA* genes (ME1 and ME2)[21,22]. Two primer pairs were also used to amplify *dsrAB* and *dsrA* genes from the sediments, but these did not yield products from any depth despite successful amplifications on other Leg 201 and near-surface sediments.

Cloning and sequencing. PCR products were screened by DGGE to ensure representative amplification as described previously[20], and five independent PCR products from each depth (6.7, 30.2, 42.03 and 86.67 m BSF) were pooled and cleaned (Wizard PCR Preps DNA Purification System; Promega). Cloning was conducted with the pGEM-T Easy Vector System (Promega) and inserts were confirmed by PCR with vector-specific M13, 16S rRNA gene or *mcrA* gene primers. Random clones were chosen and sequenced with 27F (Bacteria 16S rRNA genes), 109F (Archaea 16S rRNA genes) and ME1 (*mcrA* genes) primers by using an ABI 3100 Prism Genetic Analyzer (Applied Biosystems). Sequences were checked for chimaeras and assigned to phylogenetic groups by sequence comparison with databases (http://www.ncbi.nlm.nih.gov/); assignment was confirmed by phylogenetic tree reconstruction with, first, neighbour-joining with the Jukes and Cantor correction, and second, minimum evolution with the LogDet/Paralinear distance methods[21,22]. Sequence lengths used to construct Fig. 2a were as follows: 640–720 base pairs (bp) for Archaea, 315–828 bp for Bacteria (mean 580 bp; 92% more than 450 bp) and 605–720 bp for methanogen *mcrA* sequences.

DGGE analysis. 16S rRNA gene products from Bacteria were reamplified in a nested PCR with primers 357F and 518R; PCR products were analysed by DGGE[20]. DGGE bands were identified by visually inspecting gels through a mask consisting of 33 horizontal slices, with each slice approximately the width of the brightest band. The 24 DGGE bands obtained were scored (down to the lowest marker band in Fig. 2b) as present (score 1) or absent (score 0) and the data were analysed by principal-component analysis with Minitab Release 14 (Minitab Inc.), using depths as the variables. Other ordination approaches including multi-dimensional scaling, factor analysis and using bands as variables, scoring by the presence or absence of a band and by band density, all provided very similar results, confirming the consistency of our analysis; some cluster analysis approaches showed similar groupings to the ordinations, but others did not. Correlation of the individual principal components with prokaryotic and geochemical variables[29] was used to identify the main factors affecting the diversity of Bacteria in depth profiles. The DGGE profile of 88.4 m BSF was excluded from the correlation analysis: inclusion obscured overall relationships because the biodiversity of this depth was so different from that at all other depths.

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

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