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Microorganisms persist at record depths in the subseafloor of the Canterbury Basin

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The subsurface realm is colonized by microbial communities to depths of more than 1000 meters below the seafloor (mbsf), but little is known about overall diversity and microbial distribution patterns at the most profound depths. Here we show that not only *Bacteria* and *Archaea*, but also *Eukarya* occur at record depths in the subseafloor of the Canterbury Basin. Shifts in microbial community composition along a core of nearly 2 km reflect vertical taxa zonation influenced by sediment depth. Representatives of some microbial taxa were also cultivated using methods mimicking *in situ* conditions. These results suggest that diverse microorganisms persist down to 1922 mbsf in the seafloor of the Canterbury Basin and extend the previously known depth limits of microbial evidence (i) from 159 to 1740 mbsf for *Eukarya* and (ii) from 518 to 1922 mbsf for *Bacteria*.

Introduction

In addition to terrestrial and marine near-surface habitats, the deep biosphere is considered to be a third realm for microbial life. Subseafloor sediments provide a habitat for large numbers of microbial cells, as revealed by cell counts (Parkes et al., 2000) or CARD-FISH (Schippers et al., 2005). Although recent data have shown that the global biomass in subseafloor sediments is smaller than given by earlier estimates, the deep subseafloor biosphere still constitutes a large fraction (2.9 × 1029 cells) of Earth's living biomass (Kallmeyer et al., 2012).

The subsurface microbiota is diverse and complex, hosting metabolically active communities down to depths of more than one thousand meters below the seafloor (mbsf), as revealed by molecular, metagenomic and metatranscriptomic studies (Lipp *et al*., 2008; Roussel *et al*., 2008; Biddle *et al*., 2011; Pawlowski *et al*., 2011; Orsi *et al*.

2013a). It harbors representatives from the three domains of life, i.e., numerous endemic and/or as yet uncultured *Archaea* and *Bacteria* (e.g., Orcutt *et al*., 2011; Inagaki *et al.* 2006), in addition to bacterial endospores (Lomstein *et al.*, 2012), protists and fungi belonging to *Eukarya* (Schippers *et al.*, 2006; Edgcomb *et al.*, 2011; Orsi *et al.*, 2013a, 2013b). Occurrence of capsid-encoding organisms has also been confirmed (Engelhardt *et al.*, 2011). Although background molecular data on bacterial and archaeal lineages inhabiting subsurface sediment above 1000 mbsf exists (e.g., Orcutt *et al*., 2011; Inagaki *et al.* 2006), most deep-subsurface microorganisms detected so far were refractory to cultivation (Sass and Parkes, 2011). The diversity of deeply buried microorganisms remains untapped, as subseafloor prokaryotic culturability in most studies is less than 0.1% of all microscopically detected cells (D'Hondt *et al.*, 2004). Remarkably, when wide enrichment collections targeting different physiological groups such as fermenters, sulfate-reducers and methanogens were performed using different subseafloor sediments, these often led to the isolation of the same few "generalist" bacteria (e.g., Batzke *et al.*, 2007). In most cases, the retrieved bacterial genera were adapted to a broader spectrum of environmental conditions (e.g., broad temperature range for growth) compared to their surface counterparts (Sass and Parkes, 2011).

So far, within subseafloor sediments, active *Bacteria* have been identified down to 518 mbsf (Bale *et al.*, 1997), active *Archaea* down to 1626 mbsf (Roussel *et al.*, 2008), and active microeukaryotes down to 159 mbsf (Orsi *et al.*, 2013a), but we are still eager to know the depth limit of the deep subsurface biosphere. Limits to microbial habitability in subseafloor sediments are set by a variety of physical and chemical parameters like temperature, pH, pressure, salinity, porosity, availability of energy, nutrients and water, and maybe also by age. The present study site is not characterized by particularly

extreme conditions but stands out from sites previously examined by its depth and low porosity. The depth limit of the deep biosphere remains an important issue to place bounds on the volume of the subseafloor biosphere and to guide the search for deep life capabilities/adaptation and the role of microorganisms in global nutrient cycles. We hypothesized that life could exist in even deeper sediments if pore space was sufficient. In this study, we investigated the subsurface microbial communities from a core of 1927 m length collected in the Canterbury Basin (344 m water depth), off the coast of New Zealand at site U1352, which was drilled during the Integrated Ocean Drilling Program (IODP) Expedition 317 with DS *Joides Resolution.* Our purpose was to investigate vertical distribution of microbial communities, abundance and evenness of taxa above and below 1000 mbsf depth. We developed a highly stringent massive parallel tagged-amplicon sequencing of 16S-18S hypervariable regions of small-subunit (SSU) rRNA gene (Fig. S1-S2, Tables S1-S3), coupled with cell counts, real-time PCR (phylogenetic and functional genes) and cultivation approaches. This rigorous method was applied to sediment/carbonate rocks spanning epochs from the Holocene to late Eocene.

Materials and methods

Site description and sampling

Three holes (A, B and C) were drilled at Site U1352 (44°56'26.62''S; 172°1'36.30''E), reaching a total depth of 1927.5 m CSF-A, and thus spanning the Holocene to late Eocene epochs. Fluorescent microspheres were used as tracers for contamination during drilling. Sampling was processed under strict contamination controls onboard and offshore and only samples with no detectable contamination were used for this study (Fulthorpe *et al.*, 2011). Onboard, only the center parts of unconsolidated sediments and intact pieces of rocks that had

been exposed to UV radiation after washing were kept for microbiological analyses, as reported elsewhere (Expedition 317 Scientists, 2011). Subsamples were immediately frozen at –80°C for onshore molecular analyses, stored at 4°C under an anaerobic gas phase for later cultivation, and stored at 4°C in a 3% NaCl/3% formalin solution for cell counting. Detailed information on sampling/subsampling of sediment, contamination controls and on depth scale terminology are provided in Supplementary Text.

Lithological, physical and geochemical data

Environmental data were acquired onboard during IODP Expedition 317, as reported elsewhere (Fulthorpe *et al.*, 2011).

DNA extraction, PCR amplification and contamination controls

DNA extractions were made from 16 samples collected all along the core. In order to avoid contamination, all handling was carried out in a PCR cabinet exclusively dedicated to low biomass sediment samples (PCR cabinet; Captair®Bio, Erlab), using Biopur 1.5 mL Safe-Lock micro test tubes (Eppendorf), ultrapure PCR water (Ozyme) and UV-treated (> 40 min) plasticware and micropipettes. Negative controls (reaction mixture without DNA) were included in each set of PCR reactions. In addition, a negative control (e.g., negative DNA extraction) was prepared for each work stage, to ensure that no contamination with exogenous amplifiable DNA occurred during the different stages of sample treatment. The FastDNA™ Spin Kit for Soil (#6560-200, MP Biomedicals®) was used to perform DNA extractions, with few modifications. Detailed information on DNA extractions and PCR amplifications are provided in Supplementary Information. Primer sequences used in this study are detailed in Table S2 and primer sets for direct and nested PCR amplifications are detailed in Fig. S2.

454-Pyrosequencing

For each DNA extract, four independent 25 μL PCR amplifications were run with fusion primer pairs specific for *Bacteria*, *Archaea* and *Eukarya*, as detailed in Table S3. PCR products were pooled two by two, so as to have two independent replicates for pyrosequencing. Potential contaminants from lab reagents were excluded through the sequencing of negative-control samples and the removal of OTUs containing sequences retrieved in negative controls. Detailed information on 454-pyrosequencing, quality filtration, trimming, clustering and taxonomic affiliation are provided in Supplementary Text.

Cell counts

Total prokaryotic cells were enumerated in triplicate from 13 uncontaminated sediment samples collected all along the core, using the cell extraction protocol (protocol FCM-A) described by Morono *et al.* (2011) until step 9. Then, all supernatants containing extracted cells were filtered onto 0.2 μm filters (Anodisc, Whatman) and stained with SYBR®Green I (Invitrogen), as described elsewhere (Noble and Fuhrman, 1998). Filters were counted in epifluorescence mode, with an Olympus BX60 microscope (objective 100×, pH3, WIB filter) (details in Supplementary Text).

Real-time PCR measurements

Quantifications of different lineages and diverse functional genes were performed all along the core by quantitative, real-time PCR (Q-PCR). Quantifications of *Bacteria*, *Archaea*, *Eukarya*, JS1-*Chloroflexi*, and *Geobacteriaceae* were performed using previously described Q-PCR assays based on the detection of 16S or 18S rRNA (Schippers *et al*., 2012). These assays were carried out using *Taq*Man® or SYBR®Green chemistries. DNA copy numbers were also determined for the following functional genes: *mcrA* for alpha subunit of the methyl coenzyme M reductase, *dsrA* for the alpha subunit of the sulfite (bi)reductase, *aprA* for the alpha subunit

of the adenosine-5'-phosphosulfate reductase and *cbbL* for the large subunit of the enzyme ribulose-1.5-bisphosphate carboxylase/oxygenase (RubisCO, form I "red-like"), as described elsewhere (Schippers *et al*., 2012).

Cultures and approaches used for their analysis

A sediment slurry membrane system was used for cultivation (Ferrari *et al.*, 2008) (Fig. S8; details in Supplementary Information). Different anaerobic metabolisms found in the subsurface biosphere were targeted in culture: fermentation, sulfate-reduction and methanogenesis/acetogenesis. Media, culture conditions, viability and identification procedures of cells are described in Supplementary Text.

Statistical analyses

Principal Component Analysis (PCA) was used to help in visualization of high-dimensional data. An order abundance matrix was combined with environmental parameters, using XLSTAT, to assess relationships between microbial taxa and ecological variables (Addinsoft USA, New York, USA). A second complementary approach was based on regularized canonical correlation analyses (RCCA), which were performed to highlight correlations between the order abundance matrices (X) and the environmental parameters (Y) using the R software CCA package.

Results and Discussion

Core description

The core lithology was characterized by horizontal gradual layers, from unconsolidated sediments (clay, marl) to carbonate rocks (Fig. 1). The core was composed of three lithological units (UI, UII and UIII). Unit I (0–711 m CSF-A, meters of core depth below seafloor computed by conventional method A, corresponding to mbsf; see "IODP depth scale terminology" at www.iodp.org/program-policies/) was predominantly characterized by a transition from mud-rich sediment to marl. Unit II (711–1853 m CSF-A) consisted of hemipelagic/pelagic sediment from calcareous sandy mud to sandy sandstone. Unit III (1853–1924 m CSF-A) was characterized by a sharp change (Marshall unconformity: ~12 Ma are missing) that occurred at 1853 m CSF-A, and was formed of hemipelagic to pelagic foraminifer-bearing nannofossil limestone of early Oligocene to late Eocene age (Fig. 1). The temperature at the bottom of the hole was estimated to be in the range 60°C–100°C on the basis of thermal conductivity measurements and geochemical results (Fulthorpe *et al.*, 2011). Below 1000 m CSF-A, sediments were replaced by consolidated sedimentary calcium carbonate rocks with porous horizons of glauconite. Porosity decreased with depth and mean pore-size was around 2-4 μm at the hole bottom. In carbonate rocks, numerous fractures and stylolites were observed (Fig. S3). Organic carbon content was low (<0.6 wt %), with only a few samples having >1 wt % TOC (Fig. 1). The organic matter quality changed from relatively labile volatile material in the shallower sediments to more stable protokerogen with increasing depth. Methane and ethane both occurred below 11.7 and 18.2 m CSF-A and the relative ethane content increased with increasing burial depth and temperature (Fig. 1). Low but increasing concentrations of C3-C5 and occasionally C6 alkanes were also measured with depth (18). pH values were close to 7.5 and stable from the surface to 1164 m CSF-A (18). Sulfate concentration decreased gradually in the first meters of

196 the core and reached the detection limit at ~16 m CSF-A (the SMTZ: sulfate-methane
197 transition zone, was placed between 15.2 and 16.6 m CSF-A), then it remained close to
198 the detection limit (~0.85 mM) down to 1433 m CSF-A (Fig. 1).

199

200 *Vertical distribution of cells*

201 We analyzed and compared cell abundances and cell concentrations reported for
202 different geographic sites using a standardized procedure based on cell extraction and
203 dissolution of silicates (Noble and Fuhrman, 1998; Kallmeyer *et al*., 2008; Morono *et*
204 *al.*, 2011) (Fig. 2). Mean cell numbers decreased with depth from about $1.5 \times 10^6 \pm 4.7$
205 $\times 10^4$ cells·cm^{-3} (n = 8) at the surface (3.76 and 15.1 m CSF-A) to $2.5 \times 10^4 \pm 4.9 \times 10^3$
206 cells·cm^{-3} (n = 7) within the deepest samples (1911 and 1922 m CSF-A). The detection
207 limit, calculated in our conditions (Kallmeyer *et al*., 2008), was 2.94×10^3 cells·cm^{-3}.
208 The depth profile (down to 600 m CSF-A) was consistent with the general depth
209 distribution of prokaryotic cells from other subsurface sediments (Kallmeyer *et al.*,
210 2012).

211

212 *Vertical distribution of microbial taxa*

213 It is not clear what controls abundance of *Bacteria* and *Archaea* within deep marine
214 sediments (Schippers *et al.*, 2005; Lipp *et al.*, 2008; Schippers *et al.*, 2012). Here, a
215 real-time PCR approach was applied to quantify representatives of the three life
216 domains. Calculated detection limits for *Bacteria*, *Archaea* and *Eukarya* were
217 respectively 1.6×10^4, 1.1×10^3 and 2.9×10^3 SSU rRNA gene copies per gram of

sediment (wet weight). *Archaea* were the most abundant within the first meters, while *Bacteria* dominated the rest of the core (Fig. 2). Archaeal SSU rRNA gene copy numbers strongly decreased with depth (from 1.8×10^6 to 1×10^3 gene copies·g^{-1}, corresponding roughly to 1×10^6 to 6×10^2 cells·g^{-1}) and were no longer detectable below 650 m CSF-A. A similar depth distribution was observed for eukaryotic SSU rRNA gene copy numbers, but abundances were relatively constant with depth (~10^4 copies·g^{-1}). Bacterial SSU rRNA gene copy numbers were low (~10^6 copies·g^{-1} ≈ 2.5×10^5 cells·g^{-1}) at the surface and decreased with depth up to 1600 m CSF-A (8×10^4 copies·g^{-1} ≈ 2×10^4 cells·g^{-1}).

Along with these measures, deep sequencing allowed the detection limits to be lowered and masked lineages to be revealed. We pyrosequenced bacterial (V4-V5), archaeal (V1-V3) and eukaryotic (V1-V3) SSU rRNA gene amplicons from sixteen depth horizons and one negative control, pooled together in one single dataset with two PCR-replicates per sample to overcome PCR and sequencing errors (Fig. S1). Sequences were grouped into OTUs (Operational Taxonomic Units) with a 97% identity threshold. Sequence composition of the OTUs was then analyzed, and OTUs entirely composed of sequences that had appeared in a single PCR only were excluded from the diversity analyses. All the sequences kept appeared at least twice independently. Potential contaminants from lab reagents were excluded through the sequencing of negative-control samples and the removal of OTUs containing sequences retrieved in negative controls. The remaining OTUs were used to calculate non-parametric diversity indices (Fig. 3, Fig. S4-S6) and compared to the SILVA 111 database for taxonomic affiliation. Pyrosequencing results were congruent with the data discussed above. Archaeal sequences could not be amplified and sequenced for samples below 634 m CSF-A, as

observed with real-time PCR analyses. The non-detection of archaeal 16S rRNA genes below 650 m CSF-A using two different amplification methods suggests that *Archaea* are likely rare or absent at great depths in the Canterbury Basin. Eukaryotic sequences were detected down to 1740 m CSF-A and bacterial sequences were found up to the maximal depth of 1922 m CSF-A. The observed species richness (i.e., number of OTUs) was extremely low in comparison with other microbial habitats investigated so far, including extreme environments (e.g., Roalkvam *et al.*, 2012). Indeed, only 198, 16 and 40 unique bacterial, archaeal and eukaryotic OTUs, at 3% dissimilarity level, were detected in the entire cored sequence (Fig. S4, Tables S4-S5). Chao1 estimator revealed a vertical decrease in microbial richness with increasing depth (Fig. 3). Richness estimates for *Archaea* and *Eukarya* dropped off gradually with depth and reached only 2 and 4 OTUs respectively at the deepest depth for which a PCR signal was obtained. Beta diversity estimators (i.e., diversity among samples) revealed a strong differentiation between communities with depth and a strong vertical structuration (Fig. S5).

Archaeal diversity showed high abundances of MBG-B (Marine Benthic Group B) and MCG (Miscellaneous Crenarchaeotal Group), two archaeal groups typically found in subseafloor sediments (Lloyd *et al*., 2013). Representatives of the as yet uncultured lineages MBG-B, MBG-E (Marine Benthic Group E) and MCG were the predominating taxa in surficial layers, while MCG was the most consistently detected archaeal lineage down to 346 m CSF-A (Fig. 2). MBG-B and MCG members are heterotrophic *Archaea* frequently found in surficial marine sediments (Biddle *et al.*, 2006; Lloyd *et al*., 2013). *Thermococcales* dominated archaeal diversity of the sediment horizon at 634 m CSF-A. Methanogens and anaerobic methanotrophs (ANME) were not detected, in agreement

with the real-time PCR analysis for *mcrA*. Their absence from the dataset might be due to the intervals sampled which do not correspond to the SMTZ.

In *Eukarya*, few protist OTUs (Stramenopiles and uncultured *Eukaryota*) were detected down to 583 m CSF-A. Sequences affiliated with the bacterivorous protists *Bicosoecida* were detected at 346 m CSF-A, raising the question of the existence of a subsurface complex trophic web. In agreement with recently published papers (Edgcomb *et al.*, 2011; Orsi *et al.*, 2013a, 2013b), fungi appeared to be the most frequently detected eukaryotes in the Canterbury Basin, with 56 to 100% of the SSU rRNA gene sequences. Different shifts between *Ascomycota* and *Basidiomycota* were observed along the core (Fig. 2). *Tremellomycetes* (order *Tremellales*), *Sordariomycetes* and *Eurotiomycetes* dominated shallow depths while *Saccharomycetes* were detected at depths between 630 and 1365 m CSF-A. Deeper layers were dominated by *Wallemiomycetes*, *Microbotryomycetes* and *Tremellomycetes* (order *Filobasidiales*, not found at shallow depths). These heterotrophic fungi have been described in deep sediments of other locations (e.g., Nagano and Nagahama, 2012; Richards *et al.*, 2012) and demonstrated to be active members of microbial communities (Orsi *et al*., 2013b). So, fungi represent an important component of sediment ecosystems through their impact on nutrient cycling and mineral weathering.

Bacteria were dominated by *Chloroflexi* and *Proteobacteria,* two heterotrophic bacterial groups well-represented in subsurface sediments (Fig. 2). They comprised 67% of the sequences and 69% of the OTUs in total. However, the abundances of the two phyla were negatively correlated. *Chloroflexi* dominated microbial communities at shallow depths (above 600 m CSF-A) and their abundances and richness decreased rapidly. Reciprocally, *Proteobacteria* were found all along the core, but their relative

290 abundance showed a sharp increase below 343 m CSF-A. Among the other lineages
291 observed in this study, *Planctomycetes, Nitrospirae* and the candidate division OP9
292 were major contributors of the amplicon pool at shallow depths. Below 600 m CSF-A,
293 *Acidobacteria, Firmicutes* (a phylum containing spore-formers), and two loosely
294 defined groups of uncultured *Bacteria* (ML635J-21 and MLE1-12) were the most
295 consistently detected lineages. Real-time PCR quantification of the JS1-*Chloroflexi*
296 group confirmed these results as ~10^3 to 10^6 SSU rRNA gene copies·g^{-1} were detected
297 between the sediment surface and 1532 m CSF-A. *Deltaproteobacteria* were detected
298 above the SMTZ and at great depths. Genes encoding a functional dissimilatory sulfite
299 (bi)reductase (*dsrA*), a key enzyme of dissimilatory sulfate reduction frequently
300 encountered among *Deltaproteobacteria*, was quantified above the SMTZ and in layers
301 up to 1000 m deep in the sediment. The gene became undetectable below this depth,
302 either because it may decrease below the detection limit or because the detected
303 *Deltaproteobacteria* cannot respire sulfate.

304

305 *Diversity and environmental factors*

306 Principal component analyses (PCA) coupled with regularized canonical correlation
307 analyses (RCCA) were performed to visualize relationships between environmental
308 factors and microbial taxa. We first evaluated the relationships between all
309 environmental parameters measured (i.e., depth, pH, salinity, porosity, alkalinity and
310 concentrations of calcium, calcium carbonate, ammonium, magnesium, sulfate,
311 inorganic carbon, organic carbon, methane and ethane) to design a network of
312 correlations. Only the six most explanatory variables were kept (Fig. S6). This

complementary analysis reinforced the conclusion about microbial distribution pattern and vertical community composition, depth being defined as a main factor explaining diversity changes (Fig. S7).

Handling deeply buried microorganisms

Cultivation approaches allowed prokaryotic and eukaryotic strains to be grown, corresponding to a fraction of the microbial communities detected all along the core, underlining that these microorganisms were viable. Fungal strains were obtained at 21 to 765 m CSF-A, using elevated hydrostatic pressure to mimic *in situ* conditions (Fig. 4 A-C, Table S6). Sequencing of the ITS1 rRNA regions allowed identification of a *Cadophora* representative that had already been found in extreme environments, i.e., Antarctic environments (Tosi *et al.*, 2002) and deep-sea hydrothermal vents (Burgaud *et al.*, 2009) (Table S6). Fifty-seven anaerobic fungi, currently under description, have also been isolated from these sediments (Rédou and Burgaud, unpublished data). In addition to the important finding that living fungi could be cultivated from the sediment samples, microbial colonies were grown anaerobically at 60-70°C from calcareous chalk/limestone samples collected at 1827 and 1922 m CSF-A (Fig. 4 D-E), using a microcultivation method (Fig. S8). The microcolonies were successfully transferred to liquid media and subcultured. From the different tests performed, it was impossible to grow true methanogens and true sulfate-reducers. Only bacterial fermentative strains degrading the organic compounds supplied (i.e., low quantity of yeast extract) have grown. Within these subcultures, mean cell densities were low, around 4×10^5 cells·mL^{-1} and growth rates were slow (in 2.5 years of culture, only 6 to 9 subcultures at 1/40 or

1/50 have been performed). Cells were able to grow at atmospheric pressure and at the estimated *in situ* pressure (22 MPa). They were composed of viable very small rods, coccobacilli and cocci of 300 to 800 nm in diameter, often forming aggregates (Fig. 4 F-I). These small sizes and this cellular organization as consortia raises questions about the living conditions of these cells and their (in)dependence with regard to other cells. The smallest diameter of a cell that assures its viability was calculated as ~ 200 nm (Velimorov, 2001). The major lineages identified in DNA and RNA libraries from these subcultures belonged to *Alpha*-, *Beta*-, *Gamma-proteobacteria*, *Actinobacteria* and *Armatimonadetes* (Fig. 5). With the exception of *Armatimonadetes,* all these taxa were detected from pyrosequencing in crude samples from 1827 and 1922 m CSF-A. The majority of the sequences had relatives recovered from environments with similar physical-chemical characteristics (Lin *et al.*, 2006; Mason *et al.*, 2010) (i.e., hot and reduced habitats) compared with the Canterbury subseafloor. Considering the 'ubiquity' of these taxa, one can hypothesize that they are generalist bacteria which would have been maintained during progressive burial of sediments or by transportation through circulating fluids. They might have acquired metabolic capabilities enabling them to resist the associated environmental changes. However, this hypothesis needs to be analyzed in detail. Furthermore, similar SSU rRNA gene sequences do not automatically correspond to identical physiologies, identical phenotypes or similar functions.

Impact of potential contaminants on native microbial populations

Contamination is a crucial issue when working with subseafloor sediments. In general, contamination during drilling is still difficult to predict. During IODP Expedition 317,

361 the level of contamination during drilling was evaluated by using fluorescent
362 microspheres and only samples with no detectable contamination were kept for
363 microbiological analyses. Nevertheless, samples without microspheres are not
364 necessarily uncontaminated (Smith *et al.*, 2000). Contamination generally decreases
365 from the exterior to the interior of both sediment and rocks cores (e.g., Lever *et al.,*
366 2006). In consequence, only the interior of sediment cores and intact pieces of rocks that
367 had been exposed to UV light after washing were used for the analyses. In addition, for
368 molecular experiments deeply frozen samples of more than 1 cm in diameter were
369 sterilized by flaming. Afterwards, all possible contaminations during the wet-lab steps
370 have been strictly controlled and minimized (see Supplementary Text). The cutting-
371 edge strategy applied for the pyrosequencing and bioinformatic analyses allowed
372 removing potential spurious sequences and OTUs likely to contain contaminants, by
373 sequencing of negative controls, a duplicate procedure and an associated bioinformatics
374 pipeline. In addition to these precautions, the level of potential contamination of our
375 samples was estimated by calculating the number of contaminating cells per gram of
376 sediment and per gram of sedimentary rock based on the mean contamination values
377 with drilling fluids and mean cell abundances in surface waters reported in the literature.
378 The mean potential contamination was estimated as (i) 0.011 ± 0.018 μL of drilling
379 fluid per gram for unconsolidated sediments drilled using advanced piston coring (APC)
380 and (ii) 0.027 ± 0.029 $\mu L \cdot g^{-1}$ for rocks collected using rotary core barrel (RCB) (Lever
381 *et al.,* 2006). Considering these levels of contamination, mean cell counts of 5×10^5
382 cells$\cdot mL^{-1}$ in surface waters in the ocean (Whitman *et al.,* 1998) and average densities of
383 1.85 g$\cdot cm^{-3}$ in sediments and 1.99 g$\cdot cm^{-3}$ in sedimentary rocks at site U1352, potential
384 contamination of the interior of the core sample should be expected very low with 5 to

385 11 cells·g^{-1} of sediment only. A second reported estimate indicates that less than 50
386 cells per gram of sediment contaminated APC core centers drilled with *Joides*
387 *Resolution* and that XCB cores were generally more contaminated with contamination
388 levels 3 to 10 times higher in XCB cores than in APC core centers (House *et al.,* 2003).
389 Considering these different estimates of potential contamination, the observed cell
390 counts at site U1352 were 2 to 5 orders of magnitude higher in the studied samples. If
391 contamination cannot be excluded, in the worst case, non-indigenous cells represent
392 only up to 1% of total cells in the sample. Therefore, it is most likely that more than
393 99% of the counted cells are native to the sampled sediment/rocks. This implies that the
394 vast majority of the prokaryotic and eukaryotic DNA subjected to pyrosequencing was
395 therefore derived from the sediment native cells. By extension, assuming that most of
396 the prokaryotic DNA extracted from sediments samples is from native cells, the fact that
397 cultivated bacteria match OTUs abundant in the crude sediment samples supports the
398 idea that these cultivated strains are isolates of native bacteria. Consequently, the
399 potential impact of contaminants on each category of data (cell counts, molecular data
400 and cultures) is likely very low.
401
402 *Ecological implications and future prospects*
403 We have underlined that the subseafloor of the Canterbury basin hosts microorganisms
404 which comprise *Bacteria, Archaea* and *Eukarya.* Some of these microorganisms are
405 alive and, at least to a certain extent, revivable. The communities exhibit a quite low
406 phylogenetic diversity, but this does not necessarily correspond to a low functional
407 diversity. This poor diversity could be explained if natural selection has produced (i)
408 taxa adapted to harsh subsurface conditions (i.e., specialists), which would be expected

409 in the case of a low connectivity among habitats; and/or (ii) taxa with a broad
410 physiological plasticity, allowing them to survive in a diversity of nutritional and
411 physical-chemical conditions (i.e., generalists). In fact, some taxa detected through their
412 16S/18S rRNA gene sequences are thought to be endemic to subsurface habitats, while
413 others seem ubiquitous and are consistently encountered in common and extreme
414 environments. The bacterial strains in cultures are related to opportunistic or generalist
415 taxa isolated from a broad array of redox environments, which raises the question of the
416 existence of microbial metabolic versatility and also questions the species concept, since
417 behind a given name or a given OTU can lay a variety of microorganisms with different
418 ecological lifestyles. Metabolic versatility has already been demonstrated in well-known
419 taxa. For example, some *Thermococcales* strains, which are usually fermenters that
420 reduce sulfur compounds, can grow in oligotrophic conditions or can oxidize carbon
421 monoxide (Sokolova *et al*., 2004). Heterotrophy is likely to be the major mode of
422 carbon assimilation within microbial communities of subsurface marine sediments
423 (Batzke *et al*., 2007). Our culture data support this hypothesis. Genome and
424 metagenome analyses would allow functions to be predicted on a finer scale, to assess
425 and hypothesize the individual ecological functions within the analyzed habitat or
426 ecosystem (Vandenkoornhuyse *et al.*, 2010). The detection of fungal sequences at great
427 depths and our success in the cultivation of fungal strains leads us to ask what role they
428 play in deep carbon cycling and what involvement they have in dynamics/regulation of
429 prokaryotic populations, if they are active *in situ*.
430 The broad polyphasic approach developed in this study provides direct evidence that
431 viable microorganisms can be present in rocks that are hardened but not totally
432 cemented, where stylolites and micro-fluid circulations exist. Our data demonstrate that

433 the combination of physical, chemical and energetic constraints encountered from 0 to
434 1922 m CSF-A in the subseafloor of the Canterbury Basin still allow microorganisms to
435 persist down to at least 650, 1740 and 1922 m CSF-A for *Archaea*, fungi and *Bacteria*,
436 respectively. It extends the subseafloor sedimentary depths at which subseafloor
437 organisms are known to be present to 1740 m for fungi and to 1922 m for *Bacteria*.
438 Nevertheless, one cannot exclude that some of the detected sequences belong to
439 microorganisms in dormancy. More extensive sequencing efforts will be required, i.e.,
440 direct metatranscriptomics, to describe more directly the microbial communities along
441 with functional signatures, and to compile data on biogeochemical processes and fluxes.
442

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453

454 Supplementary information is available at the ISMEJ's website.

455 The authors declare no conflict of interest.

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Figure legends

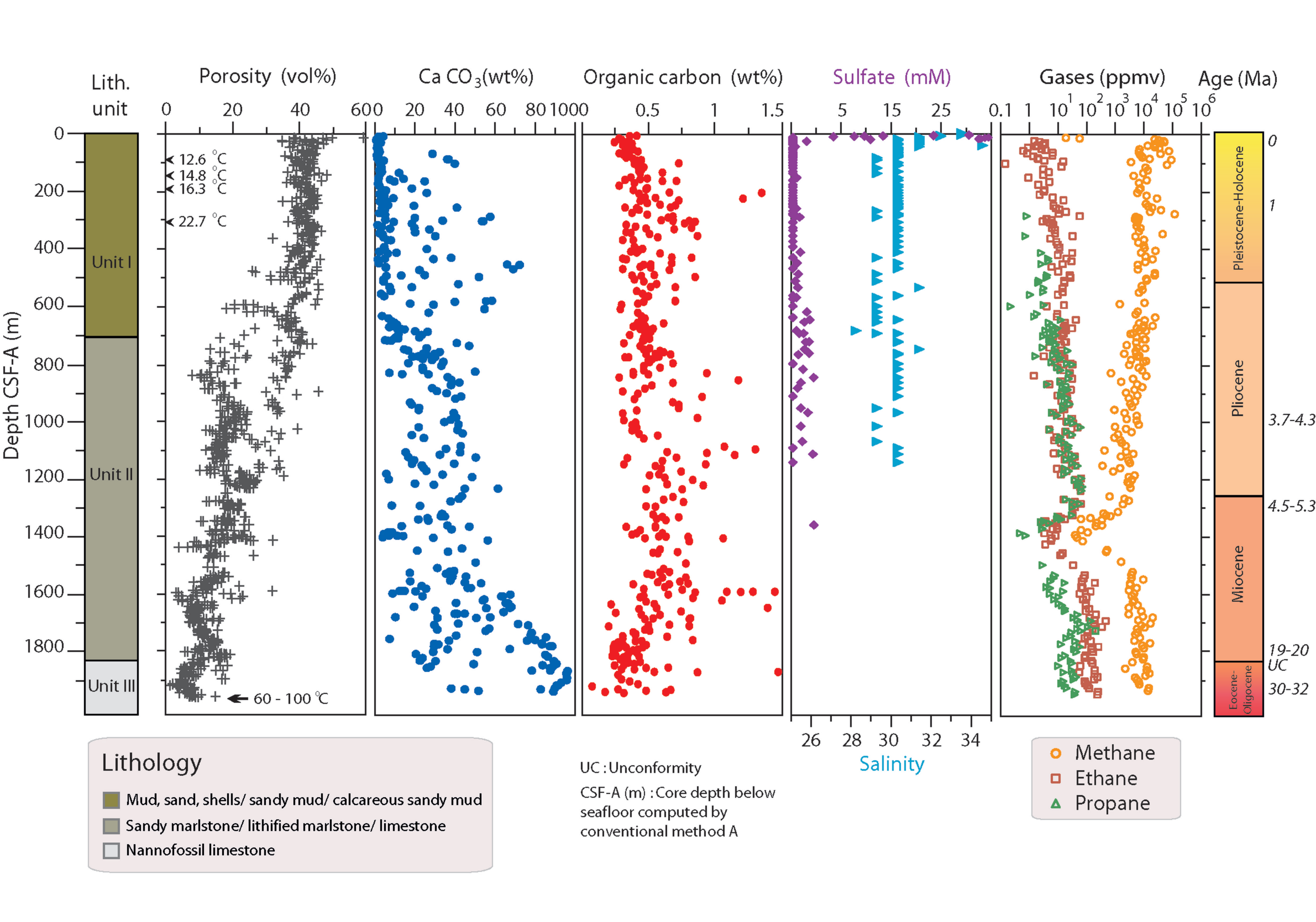
Figure 1 Main physical-chemical characteristics of the studied core. Black arrows indicate measures of *in situ* temperatures used to calculate a thermal gradient of 46°C/km. This thermal gradient together with the interpretation of the thermal maturity gradient defined by T_{max} measurements, allowed estimating a bottom-hole temperature comprised between 60 and 100 °C (Source: IODP report 317) (Fulthorpe *et al.*, 2011).

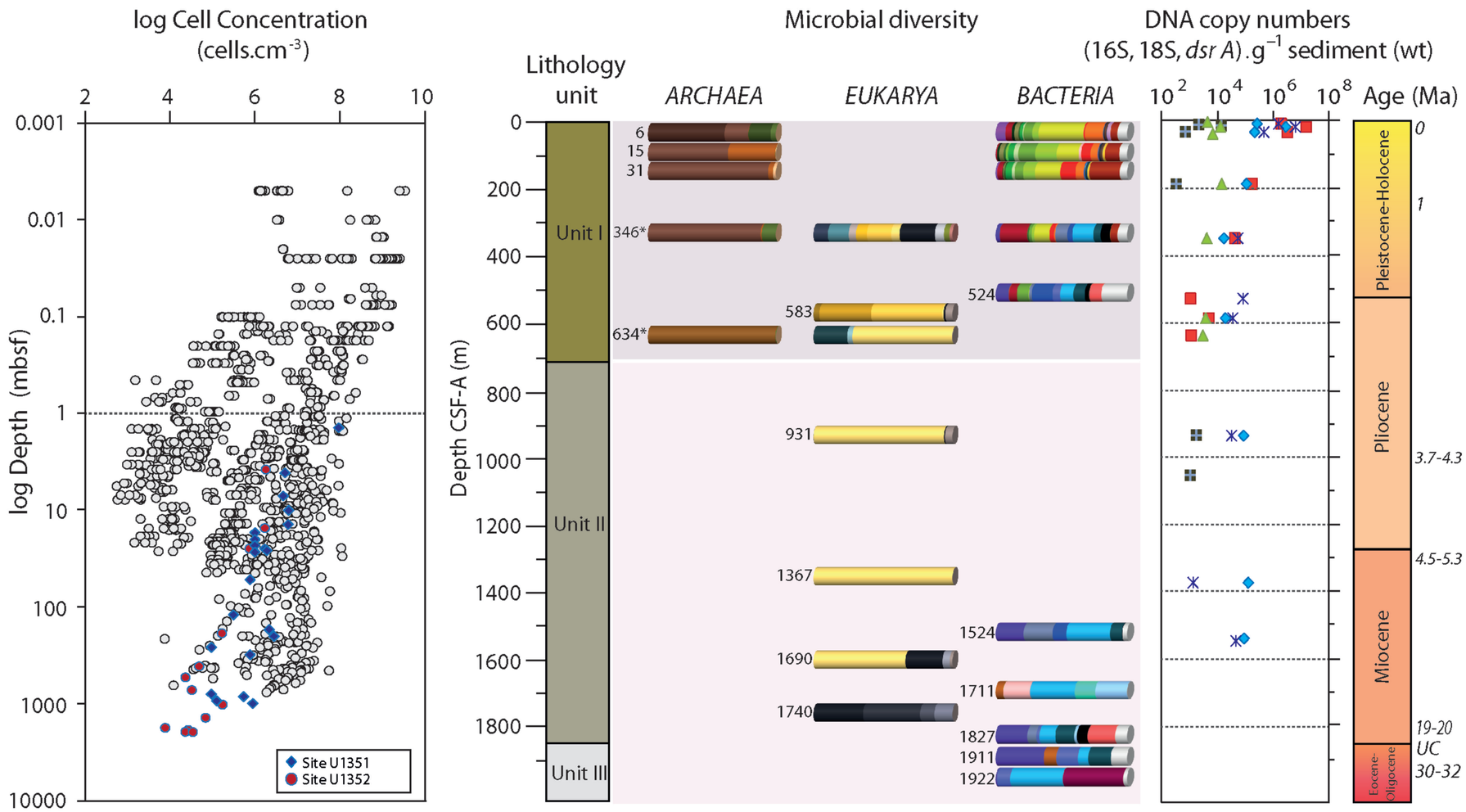
Figure 2 Lithological structure and age of lithological deposits at site U1352 in the Canterbury Basin compared with depth distribution of cell counts, 16S/18S rRNA gene-tag sequences and DNA copy numbers of genetic markers and functional genes. On the left, cell concentrations counted at site U1352 (red dots) and neighboring site U1351 (blue squares), according to depth (m CSF-A), compared with the general depth distribution of cells (grey dots) in subseafloor sediments (Kallmeyer *et al*., 2012). In the center, Phylum Class_Order distribution of archaeal, eukaryotic and bacterial 16S/18S rRNA gene-tag sequences (based on SILVA111 classification) from OTU containing 100 or more sequences (the remaining sequences were grouped into "Other"). On the right, copy numbers of the disulfite reductase genes A, of the 18S rRNA genes from total *Eukarya* and of 16S rRNA genes from total *Bacteria*, *Archaea* and *JS1/Chloroflexi*-related bacteria. *Geobacteriaceae* were detected only up to 15 m CSF-A (~ 5×10^3 copies per g). No amplification from greater depth was shown. The functional genes *mcrA, aprA* and *cbbL* were not detected at all. Legend: § In other classifications, MCG affiliate with the *Thaumarchaeota*. # Lineages of plants and algae. * Depth horizons where ≥ 100 reads of plants and Chrysophyceae were detected.

Figure 3 Community richness (Chao1 non-parametric estimator) for archaeal, eukaryotic and bacterial sequences making up the OTUs (calculated by MOTHUR at 3% difference between OTUs).

Figure 4 Epifluorescence microphotographs of initial enrichment cultures and subsequent liquid subcultures hybridized with the Cy3-labelled probe Euk516 (staining of nuclei with DAPI) (top panel), or stained with SYBR®Green I (middle panel + G) or with the dual staining LIVE/DEAD®Bacterial Viability Kit (H-I). (A-C) Identification of fungus-like eukaryotic cells with fluorescently-labeled 18S rRNA oligonucleotide probes in initial enrichment cultures with sediments from 21 m CSF-A on PDB 3%, at 4 MPa (A), with sediments from 37 m CSF-A on PDB 3%, at 11 MPa (B) and with sediments from 765 m CSF-A on PDB 0%, at 11 MPa (C). (D-E) Microcolonies observed on polycarbonate membranes (initial enrichment cultures) after 15 days of incubation with sediments from 1922 m CSF-A on H_2/CO_2 + YE. (F-G) Cellular aggregates observed in the 7th liquid subcultures performed after the initial enrichment with sediments from 1827 m CSF-A on YE + peptone + casamino acids. (H-I) Cell structural integrity was observed in the 7^{th} liquid subcultures performed after the initial enrichment with sediments from 1827 m CSF-A on acetate + YE (H) and with sediments from 1922 m CSF-A on YE + peptone + casamino acids (I). Legend: YE, Yeast Extract; PDB, Potato Dextrose Broth.

621 **Figure 5** Phylogenetic position of the bacterial 16S rRNA gene sequences detected in
622 RNA extracts from the fifth to eighth liquid subculture following the initial enrichment
623 step on a sediment substrate membrane system. The phylogenetic reconstruction was
624 performed using the Neighbor-Joining algorithm. Bootstrap values based on 1000
625 replications are indicated at the branch nodes. Representative bacterial 16S rRNA gene
626 sequences of H_2/CO_2 + yeast extract (YE)-based cultures and acetate + YE-based
627 cultures from the 137R section (1827 m CSF-A), as well as YE + peptone (pept.) +
628 casamino acids (CA)-based culture from the 148R section (1922 m CSF-A) are shown
629 in bold blue letters. Sequences from basalt or an underground aquifer where the
630 temperature is high (52<T°<80°C) are shown in brown; Sequences from environments
631 rich in hydrocarbons or radioactive metals are shown in orange. Accession numbers are
632 given in brackets.


Lith. unit
Porosity (vol%)
Ca CO3(wt%)
Organic carbon (wt%)
Sulfate (mM)
Gases (ppmv)
Age (Ma)
Depth CSF-A (m)
Unit I
Unit II
Unit III
12.6 °C
14.8 °C
16.3 °C
22.7 °C
60 - 100 °C
Pleistocene-Holocene
Pliocene
Miocene
Eocene-Oligocene
0
1
3.7-4.3
4.5-5.3
19-20
UC
30-32
Salinity
Lithology
Mud, sand, shells/ sandy mud/ calcareous sandy mud
Sandy marlstone/ lithified marlstone/ limestone
Nannofossil limestone
UC : Unconformity
CSF-A (m) : Core depth below seafloor computed by conventional method A
Methane
Ethane
Propane



Archaea

Crenarchaeota
- MBG - B
- MCG §
- Unclassified *Crenarchaeota*

Euryarchaeota
- *Thermococcales*
- MBG - E
- SAGMEG
- Other

Eukarya

Streptophyta #
- *Liliopsida_Poales*
- *Eudicotyledons_Malpighiales*
- *Eudicotyledons_Rosales*
- *Eudicotyledons_Solanales*

Stramenopiles
- *Bicosoecida_Bicosoecida*
- *Chrysophyceae_ Ochromonadales #*

Ascomycota
- *Dothideomycetes_Capnodiales*
- *Eurotiomycetes_Chaetothyriales*
- *Eurotiomycetes_Mycocaliciales*
- Unclassified_*Sordariomycetes*
- *Saccharomycetes_Saccharomycetales*

- Uncultured_*Eukaryota*
- Other

Basidiomycota
- *Tremellomycetes_Tremellales*
- *Tremellomycetes_Filobasidiales*
- *Wallemiomycetes_Wallemiales*
- *Microbotryomycetes_ Microbotryomycetes incertae sedis*
- *Exobasidiomycetes_Malasseziales*

Bacteria

- *Acidobacteria_Acidobacteria_DA052*
- *Acidobacteria_Acidobacteria_S035*
- *Actinobacteria_Actinobacteria_Corynebacteriales*
- *Actinobacteria_OPB41*
- *Firmicutes_Bacilli_Bacillales*
- *Nitrospirae_Nitrospira_Nitrospirales*
- *Planctomycetes_Phycisphaerae_MSBL9*
- *Spirochaetes_Spirochaetes_Kazan-3B-09*
- BHI80-139
- Candidate_division_OP9
- Uncultured *Bacteria*_ML635J-21
- Uncultured *Bacteria*_MLE1-12
- Other

Chloroflexi
- *Caldilineae_Caldilineales*
- GIF3
- GIF9
- JG30-KF-CM66
- MSB-5B2
- MSBL5
- Napoli4B-65
- Sh765B-AG-111
- vadinBA26

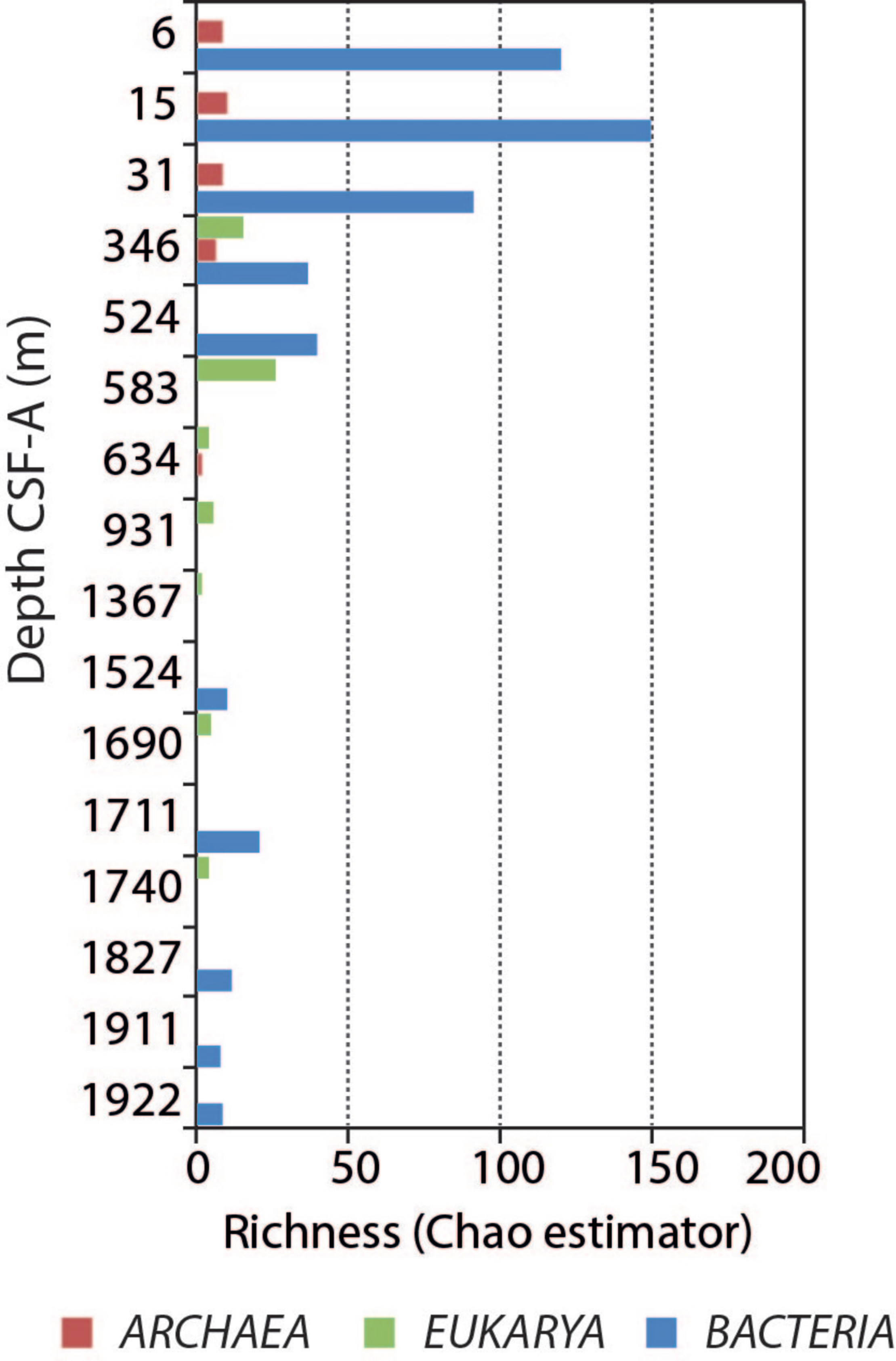
Proteobacteria
- *Alphaproteobacteria_Caulobacterales*
- *Alphaproteobacteria_Rhizobiales*
- *Alphaproteobacteria_Sphingomonadales*
- *Betaproteobacteria_Burkholderiales*
- *Betaproteobacteria_Neisseriales*
- *Deltaproteobacteria_Desulfarculales*
- *Deltaproteobacteria_Desulfobacterales*
- *Deltaproteobacteria_Myxococcales*
- *Gammaproteobacteria_Pseudomonadales*
- *Gammaproteobacteria_Xanthomonadales*

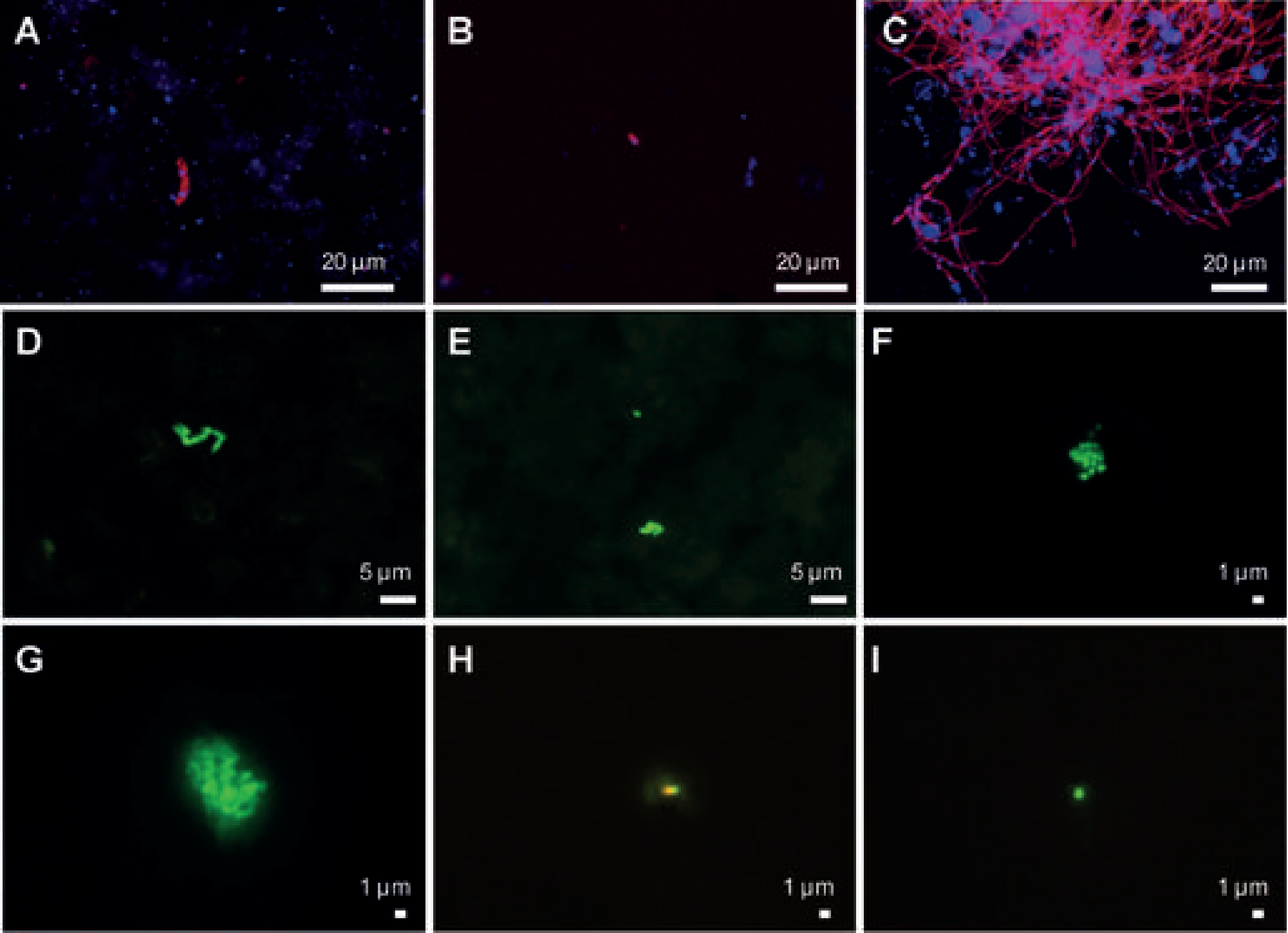
Real time-PCR
- *Bacteria*
- *Archaea*
- *Eukarya*
- JS1/*Chloroflexi*
- *dsr A*

Lithology
- Mud, sand, shells/ sandy mud/ calcareous sandy mud
- Sandy marlstone/ lithified marlstone/ limestone
- Nannofossil limestone

UC : Unconformity

CSF-A (m) : Core depth below seafloor computed by conventional method A


Depth CSF-A (m)
6
15
31
346
524
583
634
931
1367
1524
1690
1711
1740
1827
1911
1922
0
50
100
150
200
Richness (Chao estimator)
ARCHAEA
EUKARYA
BACTERIA


A
20 µm
B
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C
20 µm
D
5 µm
E
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F
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G
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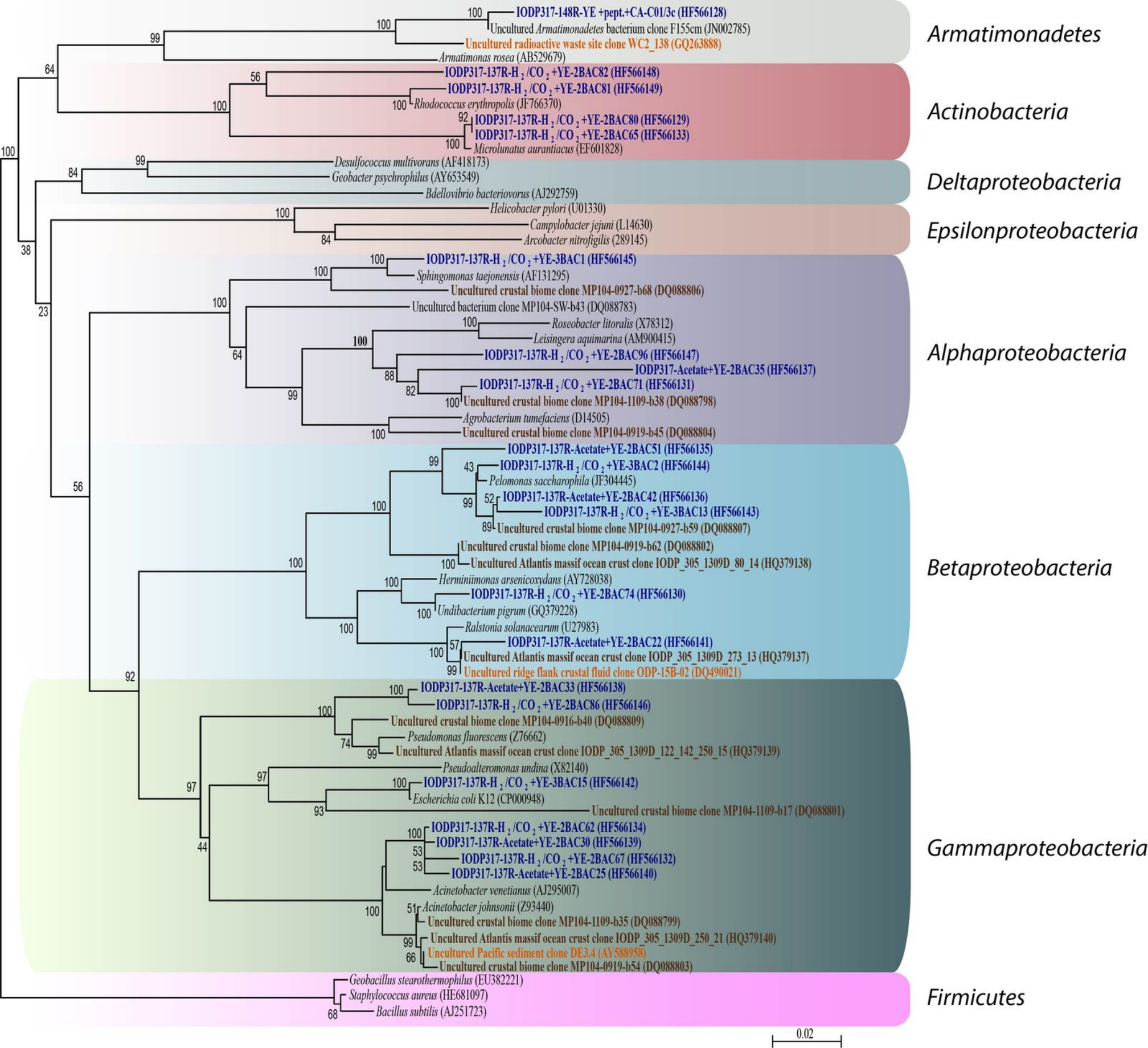
H
1 µm
I
1 µm


IODP317-148R-YE +pept.+CA-C01/3c (HF566128)
Uncultured Armatimonadetes bacterium clone F155cm (JN002785)
Uncultured radioactive waste site clone WC2_138 (GQ263888)
Armatimonas rosea (AB529679)
IODP317-137R-H2/CO2+YE-2BAC82 (HF566148)
IODP317-137R-H2/CO2+YE-2BAC81 (HF566149)
Rhodococcus erythropolis (JF766370)
IODP317-137R-H2/CO2+YE-2BAC80 (HF566129)
IODP317-137R-H2/CO2+YE-2BAC65 (HF566133)
Microlunatus aurantiacus (EF601828)
Desulfococcus multivorans (AF418173)
Geobacter psychrophilus (AY653549)
Bdellovibrio bacteriovorus (AJ292759)
Helicobacter pylori (U01330)
Campylobacter jejuni (L14630)
Arcobacter nitrofigilis (289145)
IODP317-137R-H2/CO2+YE-3BAC1 (HF566145)
Sphingomonas taejonensis (AF131295)
Uncultured crustal biome clone MP104-0927-b68 (DQ088806)
Uncultured bacterium clone MP104-SW-b43 (DQ088783)
Roseobacter litoralis (X78312)
Leisingera aquimarina (AM900415)
IODP317-137R-H2/CO2+YE-2BAC96 (HF566147)
IODP317-Acetate+YE-2BAC35 (HF566137)
IODP317-137R-H2/CO2+YE-2BAC71 (HF566131)
Uncultured crustal biome clone MP104-1109-b38 (DQ088798)
Agrobacterium tumefaciens (D14505)
Uncultured crustal biome clone MP104-0919-b45 (DQ088804)
IODP317-137R-Acetate+YE-2BAC51 (HF566135)
IODP317-137R-H2/CO2+YE-3BAC2 (HF566144)
Pelomonas saccharophila (JF304445)
IODP317-137R-Acetate+YE-2BAC42 (HF566136)
IODP317-137R-H2/CO2+YE-3BAC13 (HF566143)
Uncultured crustal biome clone MP104-0927-b59 (DQ088807)
Uncultured crustal biome clone MP104-0919-b62 (DQ088802)
Uncultured Atlantis massif ocean crust clone IODP_305_1309D_80_14 (HQ379138)
Herminiimonas arsenicoxydans (AY728038)
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Undibacterium pigrum (GQ379228)
Ralstonia solanacearum (U27983)
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Uncultured ridge flank crustal fluid clone ODP-15B-02 (DQ490021)
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IODP317-137R-H2/CO2+YE-2BAC86 (HF566146)
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Pseudomonas fluorescens (Z76662)
Uncultured Atlantis massif ocean crust clone IODP_305_1309D_122_142_250_15 (HQ379139)
Pseudoalteromonas undina (X82140)
IODP317-137R-H2/CO2+YE-3BAC15 (HF566142)
Escherichia coli K12 (CP000948)
Uncultured crustal biome clone MP104-1109-b17 (DQ088801)
IODP317-137R-H2/CO2+YE-2BAC62 (HF566134)
IODP317-137R-Acetate+YE-2BAC30 (HF566139)
IODP317-137R-H2/CO2+YE-2BAC67 (HF566132)
IODP317-137R-Acetate+YE-2BAC25 (HF566140)
Acinetobacter venetianus (AJ295007)
Acinetobacter johnsonii (Z93440)
Uncultured crustal biome clone MP104-1109-b35 (DQ088799)
Uncultured Atlantis massif ocean crust clone IODP_305_1309D_250_21 (HQ379140)
Uncultured Pacific sediment clone DE3.4 (AY588958)
Uncultured crustal biome clone MP104-0919-b54 (DQ088803)
Geobacillus stearothermophilus (EU382221)
Staphylococcus aureus (HE681097)
Bacillus subtilis (AJ251723)
Armatimonadetes
Actinobacteria
Deltaproteobacteria
Epsilonproteobacteria
Alphaproteobacteria
Betaproteobacteria
Gammaproteobacteria
Firmicutes
0.02