Molecular analysis of deep subsurface microbial communities in Nankai Trough sediments (ODP Leg 190, Site 1176)

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Abstract

The prokaryotic community inhabiting the deep subsurface sediments in the Forearc Basin of the Nankai Trough southeast of Japan (ODP Site 1176) was analyzed by 16S rDNA sequencing. Sediment samples from 1.15, 51.05, 98.50 and 193.96 m below sea floor (mbsf) harbored highly diverse bacterial communities. The most frequently retrieved clones included members of the Green non-sulfur bacteria whose closest relatives come from deep subsurface environments, a new epsilon-proteobacterial phylotype, and representatives of a cluster of closely related bacterial sequences from hydrocarbon- and methane-rich sediments around the world. Archaeal clones were limited to members of the genus Thermococcus, and were only obtained from the two deepest samples.

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1. Introduction

The deep subsurface biospheres of the world’s oceans have only recently become a main focus of scientific research. Although approximately 70% of the Earth’s surface is marine, little is known about the microbiology of the underlying sediments. Recent estimates of global bacterial biomass indicate that a large fraction of bacterial biomass resides in the deep subsurface, most of which is in the marine deep subsurface [1,2].

Although rates of metabolic processes in the deep-sea subsurface have been estimated [2–4], the microorganisms remain largely unidentified. Cultivation surveys and most probable number quantifications have detected fermentative, nitrate-reducing and sulfate-reducing bacteria as well as methanogenic archaea ([5] and references therein). Most of the pure culture isolations and characterizations from deep marine sediments have focused on sulfate-reducing bacteria from the Japan Sea [6,7] and the Cascadia Margin [8]. Diverse heterotrophic proteobacteria, Gram-positive bacteria and members of the Cytophaga-Flavobacterium-Bacteroides (CFB) phylum have also been enriched from deep sediments of the Nankai Trough southeast of Japan [9]. Likewise, extensive cultivation efforts on samples from terrestrial subsurface environments have yielded predominantly members of the alpha-, beta-, and gamma-proteobacteria, the CFB phylum and of the high G+C and low G+C Gram-positive bacteria [10]. To date, almost all cultured bacteria from the deep subsurface fall into well-known bacterial genera with many cultured species and strains from near-surface habitats.

A very different picture emerges from culture-independent surveys using polymerase chain reaction (PCR), cloning and sequencing of rRNA genes and functional genes. From the first sequencing studies of deep subsurface sediments [11,12], it has become clear that these microbial ecosystems harbor diverse bacteria and archaea that are in many cases only distantly related to cultured isolates [13–15]. This 16S rRNA survey describes the prokaryotic biodiversity of non-hydrothermal deep subsurface sediments from 1–194 m below sea floor (mbsf) at a water depth of 3017 m in the Nankai Trough southeast of Japan.
2. Materials and methods

2.1. Site description and sampling

All sediment samples for this study were obtained during Ocean Drilling Program (ODP) Leg 190, from Hole 1176A in the Nankai Trough, at a depth of 3017 m below sea level in June 2000 (Fig. 1). Sediment samples were retrieved by advanced piston coring (APC), except for the sample from 194 mbsf that was retrieved by extended core barrel (XCB) drilling [16,17]. Using cutting wires, whole-round samples were obtained from core sections immediately after core retrieval. Subcore samples of approximately 706–1178 ml were taken from sediment layers at 1.15, 51.05, 98.50 and 193.96 mbsf, and were stored at −80°C on the ship until transport to the home laboratory on dry ice.

Site 1176 is located in the upper slope basin within the large thrust-slice zone in the Nankai Trough accretionary prism, which is created by the subduction of the sediment-rich Philippine plate below the Eurasian plate at a rate of 4 cm/year [18]. All samples analyzed in the present study belong to the same quaternary sediment unit in the upper slope basin that covers the accretionary prism. At 200 mbsf, the sediments are approximately 800,000 years old [19]. The sediments consist predominantly of nannofossil-rich hemipelagic mud interlayered with volcanic ash, and sand-to-silt turbidites showing local soft-sediment folding and disruption. Diatoms become scarce in the lower part of the unit, but nannofossils are abundant throughout. This sedimentary sequence is characterized by hemipelagic settling, occasional turbidity currents, and submarine mud flows, together with air falls of volcanic ash. Porosities decrease gradually with depth, from ~65–70% at the mudline to 55–60% at 220 mbsf, with a steeper decrease by 3–5% around 200 mbsf, and remain relatively constant between 40 and 45% at 300 mbsf. Temperature increased linearly from 1.4°C at 1.15 mbsf to 12.2°C at 193.94 mbsf. Sulfate concentrations decreased with depth, and reached zero at approximately 20 mbsf. Sulfate concentrations decreased linearly with depth over the upper 10 m, followed by a local maximum at 20 mbsf. At the base of Hole 1176A (325–393 mbsf), sulfate concentrations increased again to ~17 mM, almost 60% of the seawater value. Except for sulfate, pore fluid composition at these depths remained near that of seawater, suggesting slow, ongoing bacterial sulfate reduction. Ammonium produced by microbioly mediated decomposition of organic matter in-
The most intense microbially mediated reactions occurred with a broad maximum of ~5 mM between ~20 and 200 mbsf and peaked at ~100 mbsf. Total organic carbon (TOC) ranged from 0.30 to 1.73 wt% in the first 200 mbsf (lowest at 62.48 and highest at 135.20 mbsf). Headspace gas concentrations of methane were very low in the first 11.9 mbsf within the sulfate reduction zone (7.3–11.7 ppm), increased to 3000–30000 ppm below the sulfate reduction zone, and returned to generally less than 10 ppm in the deepest portion of the core, below 325 mbsf. Methane was the most dominant hydrocarbon measured throughout Hole 1176A. In general, bacterial cell densities declined rapidly from the surface. Microbial abundances, determined by acridine orange direct counts [2,5], decreased by one order of magnitude over the sampling interval from 9.33 × 10⁷ cells per cm³ sediment at 1.19 mbsf to 1.19 × 10⁶ at 193.94 mbsf, followed by a further decrease to 1.11 × 10⁶ per cm³ in the two deepest samples at 344 and 363.5 mbsf. The most intense microbially mediated reactions occurred in the top 100 mbsf of the section [19].

2.2. DNA isolation, 16S rRNA gene amplification, cloning and sequencing

Whole-round core samples were stored at −80°C until processing. The outer layers of the deep-frozen cores were pared away aseptically in order to remove sediment that had been in contact with the drilling fluid. DNA was extracted from approximately 1.5 g of sediment from the interior of each core using the UltraClean soil DNA isolation kit (MoBio Laboratories Inc., Solana Beach, CA, USA) according to the manufacturer’s protocol, and dissolved in 25 μl PCR water. The DNA was diluted with PCR water before PCR amplification, to overcome persistent PCR inhibition problems. All samples for archaeal PCR were diluted 1:1000; samples for bacterial DNA amplification from the surface (1 mbsf) were diluted 1:10, and samples from the other layers (51, 98, 194 mbsf) were diluted 1:100. For PCR amplification, 1 μl of the diluted DNA preparation was used. The DNA input for the archaeal PCR corresponded to the DNA content of 6 × 10⁻⁵ g sediment ([1 μl/25μl] × dilution factor 0.001 × 1.5 g sediment = 6 × 10⁻⁵ g sediment); the DNA input for the 1:100 and 1:10 diluted bacterial PCR corresponded to 6 × 10⁻⁴ and 6 × 10⁻³ g sediment, respectively. With nested PCR, a 900-bp portion of the 16S rRNA genes was amplified using nested PCR on a Gene-Amp PCR Cycler (Perkin-Elmer, Foster City, CA, USA) following the manufacturer’s protocol. For each sediment layer, 24–39 clones were analyzed. Of all the clones analyzed, on average, 27% were false positive and were excluded from further analysis.

Sequence data were obtained on an ABI3700 (Applied Biosystems Inc., Foster City, CA, USA) capillary sequencing apparatus using the BigDye Terminator v 3.0 kit (Applied Biosystems Inc., Foster City, CA, USA) with the primers M13F (5’-GTAAAACGACGGCCAG-3’) and M13R (5’-CAGGAAACACGTATGAC-3’) and a cycle sequencing protocol according to the manufacturer’s protocol. For each individual clone, forward and reverse reads were assembled using Sequencher 3.0 (Gene Codes Corporation, Ann Arbor, MI, USA). The 5’ and 3’ ends of each PCR product were covered by one sequence read each, and the central 0.7- to 0.8-kb portion was covered on both strands. Sequences were checked for chimeras using the CHIMERA_CHECK function of the Ribosomal Database Project II [20].

2.3. Phylogenetic analysis

Sequence data were compared using the ARB software (www.arb-home.de) and aligned with sequences obtained from the ARB and GenBank databases, using the ARB
FastAligner utility, and followed by manual aligning according to secondary structure. Analyses were performed using minimum evolution and parsimony methods implemented in PAUP* version 4.08b [21]. Heuristic searches under minimum evolution criteria used 1000 random-addition replicates per data set, each followed by tree bisection–reconnection topological rearrangements. Models for use in minimum evolution analyses of the archaeal and bacterial data sets were selected using the likelihood ratio test implemented in Modeltest v.3.0 [22]. For both data sets, all models tested that were simpler than the general time reversible (GTR)+invariant sites (I)+gamma shape parameter (G) model had a significantly poorer fit to the data for the reference topology (based on a neighbor-joining tree according to Jukes–Cantor). Therefore, minimum evolution distance trees were inferred using a GTR model with six classes of substitutions, unequal base frequencies, proportion of invariable sites, and the evolutionary rate of the remaining portions of sites differing according to a gamma distribution (with shape parameter alpha). Model parameters estimated for minimum evolution analyses for the archaeal and bacterial data sets were: alpha of 0.620 and 0.818, and proportion of invariable sites of 0.618 and 0.194, respectively. Bootstrapping under minimum evolution and parsimony criteria was done with 200 replicates for both the archaeal and bacterial data sets. New sequences have GenBank numbers AY191324–AY191357 and AY191321–AY191323 for bacteria and archaean, respectively.

3. Results

3.1. 16S rDNA from Nankai Trough sediments

Only low amounts of DNA could be extracted from Nankai Trough sediments, using 1.5 g sediment/sample. 5-µl aliquots of the 25-µl DNA extracts could not be visualized with ethidium bromide staining, indicating that the DNA concentration was below 1-2 ng µl⁻¹, or 5-10 ng per sediment sample. After the first round of 16S rDNA-targeted PCR amplification, no visible PCR product could be observed. Visible 900-bp PCR products appeared only after the second (nested) PCR, and were absent in the negative controls for DNA blind preparation and PCR.

3.2. Bacterial sequences

The sequences of 61 bacterial and 80 archaeal clones were determined. As a caveat, the absolute numbers of clones and their depth distribution patterns cannot be interpreted as indicators of the abundance of different bacterial groups in nature; also, the frequency of rarely retrieved clones indicates that the microbial community was not sampled to exhaustion. To avoid the misleading connotation of a 100% complete microbial community survey, the abundances of clone groups are given in absolute numbers, not as percentages.

The highly diverse bacterial 16S rDNA clone libraries were dominated by proteobacterial clones (35 clones; including 16 epsilon- and 12 gamma-proteobacterial clones), members of the Green non-sulfur (GNS) bacteria, and a bacterial lineage without cultured representatives (Fig. 2). The sequences in the epsilon-proteobacteria consisted of essentially identical clones of a phylotype that was related to sulfur- and iron-reducing Sulfovibrio species [23], and the sulfur oxidizer Arcobacter sulfidicus [24]. They differ from the epsilon-proteobacterial group I sequences (related to Alvinella pompejana and Rimicaris exoculata symbionts) that are frequently found at the sediment–water interface in deep-sea sediments, hydrothermal sediments and cold seep sediments [25,26]. Sequences of the GNS division were found eight times. Their closest relatives were sequences from terrestrial and marine subsurface sediments including hydrate-containing sediments of the Nankai Trough [27,13], and the dehalogenating, anaerobic bacterium Dehalococcoides ethenogenes [28]. Eleven clones belonged to a bacterial lineage that predominantly harbors sequences from hydrocarbon- and methane-rich sediments from around the world, cold seeps, and enrichment cultures on alkanes and aromatic compounds. With the exception of Nankai subsurface clones 1 mbsf-15 and 51 mbsf-15, the members of this lineage form a well-supported cluster of closely related strains [29,30,31,13,26].

Fewer clones from other phylogenetic groups were found. Four clones grouped within the alpha-proteobacterial genera Hydrogenibacter and Pedobacteria, methylotrophic and heterotrophic bacteria who divide by hyphae formation (Fig. 2). Three clones belonged to the cyanobacteria and chloroplasts. Lineages that were represented by single clones included the delta-proteobacteria, the OP8 candidate subdivision, the CFB phylum, and the Planctomycetes phylum (Fig. 2).

In marked contrast to these subsurface clones that are only distantly related to cultured bacterial species, almost all beta- and gamma-proteobacterial sequences in the Nankai clone libraries closely match the 16S rDNA sequences of Escherichia coli, Pseudomonas stutzeri, Variovorax paradoxus, Acinetobacter junii, and near-identical sequences (MT9, MT5, MT11, MT6) that have been obtained by cloning from negative controls or from environmental samples with low DNA content [32]. Several of these sequences were obtained multiple times from different sediment layers, except the near-surface sediment (Table 1).

3.3. Depth distribution

Different lineages of bacterial 16S rRNA sequences followed different patterns in depth distribution. Some bacterial groups were only recovered from deep sediment
The epsilon-proteobacterial clones were obtained from the 51, 98.5 and 194 mbsf samples, but not from the 1 mbsf samples (Table 1). The cyanobacteria- and chloroplast-related sequences, the delta-proteobacterial clone, and the CFB clone were obtained from the 194 mbsf sample. Other sequences are associated with the 1 m surface layer of the sediment column; such as all of the alpha-proteobacterial clones, the OP8 candidate division, and most of the clones in the hydrocarbon-associated uncultured bacterial lineage. The sequences in the GNS phylum were found at all depths except 194 mbsf.
3.4. Archaeal 16S rDNA sequences

Archaeal sequences were found only at 98.5 and 194 mbsf, not in the surface layer or at 51 mbsf. All archaeal sequences from Hole 1176A belonged to a single phylogenetic type within the Thermococcales (Fig. 3); sequences of methanogens or other archaeal groups were not recovered. The archaeal sequences formed a monophyletic branch with Thermococcus strains MZ1, MZ2, MZ5 and MZ13 isolated from sulfide chimney samples, and with strain MZ3 isolated from alvinellid worms (MZ3) at the Main Endeavor and High Rise fields on the Juan de Fuca Ridge [33].

4. Discussion

4.1. Bacterial diversity and distribution patterns in the sediment column

The best candidates for specialized subsurface bacteria at Nankai Site 1176 are the members of the GNS bacterial phylum that have been repeatedly found in both the terrestrial and marine subsurface [27,34,13]. In deeply buried sediments in Eastern Mediterranean organic-rich sapropels, 70% of all bacterial clones were members of the GNS phylum; significantly fewer clones of this group were found in organic-poor sediments between the sapropel layers [34]. Apparently, these bacteria require organic carbon sources, but their substrate preferences are unknown. The closest cultured relatives of these subsurface clones within the GNS bacteria are several strains of the reductively dehalogenating bacterium D. ethenogenes. This bacterium gains energy by oxidation of hydrogen with concomitant dechlorination of tetrachloroethene, but requires extracts of mixed microbial enrichments for growth [28]. The phylogeny of the GNS phylum groups the currently known Dehalococcoides sequences and related subsurface clones (Fig. 2) into one of at least four major subdivisions within the GNS phylum [35]. Other subdivisions include clones from jet fuel-contaminated aquifers, hot geothermal springs, and subsurface sediments and soils. The relatively well-studied anoxicogenic phototrophic bacteria that became the namesake of the GNS bacterial phylum are limited to one subdivision only. The phylogenetic diversity within the GNS bacteria indicates considerable physiological and ecophysiological diversity, as evidenced by the physiological dissimilarity of the few cultured species, and the unusual range of their extreme habitats.

The representatives of the well-supported clone cluster (99 and 90% bootstrap support under minimum evolution and maximum parsimony criteria) whose members are generally found in hydrocarbon- and methane-rich sediments were retrieved mostly in the surface sediment layer of the Nankai Trough samples, except for two clones from 51 mbsf (Fig. 2). These bacteria may require a more organic carbon-rich environment than the deeper sediment layers of Site 1176 can offer. Representatives of this group are isolated either from near-surface sediment, or from methane-rich subsurface samples where methane hydrates are present, for example clone AT425 EubA5 [31] and clone MB-B2-103 [13].

The most frequently retrieved cluster of near-identical sequences, the epsilon-proteobacterial clones (represented in Fig. 2 by clone 194 mbsf-12), has to our knowledge not been found in deep subsurface environments before. Its phylogenetic position suggests similarities to the facultatively anaerobic, heterotrophic and fermentative genus Sulfurospirillum, which can use a diverse spectrum of electron acceptors (O2 in low concentrations; NO3-, S2-, S2O32-). The species Sulfurospirillum barnesii can also re- spire with Fe(III) and arsenate [23]. The epsilon-proteobacterial subsurface clones are also related to the microaerophilic, sulfide-oxidizing bacterium Candidatus A. sulfidicus. This autotrophic bacterium does not fix carbon via the Calvin cycle, but may instead use the reverse TCA cycle [24]. Thus, the closest cultured relatives of this subsurface clone group show divergent physiologies.

The alpha-proteobacterial sequences were found exclusively in the surface samples. These Hyphomicrobium- and Pedomicrobium-related sequences may represent organotrophic sediment bacteria which grow in situ near the sediment surface. As an alternative possibility, they could be derived from the overlying water column, as Hyphomicrobium and Pedomicrobium spp. are found as epibionts on settling diatoms [36].

Clones that were isolated only in a single instance are not necessarily insignificant. Near-identical sequences have been found in other molecular surveys of the Nankai deep subsurface environment and of marine sediments around Japan. This study has revealed four cases of such similarities (Fig. 2). The deeply-branching delta-proteobacterial clone 194 mbsf-22 is closely related to Nankai clone MB-C2-152, a member of a well-supported cluster from Nankai sediments at 297 mbsf below the hydrate stability zone, located ca. 350 km from Site 1176 [13]. The alpha-proteobacterial surface clone 1 mbsf-1 matches a clone

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<td>Bacterial clones obtained multiple times from different sediment layers</td>
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<td>Representative clone</td>
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<td>194 mbsf-1 (E. coli)</td>
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<td>194 mbsf-14 (E. coli)</td>
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<td>194 mbsf-18 (Hydrospira)</td>
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<td>194 mbsf-22 (Pedomicrobium)</td>
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<td>1 mbsf-3z3 (HMAC)*</td>
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<td>1 mbsf-3z16 (HMAC)*</td>
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<td>1 mbsf-3z3 (HMAC)*</td>
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<td>1 mbsf-4 (HMAC)*</td>
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*Hydrocarbon- and methane-associated cluster.
obtained by reverse transcription of rRNA from cold seep sediments on the Sanriku Escarpment of the Japan Trench at 5343 m depth, indicating the occurrence of *Pedomicrobium* and *Hyphomicrobium* spp. at the surface of these deep-sea sediments [37]. The Planctomycyes-affiliated Nankai clone 51 mbsf-20 is the closest relative of the deep-sea clone BD2-16 from Suruga Bay at 1520 m depth [38], and of Nankai clone MB-C2-147, one of two clones from Nankai subsurface sediments at 297 mbsf below the hydrate stability zone [13]. Within the OP8 candidate subdivision, the surficial Nankai clone 1 mbsf-6 is the closest relative to clone NKB18 from Nankai Trough surface sediments at

Fig. 3. 16S rRNA-based minimum evolution distance tree showing the phylogenetic relationships of archaeal Nankai subsurface clones from ODP Hole 1176A to cultured species and strains, and environmental clones within the genera *Thermococcus* and *Pyrococcus*. The tree is based on *E. coli* positions 24–906 of the 16S rRNA gene. Bootstrap support values (%) are given at nodes for minimum evolution distance (first) and parsimony (second) criteria. * indicates value < 50.
3843 m depth [39] and of reverse-transcribed clone CS8.15 from the Sanriku Escarpment cold seeps [37]. Apparently, these rarely retrieved deep-sea clones from different sites match and substantiate each other, and indicate the presence of a common deep-sea bacterial flora that can be found in several different deep-sea sediment habitats and at different depths, although it does not dominate individual clone libraries.

4.2. Contamination potential

Sediment samples with low DNA content are particularly sensitive to amplification of contaminant DNA. The gamma-proteobacterial clones obtained in this study are good candidates for contaminants, based on their close similarity to published 16S rRNA clones from negative DNA preparation controls [32]. The most frequently retrieved clones are E. coli sequences that were obtained seven times after PCR amplification and cloning from the two deepest deep sediment samples (98 and 194 mbsf). These sequences could have originated from the E. coli strain that was used for cloning the PCR products. The 98 and 194 mbsf layers had reduced cell numbers (1.5 \times 10^6 g^{-1} respectively [19] and therefore a lower DNA content compared to the surface layer, where the E. coli contaminant was not detected. A further potential source of contamination lies in the laboratory water supply. Acinetobacter spp., Alcaligenes xylosoxidans and Pseudomonas spp. are common constituents of biofilms in stainless-steel water mains [40]; P. stutzeri strains can even be isolated from ultra pure water with deionization columns [41]. The clones related to P. stutzeri, A. junii, Aquaspirillum delicatum, A. xylosoxidans and V. paradoxa were probably derived from dead cell remnants or their DNA in the water supply. The water that was used for preparing reagents, for DNA isolation and PCR reactions, was ultra filtered using a MilliQ system, and autoclaved twice before use; it is therefore unlikely to contain living cells, but could have harbored traces of their genomic DNA.

A different kind of contamination, the intrusion of seawater and pelagic microorganisms and their DNA into sediment samples, is inherent in the drilling process that uses seawater as a drilling fluid. The deepest sample, 194 mbsf, was retrieved using XCB drilling, which is more prone to seawater contamination than the drilling technique used for other sampling depths, APC [16,17,42]. The three clones related to chloroplasts of green plants and eukaryotic green algae that were found only in this sample were most likely derived from near-surface seawater. The alternative scenario, burial and long-term preservation of photosynthetic cells or their DNA in 200 m deep, 800 000 years old quaternary sediments, is unlikely.

These identifications of contaminants are based on comparisons with published studies, or on consistency arguments. Sampling and laboratory contaminations can be identified more directly by preparing control clone libraries from seawater-based drilling fluid, from the outer rim of the core samples, and from the PCR-negative DNA isolation controls [32].

4.3. Archaeal diversity

All 80 archaean clones formed a tight cluster of nearly identical sequences with 1–2% mismatches (in the sequencing error range) randomly distributed over the 16S rRNA gene, including universally conserved regions. Three representative sequences from this highly redundant clone library were included in the phylogenetic tree (Fig. 3). All retrieved archaean sequences belonged to the genus Thermococcus, the most common genus of archaean hyperthermophiles at hydrothermal vent sites, and a reliable microbial marker for hydrothermal activity [25,42]. Special attention was paid to compare the Nankai clones to Thermococcus clones from subsurface locations [43], or to Thermococcus isolates from vent plumes and warm vent fluids that could have carried subsurface strains to the surface [44,33]. The Nankai subsurface clones formed a well-supported cluster (bootstrap support above 90%) with Thermococcus strains MZ1, MZ2, MZ3, MZ5 and MZ13 (Fig. 3). These strains have been isolated from sulfidic vent chimneys and alvinellid worm tubes at the Juan de Fuca Ridge, indicating a trans-Pacific occurrence pattern of closely related Thermococcus strains. However, the Nankai clones were not specifically related to other subsurface clones or isolates. Phylogenetically distinct Thermococcus isolates (MZ4, 8, 9, 10, 11, 12, and 14) from warm vent fluids at Juan de Fuca presumably represent strains from a specific subseafloor habitat, the zone where hot hydrothermal vent fluids mix with entrained seawater in highly porous pillow basalts and lava sheets [33]. A similar subsurface origin has been inferred for Thermococcus strains Gorda 1–6, isolated from hydrothermal vent plumes at the North Gorda Ridge [44]. The Thermococcus subsurface clones (pPCA7.21 and pPCA12.6) from relatively shallow, non-hydrothermal subsurface samples in the Philippine Basin, possibly originated at southeast Asian vent sites, and could have been dispersed and subsequently deposited by sedimentation through the water column [43].

Members of the genus Thermococcus are hyperthermophiles that require a minimum growth temperature between 48 and 73°C [45]. Since the in situ temperatures in ODP Hole 1176A ranged from approximately 1 to 12°C, the Thermococcus populations in the Nankai Trough sediments examined here are presumably not metabolically active in situ. They either represent inactive relic cells from past hydrothermal activity at Site 1176, or cells that were recently introduced through interstitial fluid flow from hydrothermally active sites, for example from the nearby Nankai subduction zone and the accretionary prism. Thermococcus clones in non-hydrothermal sediments...
mments were interpreted as paleontological markers of archaeal populations that were introduced from distant, geothermally active regions millions of years ago, and buried and preserved in the sediment column [43]. These findings raise the question whether thermophilic microorganisms can survive long-term deposition and burial in cold marine sediments to the extent that their cell integrity is not damaged and their genomic DNA remains preserved. So far, thermophilic bacteria that have been found in high numbers in cold marine and terrestrial sediments belong to spore-forming bacterial genera, the anaerobic sulfate-reducing genus Desulfotomaculum [46] and the aerobic genus Geobacillus [47]. The long-term survival of spore-forming bacteria in cold environments is consistent with the observation that thermophile-containing samples from hot environments can be maintained for long periods at low temperatures without loss of viability [48]. Similar long-term survival may apply for the archaeal genera Thermococcus and Pyrococcus; cultures can be maintained in liquid culture at 4°C for years, and tolerate even oxygen exposure at cool temperatures to some extent [49]. Possibly, the Thermococcus populations in the Nankai subsurface sediments have been introduced by deep, lateral seawater influx that replenishes pore water sulfate levels below 35 mbfs in the sediment column of Hole 1176A [19]. Horizontal fluid flow through continuous channels, from the accretionary prism seawards to the deformation front and centered around the décollement depth, has been inferred for the Nankai system by comparisons of chloride profiles at ODP Sites 808, 1174, and 1173, on both sides of the deformation front [50]; fluid velocities were estimated as ca. 13 cm/year [50].

The Thermococcus clones were obtained from the methan-harbouring sediment layers, where methanogenic archaea would have been expected. This conspicuous absence of methanogen-affiliated sequences in these deep subsurface 16S rDNA clone libraries is not unprecedented. A sequencing survey of methane hydrate-rich sediments in the Cascadia Margin did not detect methanogens with general archaeal 16S rDNA primers, but found cold-water Crenarchaeota otherwise described from seawater [51]. Only PCR amplification of the highly conserved methyl coenzyme M reductase gene (mcrA), a specific key gene of methanogenesis, indicated the presence of methanogenic archaea [51]. Such results could reflect differences in the proportions of methanogens and other archaea in the sediments.

4.4. Conclusions

The Nankai subsurface sediments harbor bacterial clones without close cultured relatives, indicating that our knowledge of deep subsurface bacterial populations and their physiologies is inadequate at best. Inferences based on comparisons with cultured species become more and more unreliable with increasing phylogenetic distance. Bacterial groups whose members are consistently found in deep subsurface sediments and on the surface of deep-sea sediments (the GNS bacteria, the hydrocarbon-associated bacterial cluster, the OP8 division) point to common denominators in microbial community composition and function in these environments. The archaeal data set shows the existence of microbial populations that are unlikely to be active and growing in situ, but may have been introduced by lateral subsurface flow. The deep subsurface is not a hermetically sealed and static environment that encloses its microbial populations in perpetuity, but instead is subject to exchange processes, in particular with the deep-sea sediment surface.

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