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Diversity of prokaryotes and methanogenesis in deep subsurface sediments from the Nankai Trough, Ocean Drilling Program Leg 190

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Summary

Diversity of *Bacteria* and *Archaea* was studied in deep marine sediments by PCR amplification and sequence analysis of 16S rRNA and methyl co-enzyme M reductase (*mcrA*) genes. Samples analysed were from Ocean Drilling Program (ODP) Leg 190 deep subsurface sediments at three sites spanning the Nankai Trough in the Pacific Ocean off Shikoku Island, Japan. DNA was amplified, from three depths at site 1173 (4.15, 98.29 and 193.29 mbsf; metres below the sea floor), and phylogenetic analysis of clone libraries showed a wide variety of uncultured *Bacteria* and *Archaea*. Sequences of *Bacteria* were dominated by an uncultured and deeply branching 'deep sediment group' (53% of sequences). Archaeal 16S rRNA gene sequences were mainly within the uncultured clades of the *Crenarchaeota*. There was good agreement between sequences obtained independently by cloning and by denaturing gradient gel electrophoresis. These sequences were similar to others retrieved from marine sediment and other anoxic habitats, and so probably represent important indigenous bacteria. The *mcrA* gene analysis suggested limited methanogen diversity with only three gene clusters identified within the *Methanosarcinales* and *Methanobacteriales*. The cultivated members of the *Methanobacteriales* and some of the *Methanosarcinales* can use CO_2 and H_2 for methanogenesis. These substrates also gave the highest rates in ^{14}C-radiotracer estimates of methanogenic activity, with rates comparable to those from other deep marine sediments. Thus, this research demonstrates the importance of the 'deep sediment group' of uncultured *Bacteria* and links limited diversity of methanogens to the dominance of CO_2/H_2 based methanogenesis in deep sub-seafloor sediments.

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Introduction

Bacteria and *Archaea* in sub-seafloor sediments make up about 70% of the global number of prokaryotes (Whitman *et al*., 1998), but these have not been extensively studied. This deep biosphere extends to at least 800 m below the sea floor (mbsf) and acridine orange direct bacterial counts decrease from about 10^9 cells ml^{-1} near the sediment surface to 10^4 cells ml^{-1} in the deep layers (Parkes *et al*., 1994; Reed *et al*., 2002; Wellsbury *et al*., 2002). Prokaryote populations are highest in shallow water, high productivity sites where the organic carbon content of the sediment is high, and lowest in deep water, low productivity sites (Parkes *et al*., 2000). Despite lower population sizes in deeper layers prokaryotic activity can be measured and can account for the majority of depth integrated activity (Wellsbury *et al*., 2002).

Enrichments from deep sediments have enabled novel organisms to be isolated (Bale *et al*., 1997; Barnes *et al*., 1998), but, most probable, number counts of bacteria have been very low, with viability down to 0.0000087% (Cragg *et al*., 1990). Consequently the limitations of current culture methods mean that molecular approaches must be used if the full diversity of prokaryotes in the deep biosphere is to be explored. A few studies of this type have been carried out using 16S rRNA gene-based approaches from deep sediments with relatively high densities of bacteria (e.g. Rochelle *et al*., 1994; Marchesi *et al*., 2001). This research showed comparatively low diversity compared with near-surface marine sediments (e.g. Li *et al*., 1999a; Ravenschlag *et al*., 1999; Teske *et al*., 2002; Bowman and McCuaig, 2003). This is especially true for methanogens as only three methanogenic species were identified with methanogen specific primers from deep, methane-hydrate-rich sediment from the Cascadia Margin (Marchesi *et al*., 2001).

The current study aimed to compare the diversity of prokaryotes with methanogenic activity in low organic carbon, deep-sediments from the Nankai Trough, Japan. It is the first study to directly relate methanogen methyl coenzyme M reductase gene (*mcrA*) diversity with rates of methanogenesis in deep sediments. Clone libraries for *Archaea* and *Bacteria* with general 16S rRNA gene primers were examined to place the methanogen diversity and activity in context. During this study we used a cautious approach to the molecular methodology to minimise amplification of contaminating sequences (Webster *et al*., 2003). This is important because recent studies using 16S rRNA gene approaches with deep sediments from nearby locations have shown problems with PCR amplification of negative controls (Reed *et al*., 2002) and probable contamination from γ-*Proteobacteria* (Kormas *et al*., 2003).

Results

DNA extraction and PCR amplification

It proved difficult to extract amplifiable DNA from these sediments, despite several methods and modifications being used (Webster *et al*., 2003). The FastDNA Spin kit for Soil extracted amplifiable DNA from the upper three depths of site 1173 (4.15, 98.29 and 193.29 mbsf). DNA was also extracted by a second method (Rochelle *et al*., 1992; Marchesi *et al*., 1998) from site 1174 at 412.6 mbsf but it could not be amplified. No DNA that was visible by gel electrophoresis could be extracted from site 1177 or other depths at sites 1173 or 1174.

Diversity of Bacteria

Primary bacterial 16S rRNA gene amplifications using primers 27F, 1492R gave no apparent product when visualized by gel electrophoresis. However, using nested PCR with general primers in the first (27F, 1492R) and second (63F, 1387R) stage sufficient PCR product was obtained to produce a satisfactory clone library ($n = 72$) after 1 in 100 dilution of DNA from the 4.15 mbsf sediment. Libraries from deeper sediments were not obtained. This was consistent with the results from DGGE which also used a nested approach and revealed a high diversity of *Bacteria* in the 4.15 mbsf sediment (Fig. 1A). Clones screened using restriction fragment length polymorphism analysis (RFLP) with *Hae*III and *Hin*fI restriction enzymes gave 11 unique patterns, representatives of which were sequenced ($n = 40$).

Phylogenetic analysis (Fig. 2) of the sequences from this oceanic sediment showed a diverse population of *Bacteria* (coverage = 75%). Some sequences were related to the *Planctomycetes* (4%), *Cyanobacteria* and chloroplasts (8%), β-*Proteobacteria* (11%) and γ-*Proteobacteria* (14%). Other sequences were chimeric (5%) and were therefore excluded from the analysis. However, most sequences (53%) were in a tightly clustered, deep-branching group, which contained several sequences from other deep-sea marine sediments. Clone JTB138 (and JTB243, not included) is from deep sea, cold seep sediment from the Japan Trench (Li *et al*., 1999b) and CS-B013 is from sediment at a hydrothermal site in the Guaymas Basin (Teske *et al*., 2002). Clones MB-B2-103 and MA-A2-104 were both isolated from deep sediment (165–200 mbsf) from the forearc basin near the Nankai Trough (Reed *et al*., 2002). Clone SB-15 (and SB-45, not included) is from an anoxic benzene-degrading, sulphate-reducing enrichment also from Guaymas basin sediment (Phelps *et al*., 1998). This 'deep sediment group' appears phylogenetically closely related to the OP9 candidate division. The OP9 and deep sediment sequences form a group, which were consistently separate from the δ-*Proteobacteria*, Nitrospina Division (designated in the RDP database, version 8.1) and *Firmicutes* in different phylogenetic reconstructions. Although the bootstrap value linking these groups is <50% in the tree presented (Fig. 2), in other trees values of up to 71% were obtained (data not shown). Furthermore, these clones show 79–80% sequence similarity to the OP9 clones and so are most closely associated with the OP9 group, but cannot

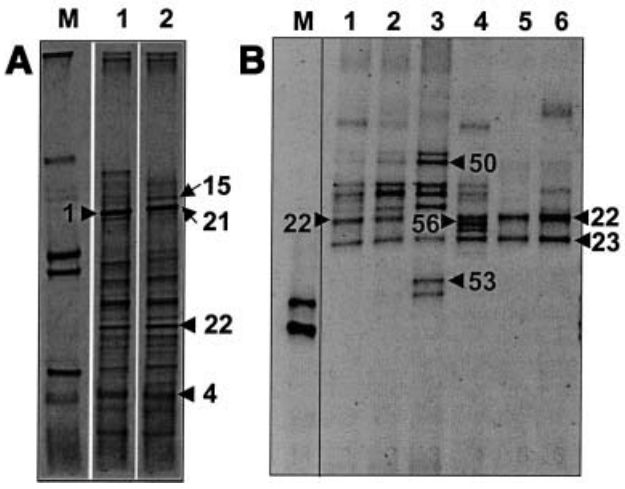


Fig. 1. DGGE profiles of 16S rRNA genes from deep subseafloor sediment samples from ODP Nankai Trough Leg 190, Site 1173.
A. Nested PCR with general *Bacteria* primers 27F, 1492R followed by 357F-GC, 518R. Lane M, DGGE marker (Webster *et al*., 2003). Lane 1 and 2 are replicate DGGE patterns from 4.15 mbsf. Note that these three lanes were on the same gel, but were not beside each other, so the panel has been reconstructed to show only the three lanes of interest.
B. Nested PCR with general *Archaea* primers from 4.15 mbsf sediment. Dilutions of sediment DNA: Lane 1, 1/50; Lane 2, 1/100; Lane 3, 1/200; Lane 4, 1/500; Lane 5, 1/1000; Lane 6, 1/2000. Lane M, *Halobacterium* sp., control. Primers used = Ar3f, Ar9r then Saf, PARCH519r. Both gels were stained with SYBR Gold nucleic acid gel stain (Molecular Probes). Arrows indicate bands excised and sequenced (see Table 1).

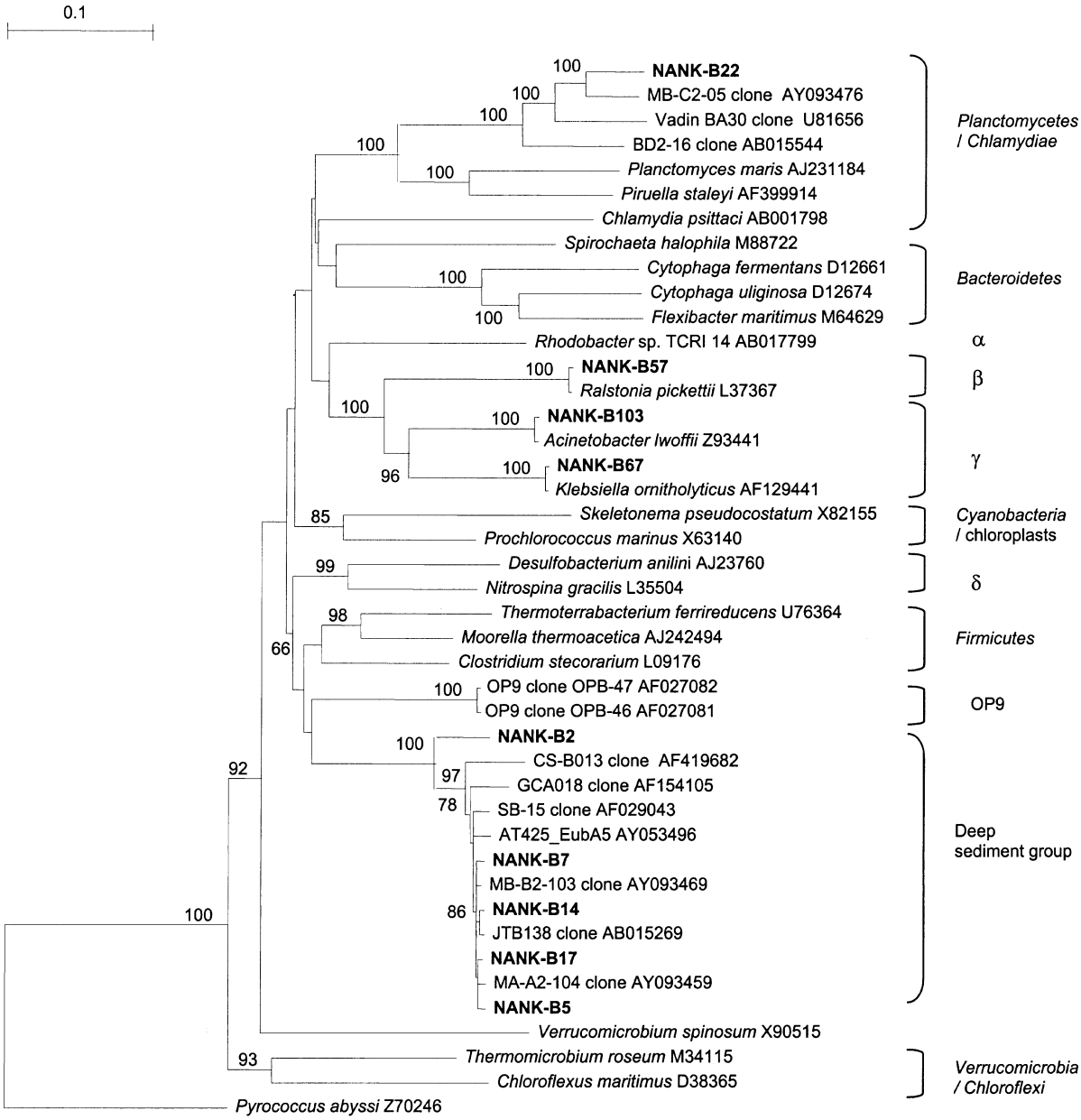


Fig. 2. Phylogenetic tree reconstructed from representative *Bacteria* 16S rDNA sequences ($n = 40$) from Nankai Trough ODP Leg 190, Site 1173 sediment (4.15 mbsf); using primers 63F, 1387R, nested within 27F, 1492R. The scale bar represents 10% sequence similarity, the bootstrap values were derived from 100 analyses and the tree was reconstructed from 1285 aligned bases. The codes α, β, γ and δ are used to designate classes within the phylum *Proteobacteria*.

be confirmed as part of the division. This group has also been called the 'hydrocarbon associated bacterial cluster' (Kormas *et al*., 2003), and based on phylogenies using about 330 bp, is phylogenetically coherent with the JAP504 cluster from the Japan Sea (data not shown; Rochelle *et al*., 1994).

Sequencing representative DGGE bands showed sequences whose nearest relatives in BLAST searches were MB-B2-103 and JTB138 (Table 1) and so were from the deep sediment group. When *Bacteria* 16S rDNA clones isolated in the present study from this group were analysed using DGGE they co-migrated with the OP9-like excised DGGE PCR products (data not shown). Comparisons of sequences from excised DGGE bands with those from clones showed high levels of similarity. For example, NANK-B-GW-21 was 98% similar to clones NANK B7, B14 and B17, and NANK-B-GW-1 was identical to the same three clones. This confirmed the close relationship of these independently amplified 16S rRNA gene sequences.

Table 1. Nearest matches for BLAST search results from 16S rDNA sequences excised from bands on DGGE gels.

Sequence identifier[a]	Nearest match by BLASTN search (accession number)	Sequence similarity (%)	Phylogenetic affiliation	Isolation environment of nearest match
NANK-B-GW-1	Uncultured *Bacteria* clone MB-B2-103 (A7093469)	99	OP9 related	Deep sea sediment, Japan
NANK-B-GW-4	*Spirochaeta* sp. Buddy (AF357916)	97	*Spirochaetes*	River sediment, dechlorinating culture
NANK-B-GW-15	*Carnobacterium* sp. FTR-1 (AF450136)	100	*Firmicutes*	Permafrost, Alaska
NANK-B-GW-21	Uncultured *Bacteria* clone JTB138 (AB015269)	98	OP9 related	Cold seep sediment, Japan Sea
NANK-B-GW-22	Uncultured *Bacteria* clone LCK-41 (AF107329)	93	Unknown	Alpine Lake Cadagno water
NANK-A-GW-22	Uncultured *Archaea* clone no. 15 (D87350)	99	*Crenarchaeota*	Sediment, Mariana Trench
NANK-A-GW-23	Uncultured *Archaea* clone no. 15 (D87350)	99	*Crenarchaeota*	Sediment, Mariana Trench
NANK-A-GW-50	Uncultured *Archaea* clone MN13BT4-97 (AF361693)	97	*Crenarchaeota*	Deep sea carbonate crust
NANK-A-GW-53	Uncultured *Archaea* clone TA1c9 (AF134388)	95	*Euryarchaeota*	Methane using marine sediment
NANK-A-GW-56	Uncultured *Archaea* clone 33-FL49A00 (AF355958)	91	*Crenarchaeota*	Mid-ocean ridge sediment

a. Sequences named NANK-B-GW-*nn* are from the bands in Fig. 1(a) and those named NANK-A-GW-*nn* are from the bands in Fig. 1B; where *nn* is the band identifier on the gel.

Some clones within other phyla were also related to uncultured clones from deep-sea sediments and other anaerobic habitats. For example, NANK-B22 was assigned to the *Planctomycetes* (Fig. 2) and was closely related to MB-C2-05 from the forearc basin (Reed *et al.*, 2002). The deeper branching clone BD2-16 was from sediment in 1521 m of water from Surruga Bay near Tokyo, Japan, which is also close to the Nankai Trough (Li *et al.*, 1999a) and BA30 was from an anaerobic digester (Godon *et al.*, 1997).

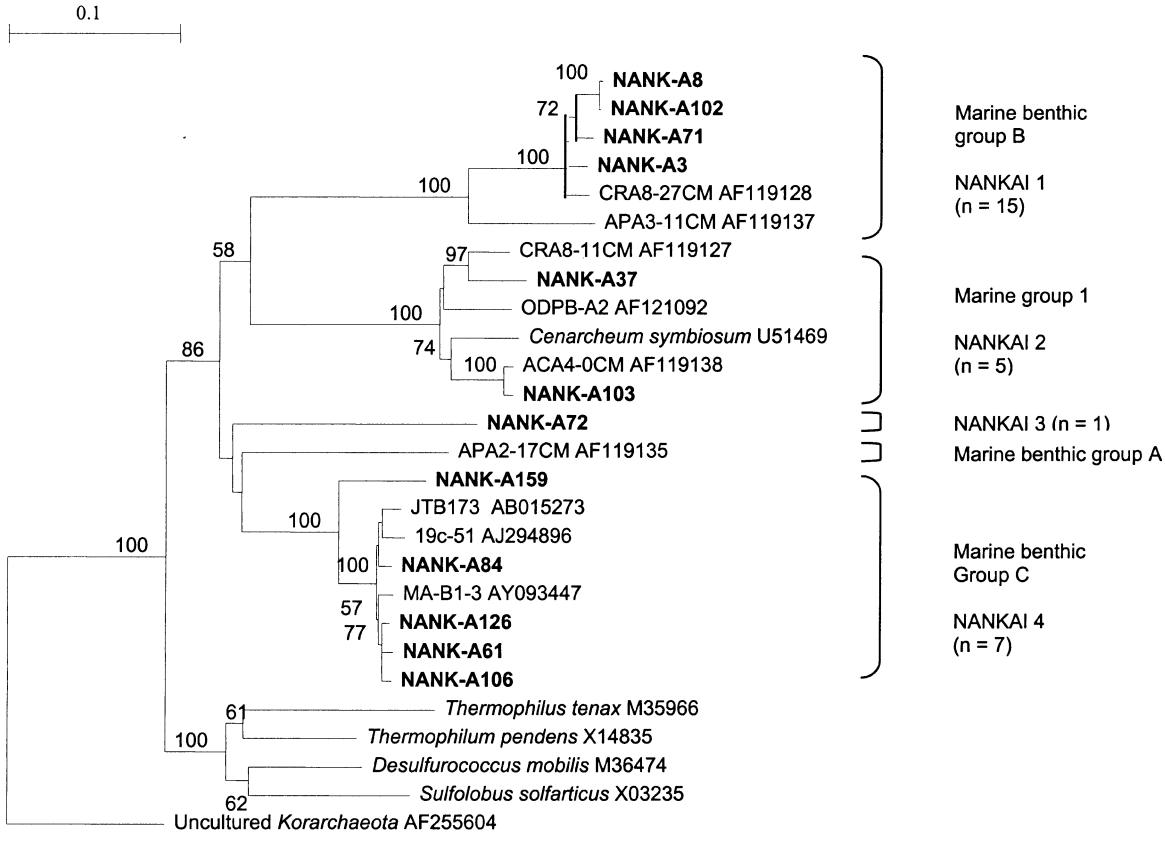
Diversity of Archaea

Polymerase chain reaction products using general *Archaea* primers (21F, 958R) were only obtained from the DNA extracted from the 4.15 mbsf sediment. The resulting 16S rRNA gene clone library ($n = 59$) was screened by RFLP using *Hae*III and *Hin*fI, which gave seven different restriction patterns. Sequencing representative clones ($n = 34$; coverage = 91%) followed by phylogenetic analysis showed that most clones fell into five clusters, four were within the *Crenarchaeota* (Nankai 1–4, Fig. 3A), and one within the *Euryarchaeota* (Nankai 5, Fig. 3B). All the *Crenarchaeota* clones fell within the uncultured Group 1 (DeLong, 1998), which includes a wide variety of clones from natural habitats including sediments, forest soils and paleosols (Jurgens *et al.*, 1997; Pesaro and Widmer, 2002). The *Euryarchaeota* were in an uncultured group positioned between Groups 2 and 3 (DeLong, 1998; Vetriani *et al.*, 1999). Unfortunately, comparisons with other work are made difficult because there is substantial confusion in the literature regarding the names used to denote the groups of uncultured *Archaea* by different authors.

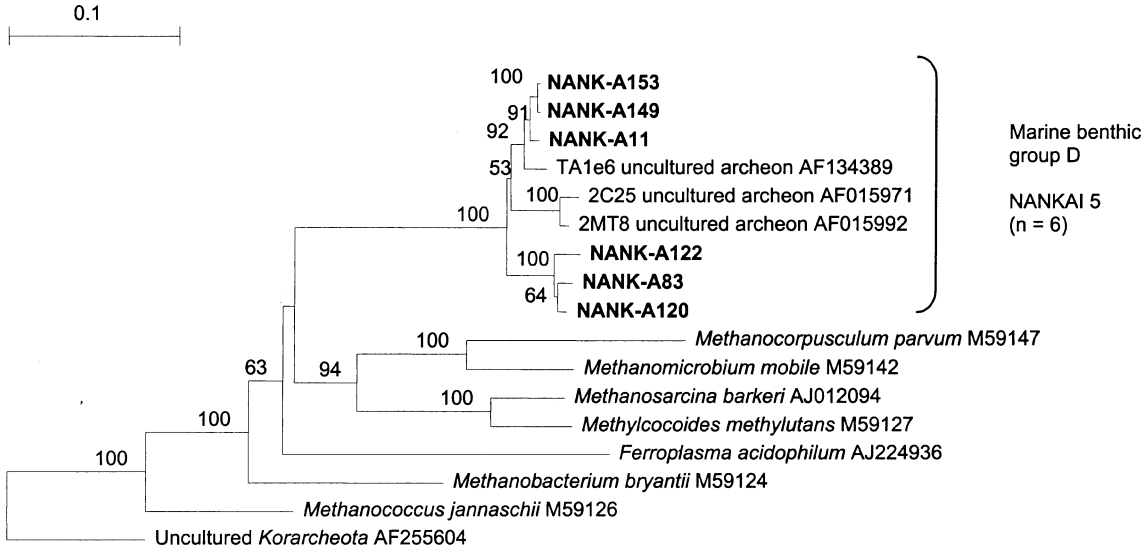
Nankai 1 ($n = 15$) grouped with clones from abyssal plain sediments from the north-western Atlantic Ocean previously assigned to Marine Benthic Group B (CRA8-27 cm; APA3-11 cm; Vetriani *et al.*, 1999). Nankai 2 clones ($n = 5$) were phylogenetically similar to several other deep-sea sediment clones. For example, from the Atlantic (ACA4–0 cm; Vetriani *et al.*, 1999), the Cascadia Margin (ODPB-A; Bidle *et al.* 1999) and the Juan de Fuca ridge (33-F120A00, not included; Huber *et al.*, 2002). This cluster has been designated Marine Group 1 (Vetriani *et al.*, 1999), but has previously been called Group 1.1a (DeLong, 1998). The remainder of the Nankai Trough crenarchaeote clones fell into two clusters (Nankai 3 and 4). Nankai 3 contained only one clone, which was most closely associated with an unidentified crenarchaeote (BBA6; 94% similar; not included) from Buzzards Bay, Cape Cod, USA (Vetriani *et al.*, 1998) and clones from Antarctic continental shelf sediments (e.g. Mertz 21 CM 232; not included; Bowman and McCuaig, 2003). This group has been called Group 1.2 (DeLong, 1998; Jurgens *et al.*, 2000). More clones ($n = 7$) fell into Nankai 4 which was within a grouping called Marine Benthic Group C (Vetriani *et al.*, 1999), also called Group 1.3 (DeLong, 1998; Jurgens *et al.*, 2000; Pesaro and Widmer, 2002). This cluster included clones JTB173 (Li *et al.*, 1999b) from 6400 m deep Japan Trench surface sediment, 19c-51 from Aegean Sea sediment (unpublished) and CRA9-17 cm from Atlantic Ocean deep-sea sediments (Vetriani *et al.*, 1999; not included). The *Euryarchaeota* clones in Nankai 5 ($n = 6$), were phylogenetically most closely related to the Eel-TA1e6 (95%) and similar clones from methane seep sediments from the Eel River in offshore Northern California (Hinrichs *et al.*, 1999). This group of uncultured *Archaea* has been called Marine Benthic Group D (Vetriani *et al.*, 1999), Group 3 (DeLong, 1998; Jurgens *et al.*, 2000) and *Thermoplasmales* and relatives (Orphan *et al.*, 2001; Pesaro and Widmer, 2002). The group includes some Buzzard Bay sediment (e.g. BBA10, not included; Vetriani *et al.*, 1998) and some saltmarsh clones (2C25, 2MT8; Munson *et al.*, 1997).

Amplification 16S rRNA genes from *Archaea* for DGGE analysis using other general PCR primers (Ar3F, Ar9F; Jurgens *et al.*, 1997) was also only successful with the 4.15 mbsf sediment. Therefore, nested PCR was applied

A


0.1
NANK-A8
NANK-A102
NANK-A71
NANK-A3
CRA8-27CM AF119128
APA3-11CM AF119137
CRA8-11CM AF119127
NANK-A37
ODPB-A2 AF121092
Cenarcheum symbiosum U51469
ACA4-0CM AF119138
NANK-A103
NANK-A72
APA2-17CM AF119135
NANK-A159
JTB173 AB015273
19c-51 AJ294896
NANK-A84
MA-B1-3 AY093447
NANK-A126
NANK-A61
NANK-A106
Thermophilus tenax M35966
Thermophilum pendens X14835
Desulfurococcus mobilis M36474
Sulfolobus solfarticus X03235
Uncultured Korarchaeota AF255604
Marine benthic group B
NANKAI 1 (n = 15)
Marine group 1
NANKAI 2 (n = 5)
NANKAI 3 (n = 1)
Marine benthic group A
Marine benthic Group C
NANKAI 4 (n = 7)

B


0.1
NANK-A153
NANK-A149
NANK-A11
TA1e6 uncultured archeon AF134389
2C25 uncultured archeon AF015971
2MT8 uncultured archeon AF015992
NANK-A122
NANK-A83
NANK-A120
Methanocorpusculum parvum M59147
Methanomicrobium mobile M59142
Methanosarcina barkeri AJ012094
Methylcocoides methylutans M59127
Ferroplasma acidophilum AJ224936
Methanobacterium bryantii M59124
Methanococcus jannaschii M59126
Uncultured Korarcheota AF255604
Marine benthic group D
NANKAI 5 (n = 6)

to a dilution series of sample DNA using these general primers in the first stage and DGGE primers (Ovreas *et al*., 1997; Nicol *et al*., 2003) in the second stage. This approach showed good reproducibility between replicate DGGE experiments as indicated, for example, in Fig. 1B. The numbers of bands increased with dilution until 1/100 or 1/200 and reduced thereafter. This indicated that diluting out PCR inhibitors allowed the major and minor components of the community to be discerned, identifying the most dominant sequences in the highest dilutions. Sequence analysis of selected bands (Fig. 1B; Table 1) showed good agreement with the cloning results. All six DGGE sequences were closely related to those from other marine sediment studies. Furthermore, two sequences were similar to the Nankai 2 clones within Marine Group 1, and one was closely matched to the *Euryarchaeota* clones in the Nankai 5 cluster (Fig. 3B), confirming the presence of these two groups of *Archaea* in Nankai Trough sediments.

Amplification with *mcrA* gene primers was more successful, as all three depths from which DNA was extracted from site 1173 gave PCR products using undiluted DNA as template. After pooling product from five replicate PCR reactions a *mcrA* gene clone library ($n = 450$) was obtained, and 150 clones selected from each depth for analysis. Clones were screened by RFLP with *Sau*961, which gave seven restriction groups, and representatives of each group were sequenced ($n = 62$). A phylogenetic reconstruction from these sequences showed that they fell into three tightly grouped clusters (Fig. 4). The number of RFLP groups was greater than the actual clusters found, but comparison of restriction patterns with sequences obtained showed multiple patterns within Nankai cluster 1, possibly resulting from microheterogeneity. As there were no sequences that occurred only once the calculated coverage was 100%, indicating low methanogen diversity. Cluster 1 included the sequence for the cultured species *Methanobrevibacter arboriphilus* within the *Methanobacteriales*. The other two clusters were separated by *Methanosarcina barkeri* and *Methanosarcina mazei*, within the *Methanosarcinales*, and did not match closely with cultured species, so these could represent new species of *Methanosarcina*. These *mcrA* clones were phylogenetically related to clones (e.g. ODP8-ME6 and RS MCR34; see Fig. 4) from the *Methanosarcinales* identified in other studies (Bidle *et al*., 1999; Lueders *et al*., 2001). The clones in these three clusters were not evenly distributed with sediment depth. Most clones were in Cluster 1 (61%), which dominated (73–74%) at 4.15 and 193.29 mbsf, while Clusters 2 and 3 made up 62% of the clones at 98.29 mbsf and 26–27% of the clones at the other two depths.

Methanogenesis

Active methanogenesis was detected at all sites and depths investigated, except at one depth, using the following ^{14}C-tracers: bicarbonate, acetate, methanol, hexadecane and benzoate (Fig. 5). Benzoic acid and hexadecane were used, although they are not direct substrates for methanogens, they can be degraded to methane by syntrophic consortia (Zengler *et al*., 1999; Elshahed *et al*., 2001). Methane formation by such consortia may not be measured from either ^{14}C-labelled CO_2 or acetate. At all sites potential activity was dominated by bicarbonate-methanogenesis (data not shown). From the 23 sample depths over the three sites examined, 17 were dominated by methanogenesis from bicarbonate (maximum rate 102 pmol cm^{-3} day^{-1}), four from methanol (maximum rate 35 pmol cm^{-3} day^{-1}) and two from acetate (maximum rate 0.11 pmol cm^{-3} day^{-1}). Benzoate and hexadecane were of negligible importance representing quantitatively together less than 1% of total methanogenesis in all samples. Total rates of methanogenesis typically ranged between near-zero and 20 pmol cm^{-3} day^{-1} (Fig. 5).

Considering in detail the depths from which we amplified *mcrA* genes, bicarbonate methanogenesis dominated at 98.29 mbsf and 198.29 mbsf (respectively: rates = 9.44, 4.26 pmol cm^{-3} day^{-1}; sulphate = 0, 0; methane = 10,200, 227 μM; acetate = 56.9, 18.2 μM). However, acetate methanogenesis dominated at 4.15 mbsf (rate = 0.0315 pmol cm^{-3} day^{-1}; sulphate = 4.22 mM; methane = 747 μM; acetate = 6.11 μM). Generally, for all the sites and depths investigated there was a negative relationship between rates of methanogenesis and the pore water sulphate concentrations reported elsewhere (Shipboard Scientific Party, 2001), reflecting the competition between methanogens and sulphate reducing bacteria.

Discussion

The difficulty experienced in isolating amplifiable DNA for production of gene clone libraries was not unexpected. Several previous studies reported similar difficulties for deep sediments from ODP legs in the Japan Sea (Rochelle *et al*., 1992; 1995) and Saanich Inlet, British Columbia

Fig. 3. Phylogenetic tree reconstructed from representative 16S rDNA sequences ($n = 59$) of *Archaea* from Nankai Trough Leg 190, Site 1173 sediment (4.15 mbsf) using primers Arch21F, Arch958R. The scale bar represents 10% sequence similarity and the bootstrap values were derived from 100 analyses. (A) A tree reconstructed from 925 aligned bases of *Crenarchaeota* and (B) a tree reconstructed from 916 aligned bases of the *Euryarchaeota*.

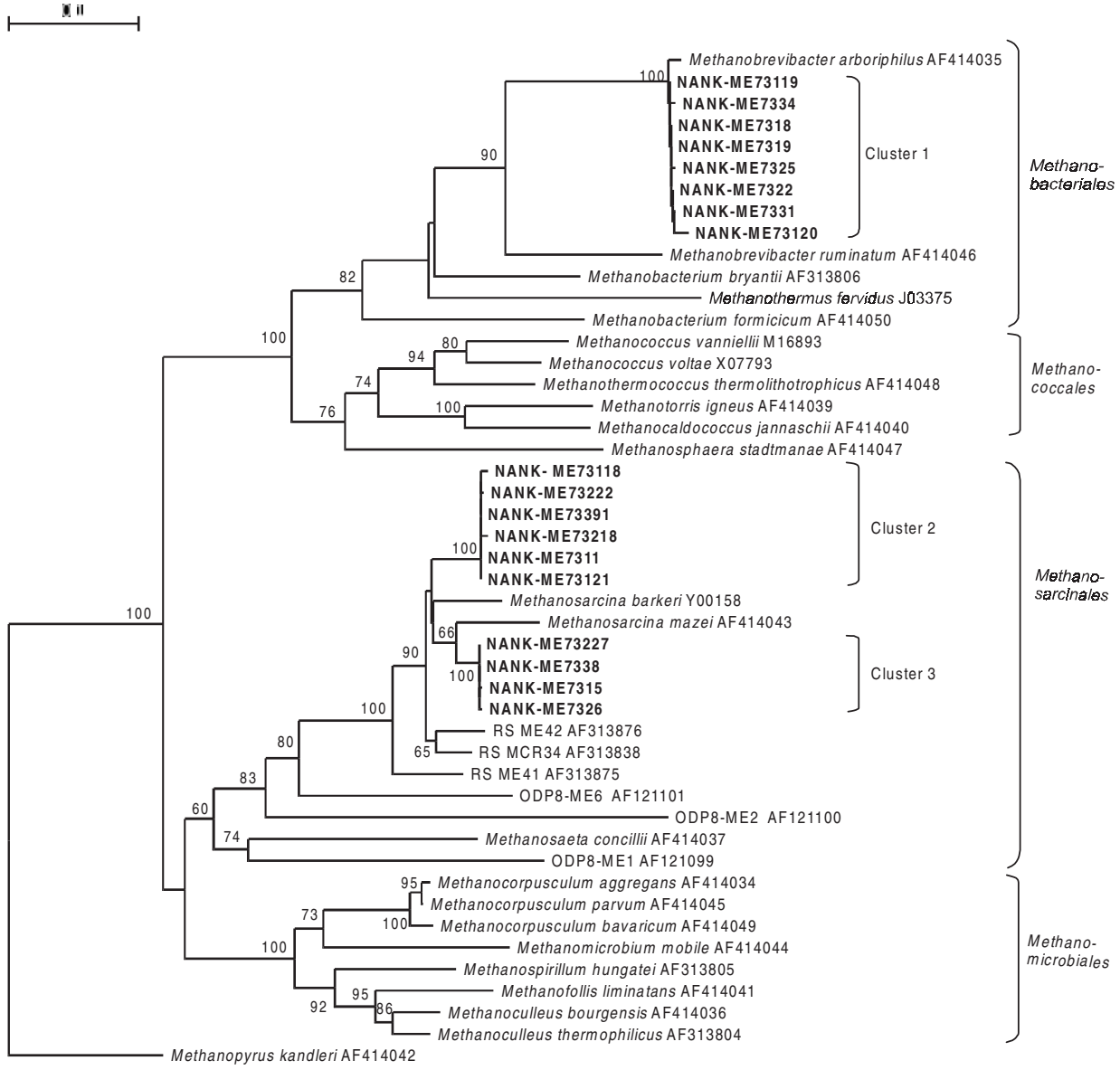


Fig. 4. Phylogenetic tree reconstructed from some methanogen methyl coenzyme M reductase sequences (*mcrA* gene) from ODP Nankai Trough ODP Leg 190 sediments from Site 1173 (4.15, 98.29 and 193.29 mbsf). The scale bar represents 10% sequence similarity, the bootstrap values were derived from 100 analyses and the tree was reconstructed from 760 bases.

(Juniper *et al*., 2001). There are many biases in the molecular approach to studying diversity (von Wintzingerode *et al*., 1997), but it remains the best current method for cataloguing diversity of predominant prokaryotes in natural habitats. Extracting DNA from environments with very low bacterial populations can cause problems caused by co-extraction of PCR inhibitors (Rochelle *et al*., 1992), can lead to random PCR amplification (Chandler *et al*., 1997) and give false impressions of diversity by cloning *Escherichia coli* sequences within *Taq* polymerase (Kormas *et al*., 2003) and other contaminants (Tanner *et al*., 1998). We also report in more detail indications of similar problems with *Bacteria* 16S rRNA gene primers in Nankai Trough sediments elsewhere (Webster *et al*., 2003). Furthermore, our approach of screening PCR products with DGGE and cloning only from PCR reactions without product in negative control tubes almost certainly resulted in cloning fewer contaminating sequences than in other studies (Reed *et al*., 2002; Kormas *et al*., 2003). Furthermore, the similarity of sequences obtained by cloning and DGGE using different primers gives confidence in our results.

Methanogenesis was measurable at all the three sites investigated and in all but one sample. The overall domi-

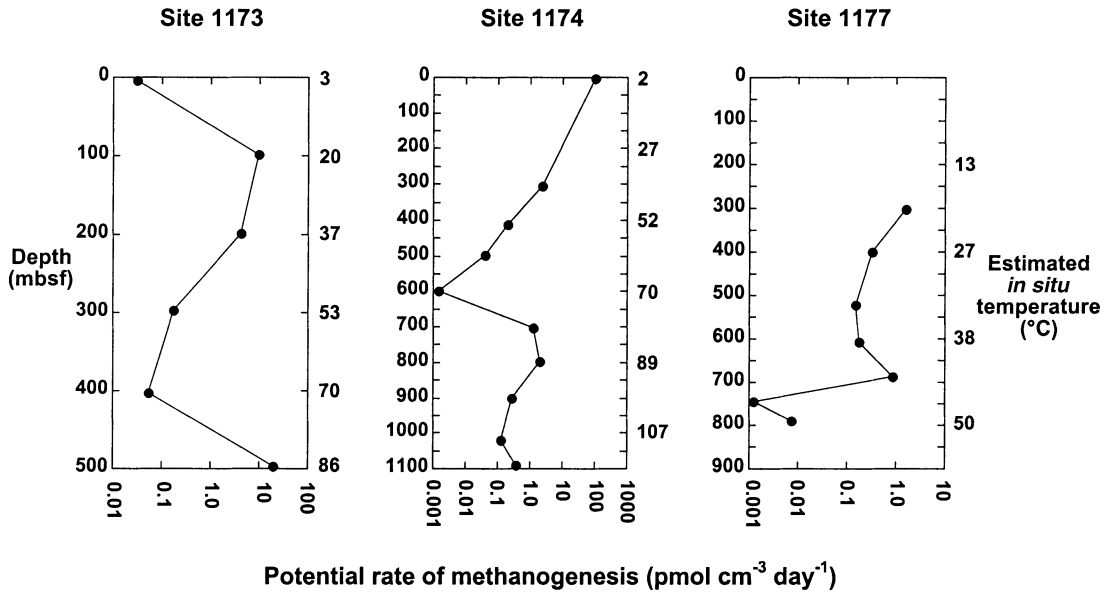


Fig. 5. Potential rates of methanogenesis (pmol cm^{-3} day^{-1}) at three sites from ODP Leg 190 Nankai Trough (data are combined rates from the substrates bicarbonate, acetate, benzoate, methanol and hexadecane).

nance of H_2/CO_2 methanogenesis is consistent with methane stable isotope evidence indicating that this is a major methanogenic pathway in these and other marine sediments (Whiticar *et al*., 1986; Whiticar, 1999). Rates of methanogenesis were comparable with bicarbonate-methanogenesis rates measured in other deep sea sediments: 1–50 pmol cm^{-3} day^{-1} in the Cascadia Margin (Cragg *et al*., 1996); 100–2730 pmol cm^{-3} day^{-1} in the Blake Ridge (Wellsbury *et al*., 2000); 1–20 pmol cm^{-3} day^{-1} in the Woodlark Basin (Wellsbury *et al*., 2002). The majority (61%) of *mcrA* clones isolated above 193.29 mbsf at site 1173 indicated the dominance of members of the *Methanobacteriales*, which use H_2 and CO_2, and sometimes formate, to produce methane. Genes from members of the *Methanosarcinales* were also present in the clone libraries, which grow by dismutating methyl compounds and use acetate as major substrates for methanogeneisis, although some can also use H_2 and CO_2. However, the dominance of H_2/CO_2 activity in all these sediments suggested that the *Methanobacteriales* were the most active methanogenic populations. This would also be consistent with the low pore water acetate concentrations; however, in deeper, hotter layers increases in acetate concentration have been predicted (Wellsbury *et al*., 1997). Interestingly, shallow samples from site 1173, which was the most successful for molecular diversity analysis, did not have the highest rates of methanogenic activity (Fig. 5). This suggests that factors other than bacterial density or activity have an impact on the success of molecular approaches.

The methanogen clones isolated here were all assigned to three tight clusters. These appeared phylogenetically almost identical to the three clusters of methanogen 16S rRNA genes isolated from deep Cascadia Margin sediments (Marchesi *et al*., 2001). This again implied the presence of *M. arboriphilus* and *M. mazei* related methanogens from another site near an accretionary prism at a continental plate boundary. Marchesi *et al*. (2001) did not clone *mcrA* genes. However, Bidle *et al*. (1999) reported the isolation of three *mcrA* clones belonging to the *Methanosarcinales* from a Cascadia Margin ODP bore hole, although these were phylogenetically more distant from *M. mazei* (Fig. 4) than our Nankai *mcrA* sequences. Clones ODP8-ME6 and ODP8-ME2 appeared deeply branching within the family *Methanosarcinaceae*, and ODP8-ME1 was assigned to the family *Methanosaeta* in our phylogenetic reconstruction. Recently, *mcrA* genes have been found in clones and fosmids from methane oxidising environments and it has been proposed that the ANME-1 and ANME-2 groups of *Archaea*, prevelant in these methane oxidising habitats, are able to reverse the normal methanogenesis pathway to oxidise methane anaerobically (Hallam *et al*., 2003). However, we did not isolate ANME related sequences in either our *Archaea* 16S rRNA gene or *mcrA* clone libraries, despite using the same *mcrA* primers as Hallam *et al*. (2003). The relative placement of our cluster 2 and 3 *mcA* clones and the ANME groups in the reconstructions of Hallam *et al*. (2003) make it unlikely, but not impossible, that these unidentified clones are methane oxidising archaea or organisms related to methanogens, but with different physiology.

The diversity of the Nankai *mcrA* sequences was con-

siderably less than that found by Lueders *et al*. (2001) in rice paddy field soil, and sequence identities were closer to cultured organisms than found by Bidle *et al*. (1999). The results discussed above, imply that the groups of methanogens we have identified in Nankai sediments are likely to be important in deep oceanic sediments. They also show that methanogen diversity in the deep biosphere may well be less than in some other more nutrient rich anaerobic environments (e.g. Munson *et al*., 1997; Lueders *et al*., 2001).

Given the above it appears surprising that we did not isolate 16S rRNA genes from methanogens in the Nankai Trough sediments; however, neither did Bidle *et al*. (1999) in their study of Cascadia Margin sediments. In both studies the general *Archaea* 16S rDNA primers described by Lane (1991) were used. Comparison of these primer sequences (Ashelford *et al*., 2002) with those held in the RDP database (release 8.1; Maidak *et al*., 2001) showed that these primers would amplify >80% of the recorded archaeal sequences, but had too many base mismatches to amplify the three methanogen groups we found. Therefore, primer deficiencies could account for this apparent contradiction. Recently, a new *Methanoculleus* sp. was isolated from deep sediment near the Nankai Trough (Mikucki *et al*., 2003). However, this genus has >7 base mismatches in the forward primer that we used and so would also not be amplified by our protocols. So there is a clear need for more effective PCR primers for 16S rRNA genes for methanogens.

In fact, few methanogens have been identified from deep sediments by 16S rRNA gene based molecular methods and most *Archaea* identified in this way have been *Crenarchaeota* or uncultured groups of *Euryarchaeota* (Hinrichs *et al*., 1999; Li *et al*., 1999b; Vetriani *et al*., 1999; Reed *et al*., 2002). The results obtained here fit this overall pattern, but further imply that a mixture of molecular methods will be needed if the prokaryotes responsible for methanogenesis are to be investigated effectively. We recommend that at present, a mixture of *mcrA* (Hales *et al*., 1996) and methanogen specific 16S rRNA gene primers (Marchesi *et al*., 2001) should be used to investigate methanogen diversity.

We have found a more diverse range of *Bacteria* in the Nankai Trough sediments than were found in either sediments from the Japan Sea (Rochelle *et al*., 1994) or the Cascadia Margin (Bidle *et al*., 1999; Marchesi *et al*., 2001). *Proteobacteria* sequences were found in all these studies and are common in other sediments; for example, in surface sediments from deep waters west of Japan *Proteobacteria* made up 76% of the clone library (Li *et al*., 1999a). Most of the increased diversity of *Bacteria* in the sediments studied here was the result of large numbers of 'deep sediment group' clones, which were phylogenetically close to many other clones from surface and deep oceanic sediments (Li *et al*., 1999b; Reed *et al*., 2002; Teske *et al*., 2002; Kormas *et al*., 2003). So it would seem likely that this group is common and maybe important in the deep-sea sedimentary environment, but as there are no cultured representatives we cannot speculate on the physiology of the bacteria from which the sequences derive.

Experimental procedures

Site description and sampling

Sample sites for ODP Leg 190 were drilled in the Pacific Ocean, south of Shikoku Island, which lies south of Honshu Island. The sites spanned the Nankai Trough and were between 40 km and 180 km offshore. Details of the sites and initial scientific data collected during the cruise are documented elsewhere (Moore *et al*., 2001; Shipboard Scientific Party, 2001; Kormas *et al*., 2003). Sediment samples were obtained from three of the six sites drilled on ODP Leg 190. Two sites were south-east of Cape Muroto. Site 1174 (32°20.54′N, 134°57.39′E) was close to the trough in 4751 m of water. Site 1173 (32°14.66′N, 135°01.51′E) was in 4791 m of water about 50 km south-east of the trough in the Shikoku Basin of the Philippine Sea Plate. Site 1177 (31°39.15′N, 134°0.71′E) was south-east of Cape Ashizuri in 4844 m of water about 30 km south of the trough. The sediment had comparatively low total organic carbon (mean = 0.35–0.45%, w/w), a steep temperature profile and all but site 1177 had very low acridine orange direct counts of bacteria ($<10^5$ cells cm^{-3}) below about 400–500 mbsf. The counts at site 1177 and in shallower depths at sites 1173 and 1174 were close to the predicted values for all deep ocean sediments (Parkes *et al*., 1994; 2000; Shipboard Scientific Party, 2001).

Sediment samples were taken between 29 June, 2000 and 4 July, 2000 as 10 cm whole round cores (WRC). Cores were obtained by three different methods on the ship, namely as advanced piston cores, extended barrel cores and rotary cores (Shipboard Scientific Party, 2001). These were cut aseptically from the plastic-encased ODP sediment cores under oxygen-free nitrogen in a specially constructed cutting rig modified from that previously described (Parkes *et al*., 1995) and stored anaerobically at −20°C (Cragg *et al*., 1992) for molecular analysis and at 4°C for rate measurements. In November 2000 the outer, potentially contaminated layer (Smith *et al*., 2000) of the frozen WRC obtained by rotary coring was removed aseptically. This was done by hydraulically forcing the WRC through a sterile cutting shoe to remove the outer layers, under nitrogen, into a pregassed sterile nylon bag (modified from Wellsbury *et al*., 2001). The bag contents were then broken into small pieces with a hammer and stored at −70°C before use, only the central, uncontaminated parts of the core (Smith *et al*., 2000) were used for molecular analysis. The following depths were analysed: 1173, eight WRC at 4.15–641.89 mbsf; 1174, 13 WRC at 2.65 and 306.7–1091.7 mbsf; 1177, eight cores at 302.99–790.79 mbsf.

For rate measurements, the cores were treated as follows. Advanced piston core WRC were subcored in a laminar flow cabinet, under nitrogen, using 5 ml sterile syringes with their

ends removed (Parkes *et al*., 1995). Extended core WRC were dismantled in an anaerobic cabinet with sterile scalpels to remove the potentially contaminated outer layers of the core (Smith *et al*., 2000). Rotary WRC were treated as described above but stored at 4°C before use. Potential rates of methanogenesis were estimated in 23 WRC from a range of depths.

DNA extraction and purification

Several different DNA extraction methods were used in the initial experiments of this study (Webster *et al*., 2003). Two main methods were used, the FastDNA Spin Kit for Soil (Bio101, Vista, CA, USA) with modifications (Webster *et al*., 2003) and that of Rochelle *et al*. (1992), with the modifications described by Marchesi *et al*. (1998). Extractions were carried out on 5 × 1 g of sediment samples and DNA was pooled, concentrated and purified by dialysis in Microcon YM-100 centrifugal filter devices (Millipore, MA, USA) to give a final volume of 50 μl. Visualization of DNA for yield and size was by agarose (1.0% w/v) gel electrophoresis with 0.5 μg ml^{-1} ethidium bromide and compared to a HyperLadder I DNA quantification marker (Bioline, London, UK).

PCR reaction conditions

The PCR primers used in this study are listed in Table 2. All amplifications were carried out under sterile conditions using a Primus 96 (MWG Biotech, Milton Keynes, UK), PTC200 DNA Engine (MJ Research, Boston, MA, USA) or a Dyad DNA Engine (MJ Research) thermal cyclers. Disposable plasticware was autoclaved and UV treated before use. Polymerase chain reaction products for cloning were visualized with UV illumination following gel electrophoresis in 1.4% agarose with 0.5 μg ml^{-1} ethidium bromide. Negative controls were included in all reaction mixes by substituting 1.0 μl of molecular grade water for template DNA. In nested PCR reactions negative control tubes from the first stage were diluted 1/10 for use as template in second stage negative controls. Positive controls used DNA extracted from appropriate pure cultures and sediment from Cardiff Bay (Ordnance Survey Reference ST 192 741). Only PCR reactions giving the expected results in all controls were used. Positive control reactions always gave PCR product. Negative control reactions never showed product with archaeal or *mcrA* primers, but did show a product in about 10% of cases with *Bacteria* in nested PCR reactions and low DNA template concentrations; this was not surprising as product from the residual DNA from the *E. coli* used to produce *Taq* polymerase is not uncommon (Rochelle *et al*., 1992; Kormas *et al*., 2003).

Amplification of *Bacteria* 16S rDNA was performed on 1/100 dilutions of template DNA using initial primers 27F, 1492R followed by a nested PCR with 63F, 1387R on 1/10 dilutions of the first round products. Reaction mixtures contained 1× reaction buffer (Amersham, Little Chalfont, UK), 0.2 pmol ml^{-1} primers, 0.25 mM each dNTP, 1.8 mM $MgCl_2$, 1.25 U *Taq* DNA polymerase (Amersham), 1.0 μl template DNA, made up to 50 μl with molecular grade water. Reaction conditions included an initial 2 min at 95°C, followed by 35 cycles of 92°C for 45 s, 50°C for 2 min and 72°C for 45 s, with a final elongation step of 72°C for 5 min.

Archaea 16S rDNA was amplified with 1/10 dilutions of template DNA using the 21F, 958R primer pair. Reaction mixtures (50 μl in molecular grade water) contained 1× reaction buffer (Bioline Ltd, London, UK), 0.4 pmol ml^{-1} primers, 1.5 mM $MgCl_2$, 1.25 U *Taq* DNA polymerase (Bioline), 10 μg ml^{-1} bovine serum albumen (BSA) and 1.0 μl of template DNA. Reaction conditions included an initial 30 s at 98°C followed by 35 cycles of 92°C for 45 s, 45°C for 45 s and 42°C for 42 s with a final elongation step of 72°C for 5 min.

Table 2. PCR primer pairs used in this study.

Amplification target	Primer code	Sequence[a] 5′-3′	Binding position[b]	Approximate product size (bp)	Reference
General 16S rDNA for *Bacteria*	27F	AGA GTT TGA TCM TGG CTC AG	8–27	1502	Lane (1991)
	1492R	GGT TAC CTT GTT ACG ACT T	1492–1510		
General 16S rDNA for *Bacteria*	63F	CAG GCC TAA CAC ATG CAA GTC	43–63	1362	Marchesi *et al*. (1998)
	1387R	GGG CGG WGT GTA CAA GGC	1387–1404		
General 16S rDNA for *Archaea*	Arch21F	TTC CGG TTG ATC CYG CCG GA	7–26	970	DeLong (1992)
	Arch958R	YCC GGC GTT GAM TCC AAT T	958–976		
General 16S rDNA for *Archaea*	Arf3	TTC CGG TTG ATC CTG CCG GA	7–26	921	Jurgens *et al*. (1997)
	Ar9r	CCC GCC AAT TCC TTT AAG TTT C	906–927		
General 16S rDNA for *Bacteria* for DGGE	357F-GC[b]	CCT ACG GGA GGC AGC AG	341–357	194	Muyzer *et al*. (1993)
	518R	ATT ACC GCG GCT GCT GG	518–534		
General 16S rDNA for *Archaea* for DGGE	SA1f[c,d]	CCT AYG GGG CGC AGC AGG	341–358	150	Nicol *et al*. (2003)
	SA2f[c,d]	CCT ACG GGG CGC AGA GGG			
	PARCH519r	TTA CCG CGG CKG CTG	519–533		Ovreas *et al*. (1997)
General α-subunit from methyl coenzyme M reductase (*mcrA*)	ME1	GCM ATG CAR ATH GGW ATG TC	684–703	760	Hales *et al*. (1996)
	ME2	TCA TKG CRT AGT TDG GRT AGT	1424–1444		

a. D = G or A or T, H = A or T or C, K = G or T, M = A or C, R = A or G, W = A or T, Y = C or T.
b. Based on *Escherichia coli* numbering (Brosius *et al*., 1981), except for the *mcrA* gene which corresponds to *Methanothermobacter marburgensis* numbering (Lueders *et al*., 2001).
c. For DGGE, this primer has the following GC-clamp at the 5′ end, CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG (Muyzer *et al*., 1993).
d. Primer SAf is a mixture of primers SA1f and SA2f at a molar ratio of 2:1 (Nicol *et al*., 2003).

Methanogen specific PCR for the *mcrA* gene was performed using the ME1 and ME2 primer pair, using undiluted template DNA. Reaction mixtures (50 μl in molecular grade water) contained 1× *Taq* extender reaction buffer (Amersham), 0.2 pmol ml^{-1} primers, 15 μg ml^{-1} T4 Gene 32 Protein (Stratagene, Amsterdam, the Netherlands), 0.25 mM each dNTP, 2.0 U *Taq* extender (Amersham), and 1.25 U *Taq* DNA polymerase (Amersham). Reaction conditions included an initial 5 min at 94°C followed by 31 cycles of 92°C for 45 s, 50°C for 45 s and 72°C for 1 min 45 s with a final elongation step of 72°C for 5 min.

Amplification of 16S rRNA genes for DGGE analysis were performed using the following primer combinations in nested PCR reactions, 27F, 1492R with 357F-GC, 518R for *Bacteria* and Ar3f, Ar9r with SAf, PARCH519R for *Archaea*. Primary amplification reactions for *Bacteria* were as described above. Amplification reaction mixtures for Ar3f, Ar9r were with primer pair 21F, 958R using PCR conditions as described in Jurgens *et al*. (1997). Secondary PCR amplification (*Bacteria* 357F-GC, 518R, and *Archaea* SAf, PARCH 519R) were carried out without BSA in a 50 μl reaction mix (Webster *et al*., 2003). The PCR conditions were as previously described (Muyzer *et al*., 1993; Ovreas *et al*., 1997).

Denaturing gradient electrophoresis (DGGE)

Polymerase chain reaction products were analysed by DGGE as described previously (Schafer and Muyzer, 2001; Webster *et al*., 2002). Representative bands were excised from gels and re-amplified for sequencing with the 518R primer (Webster *et al*., 2002) to compare sequences observed by DGGE with those derived from clone libraries. Partial 16S rRNA gene sequences were subjected to a BLAST® search in the National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/BLAST/) to identify sequences with highest similarity.

Cloning and restriction fragment length polymorphism

All 16S rRNA gene PCR reactions were screened with DGGE before cloning as recently recommended for deep biosphere samples (Webster *et al*., 2003). Only products from reactions showing the highest diversity and without PCR product in the negative control reactions were cloned. For each DNA extract and primer pair five independent PCR products were pooled and cleaned with Wizard® PCR Preps DNA purification system (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. Cloning pooled PCR products was carried out using pGEM®-TEasy Vector System 1 (Promega) according to the manufacturer's instructions, using optimised insert:vector ratios and an overnight ligation at 4°C. Plasmid preparations were made using a Wizard® Plus SV Mini Prep kit (Promega). To confirm inserts and verify size PCR reactions were carried out with M13 primers (M13F 5′- GTA AAA CGA CGG CCA GT- 3′; M13R 5′- CAG GAA ACA GCT ATG AC- 3′), 16S rRNA gene and *mcrA* primer pairs (Hales *et al*., 1996).

Clones were screened before sequencing with RFLP to reduce the number of very similar clones sequenced. Restriction enzymes *Hae*III and *Hin*fI were used for *Bacteria* and *Archaea* 16S rRNA gene clones and *Sau*961 for *mcrA* clones (Lueders *et al*., 2001). Unique restriction patterns were identified and representative clones selected for sequencing.

Sequencing and analysis

Sequences of 16S rRNA and *mcrA* genes were obtained using an ABI 3100 Prism Genetic Analyzer automated capillary sequencer (Applied Biosystems, Foster City, CA, USA) using BigDye terminator chemistry. Sample DNA was sequenced with M13 forward and reverse primers and resulting chromatograms edited using the BioEdit Sequence Editor, version 5.0.9 (Hall, 1999). In addition, selected 16S rRNA gene clones were also sequenced with 530F (5′-GTGCC AGCMGCCGCGG-3′), and 907R (5′-CCGTCAATTCMTT TRAGTTT-3′) (Lane, 1991). Sequences were assembled using DNASIS for Windows, version 2.5 (Hitachi Software Engineering, San Francisco, CA, USA), and checked for chimeras with Chimera Check from the Ribosomal Database Project II (RDP) (Maidak *et al*., 2001). Alignments against closest relatives identified in the NCBI BLAST® and RDP database searches, and known taxonomic sequences, were carried out using CLUSTALW, version 1.74 (Thompson *et al*., 1994), and files edited with BioEdit Sequence Editor, version 5.0.9 (Hall, 1999). Phylogenetic analysis of edited alignments was carried out using Treecon for Windows version 3.1 (Van de Peer and De Wachter, 1994) with evolutionary distances calculated by the Jukes-Cantor algorithm. Tree topology was inferred through the neighbour joining method (Saitou and Nei, 1987). Bootstrap analyses (Felsenstein, 1993) of 100 replicates were used to estimate the reproducibility of tree topologies, values over 50% are shown on the tree reconstructions. Comparisons of sequence similarity were made using DNADIST within the PHYLIP software package (Felsenstein, 1993). The percentage coverage of the clone libraries was estimated as described previously (Marchesi *et al*., 2001), using ≥98% sequence similarity to indicate identical clones.

The new sequences reported here have been submitted to the GenBank/EMBL database under accession numbers AY436526-AY436534 for 16S rRNA gene sequences from *Bacteria*, AY436508-AY436525 for 16S rRNA gene sequences *Archaea*, AY436535-AY436552 for *mcrA* sequences and AJ585415-AJ585424 for sequences of 16S rRNA gene bands excised from DGGE gels. Accession numbers for other sequences used in the tree reconstructions for phylogenetic comparison are given in the figures.

Potential rates of methanogenesis

After initial handling all samples were moved to an anaerobic cabinet and 1.25 ml aliquots of sediment transferred to sterile 30 ml glass bottles containing a small magnetic stirring rod, which were then tightly sealed with a sterile rubber bung. The vial headspaces were flushed in a laminar flow cabinet with sterile N_2/CO_2 (80:20) to remove any hydrogen. Sterile, anoxic basic mineral salts medium (KH_2PO_4 0.2 g, NH_4Cl 0.25 g, NaCl 25.75 g, $MgCl_2.6H_2O$ 5.15 g, KCl 0.5 g, $CaCl_2.2H_2O$ 0.15 g, $NaHCO_3$ 2.52 g, $Na_2S.XH_2O$ 0.36 g, water 1000 ml, and pH 7.5) was added at 5 ml per bottle to

make a 20% slurry. One of five ^{14}C isotopes (10 μl, supplied by Amersham Biosciences, Amersham, UK and ICN Pharmaceuticals, Basingstoke, UK) was injected into each bottle (sodium [^{14}C]-bicarbonate 185 kBq, [7–^{14}C]-benzoic acid 74 kBq, sodium [1,2–^{14}C]-acetate 74 kBq, n-[1–^{14}C]-hexadecane 37 kBq, or [^{14}C]-methanol 92.5 kBq diluted 1:2000 with cold methanol). The bottles were incubated, inverted, at a range of temperatures (7°–90°C), approximating the *in situ* temperatures, for between 0.25 and 56 days. Time-zero controls and all incubations were terminated by injection of 2 ml of 1 M NaOH.

Production of $^{14}CH_4$ was determined by flushing the bottle headspace for 20 min through a furnace at 800°C over copper oxide using air as a carrier gas. Any $^{14}CO_2$ produced by oxidation of $^{14}CH_4$ was collected in two sequential vials of scintillation fluid containing 7% β-phenylethylamine. Total ^{14}C-activity was determined by liquid scintillation counting and multiplied by the substrate pool size and incubation time to provide a rate. Bicarbonate, sulphate and acetate pool sizes were measured (Shipboard Scientific Party, 2001 and unpublished data), whilst for methanol, benzoate and hexadecane the added ^{14}C-tracer was taken as the total pool size.

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