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FEMS Microbiology Ecology 34 (2001) 221–228

FEMS MICROBIOLOGY Ecology

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Methanogen and bacterial diversity and distribution in deep gas hydrate sediments from the Cascadia Margin as revealed by 16S rRNA molecular analysis

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Received 10 May 2000; received in revised form 14 September 2000; accepted 29 September 2000

Abstract

The microbial community of a deep (to 234 m below the sea floor) sediment gas hydrate deposit (Cascadia Margin Ocean Drilling Program Site 889/890, Leg 146) was analysed for the first time by molecular genetic techniques. Both bacterial and methanogen diversity were determined by phylogenetic analysis of ribosomal DNA sequences. High molecular mass DNA, indicative of active bacteria, was present in all of the samples. Ribosomal RNA genes were amplified from extracted DNA extracted from sediment using bacteria, and methanogen specific PCR primers, the latter designed in this study. Phylogenetic analysis of approximately 400 bacterial clones demonstrated that 96% were members of the *Proteobacteria*. These clones were affiliated with the α, β and γ subdivisions, with *Caulobacter* (*Zymomonas* group), *Ralstonia* and *Pseudomonas* phylotypes predominating. The methanogen clones were of low diversity and clustered in three sub-groups. Two of these sub-groups (contained 96% of the 400 clones) were closely related to *Methanosarcina mazeii*, while the third sub-group clustered in the *Methanobacteriales*. This analysis of a deep sediment gas hydrate environment shows a bacteria and methanogen community of limited diversity and confirms that the gas hydrate zone is biogeochemically active. These results are consistent with the presence of bacterial populations capable of methanogenesis throughout the core, and suggest that the methane hydrate at this site is at least partially biogenic in origin. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Deep subsurface environment; 16S rRNA; Methanogen diversity; Bacterial diversity; Marine sediment; *Archaea*; Gas hydrate; Methane

1. Introduction

Recent evidence has demonstrated the presence of significant bacterial populations to depths of at least 750 m in marine sediments [1,2] and probably much deeper [3]. Sites studied include sediments with deep gas hydrate deposits [2,4], where bacterial activity is stimulated within the hydrate zone to such an extent that rates for some activities in the subsurface are greater than those near the sediment surface. These results suggest that gas hydrates may represent a uniquely active deep sediment habitat [3]. In addition, it has been estimated that the methane contained in gas hydrates world-wide represents more than double the total amount of carbon in known fossil fuel reserves [5] and thus gas hydrates are of global significance. As the bulk of the methane in gas hydrates is of biogenic origin [6], these deposits are also of microbiological interest. Despite the evidence demonstrating that bacterial populations and activities are stimulated in deep gas hydrate deposits, there is no detailed information about the types of bacteria present or their diversity. Culturing approaches only reveal a small subset of the total bacteria and only a few isolates have been obtained from deep sediments [7,8]. A recent investigation [9] of archael diversity in marine sediments in an area with abundant gas hydrates identified a unique cluster of 16S rRNA sequences. However, samples were obtained only 22 cm below the sediment surface. Methanogens, of particular interest for gas hydrate deposits, have proved particularly difficult to isolate [10]. To overcome these problems, we designed PCR primers specific for the 16S rRNA gene of this group

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PII: S0168-6496(00)00099-4

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of the *Euryarchaeota*. In this study, we used these primers to assess the diversity of the methanogens and other primers to examine bacteria in this unique deep gas hydrate ecosystem.

2. Materials and methods

2.1. Collection of sediment samples

Sediment samples were collected from Hole 889/890 on Leg 146 (48°41.958′N, 126°52.098′W and 48°39.750′N, 126°52.890′W) by the Ocean Drilling Program (ODP) drill ship, JOIDES Resolution, for further details see Cragg et al. [4]. Intact whole round cores (WRC) were sampled aseptically [11] for microbiological analysis and sub-sections (5 cm) for DNA analysis were stored frozen. A full description of the sites and core handling information is given by Cragg et al. [4] and Parkes et al. [12], respectively. The results from four WRC are reported here, Cas14 from 9 mbsf (metres below sea floor), deposited 100 000 years ago and Cas18, Cas19 and Cas20, from 198 mbsf, 222 mbsf and 234 mbsf, respectively, all deposited between 2 and 3×10^6 years ago. Cas14, a non-gas hydrate sample, was used as a control section, whereas Cas18 is just above the methane gas hydrate zone (215–225 mbsf), Cas19 is in the hydrate zone and Cas20 just below it (Fig. 1). The bottom of the hydrate zone was determined by the presence of a bottom simulating reflector, which is characteristic for gas hydrate layers [13].

2.2. Bacterial cultures and growth conditions

Pure cultures of methanogens, from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) culture collection, were used as positive controls for DNA extraction and PCR amplification; all were grown using recommended conditions and media (Table 1). Prior to DNA extraction by the methods described below, total bacterial numbers were determined by acridine orange staining and epifluorescence microscopy [4] to ensure that the cultures had grown.

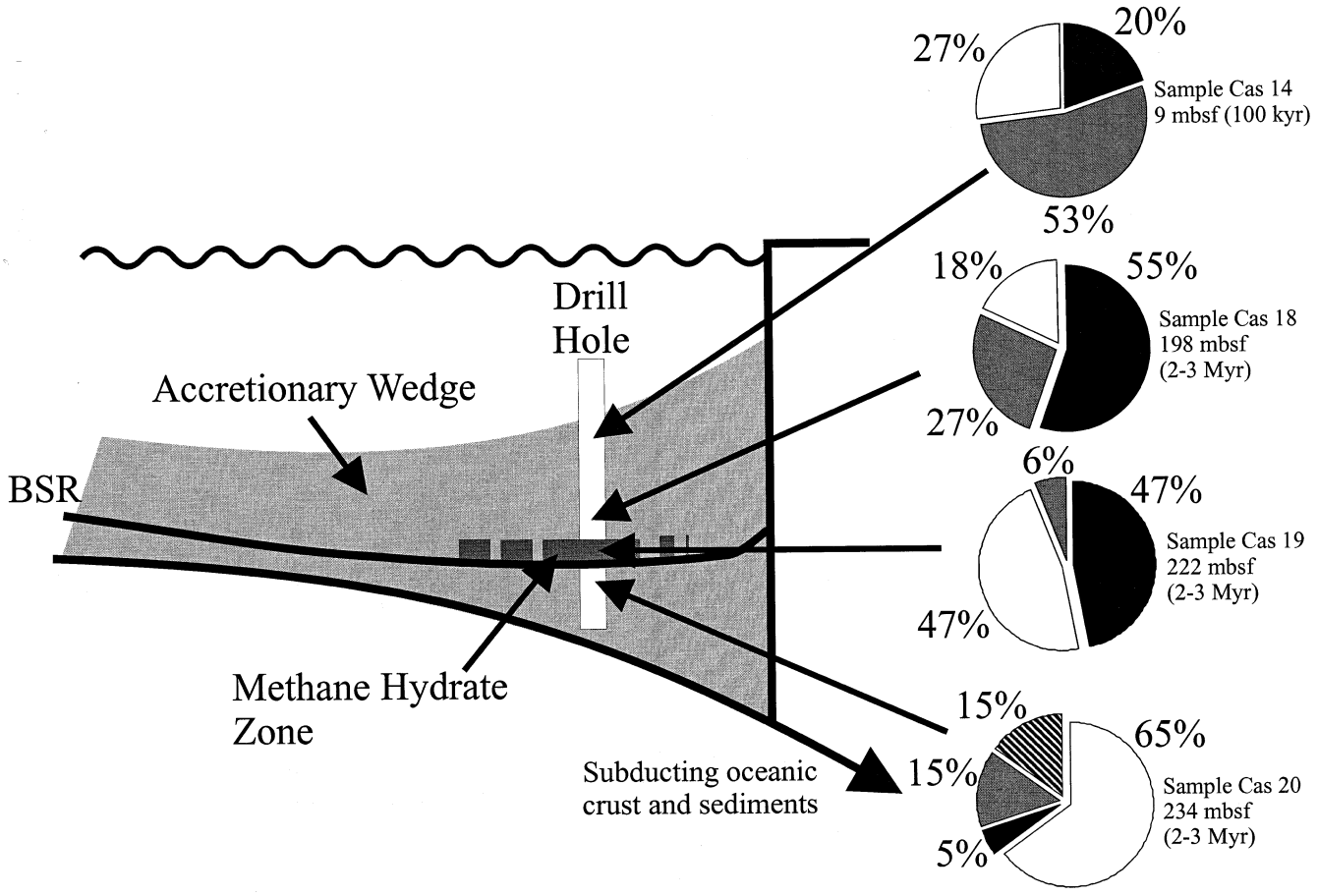

Fig. 1. Schematic representation of the sampling site on the subduction zone in the Cascadia Margin (ODP Leg 146, Site 889/890). The four pie charts on the right show the percentages of bacterial 16S rRNA clones for the four sample depths ($n=100$ for the four libraries). The explanation of the symbols is as follows: white, α – % clones were affiliated with the α-*Proteobacteria*; black, β – % clones were affiliated with the β-*Proteobacteria*; grey, γ – % clones were affiliated with the γ-*Proteobacteria*; hatched, CFB – % clones were affiliated with the *Cytophagales* (*Bacteroides-Cytophaga-Flexibacter* group), kyr = $\times10^3$ years and Myr = $\times10^6$ years. BSR-bottom simulating reflector which indicates the base of the hydrate zone.

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2.3. DNA extraction from pure culture test strains

Genomic DNA was extracted from cultures by a modification of the method of Ausubel et al. [14]. Cultures (1.5 ml) were centrifuged at $13\,000 \times g_{av}$ for 1 min and the cell pellet was resuspended in 310 μl of HTE buffer (50 mM Tris–HCl pH 8.0, 20 mM EDTA) plus 5 μl of RNase (5400 U ml^{-1}). Lysozyme (50 μl of a 1 mg ml^{-1} stock in HTE solution) was added to the suspension which was vortex-mixed and incubated at 37°C for 45 min. Subsequently 50 μl proteinase K (10 mg ml^{-1} in HTE) was added to the suspension, vortex-mixed and the mixture was further incubated for 45 min at 37°C. To lyse the cells, 200 μl of *n*-lauryl sarcosyl (2% w/v) was added and the mixture was vortex-mixed and incubated at 37°C for 15 min. NaCl (175 μl of a 5 M solution) was added and the suspension was vortex-mixed. Then 85 μl of cetyltrimethylammonium bromide (10% w/v; dissolved in 0.7 M NaCl) was added and vortex-mixed for 1 min. The mixture was incubated for 30 min at 65°C, after which an equal volume of chloroform/3-methyl butanol (24:1) was added and vortex-mixed. Samples were centrifuged for 15 min at $13\,000 \times g_{av}$ and the supernatants were removed to a new microcentrifuge tube. DNA was precipitated by adding 0.6 volume of isopropanol to the supernatant, placing the tube on ice for 10 min followed by centrifugation for 5 min at $13\,000 \times g_{av}$. The DNA pellet was washed twice with cold (−20°C) 70% (v/v) ethanol, air–dried and finally resuspended in 200 μl of sterile 10 mM Tris–HCl buffer, pH 8.0.

2.4. PCR primer design and conditions

Two PCR primers were designed, using the primer design program Oligo v3.4 (National Biosciences Inc., USA), to amplify approximately 1200 bases of a consensus methanogen 16S rRNA gene. The forward primer, designated 146f (numbering based on *Escherichia coli* 16S rRNA gene), had the sequence: 5′-GGS ATA ACC YCG GGA AAC-3′. The reverse primer, designated 1324r, had the sequence: 5′-GCG AGT TAC AGC CCW CRA-3′. The specificity of the primers was tested on members of the three major methanogenic groups (Table 1), two non-methanogen *Archaea* (*Halobacterium salinarium* and *Sulfolobus acidocaldarius*) and a γ-*Proteobacterium* (*E. coli*). The PCR mixture contained 20 pmol of the appropriate primer pairs, 200 μM dNTPs, PCR buffer (*Taq* extender buffer, Stratagene), 0.5 U *Taq* extender (Stratagene) and 0.5 U *Taq* polymerase (Boehringer Mannheim). Approximately 50–100 ng of DNA from cultures and <10 ng of sediment DNA were used in the PCR. In addition to the components described above, 2 μg T4 Gene 32 protein (Amersham Pharmacia Biotech UK Limited) was included in the PCR with sediment DNA, to overcome problems due to co-extraction of PCR inhibiting agents [15]. The PCR was performed using a Hybaid Omni-E thermal cycler programmed to perform 30 cycles of 95°C for 1 min, 40°C for 1 min and 72°C for 1 min 30 s. A final elongation step was included for 5 min at 72°C after the 30 cycles.

Table 1
Alignment of PCR primers with the corresponding regions of 16S rDNA genes of representative species used in PCR

Pure culture test organism	16S rRNA group	PCR[a]	146f[b] 5′ GGSATAACCYCGGGAAAC 3′	1324r[b] 3′ ARCWCCCGACATTGAGCG 5′
Methanosarcina frisia DSM 3318	Methanomicrobiales	+	CCGGCATAACCCCGGGAAACTGG	GATCGAGGGCTGTAACTCGCC
Methanobacterium palustre DSM 3108	Methanobacteriales	+	CTGGGATAACCCCGGGAAACTGG	GATTGAGGGCTGTAACTCGCC
Methanosarcina barkeri DSM 800	Methanomicrobiales	+	CTGGCATAACCCCGGGAAACTGG	GATCGAGGGCTGTAACTCGCC
Methanobrevibacter ruminantium DSM 1093	Methanobacteriales	+	AGGGGATAACCCCGGGAAACTGG	GATTGAGGGCTGTAACTCGCC
Methanococcus vannielii DSM 1224	Methanococcales	+	GG**A**GCATAACCT**T**GGGAAACTGG	GATCGTGGGCTGTAACTCGCC
Methanoculleus marisnigri DSM 1498	Methanomicrobiales	+	CTGGGATAACCC**T**GGGAAACTGG	GATTGTGGGCTGTAACTCGCC
Methanobacterium uliginosum DSM 2956	Methanobacteriales	+	CTGGGATAACCC**T**GGGAAACTGG	GATCGAGGGCTGTAAC**CT**GCC
Methanoculleus bourgensis DSM 4307	Methanomicrobiales	+	AGGGGATAACCCCGGGAAACTGG	GATTGTGGGCTG**C**AACTCGCC
Halobacterium salinarium DSM 3754	Euryarchaeota	-	CGGG**A**ATA**CT**CTCGGGAAACTGG	GATTGAGGGCTG**A**AACTCGCC
Sulfolobus acidocaldarius DSM 639	Crenarchaeota Group 1	-	CGGGGATAACCCCGGGAAACTGG	GATCGAGGGCTG**A**AAC**C**CGCC
Escherichia coli	γ Proteobacteria	-	GGGGGATAAC**TA**C**T**GGAAACGGT	GATTG**GA**G**T**CTG**C**AACTCG**A**C

[a] + = positive reaction giving a PCR product of expected size; - = negative reaction with no PCR product.

[b] Mismatches between the target 16S rDNA and PCR primer are shown in bold typeface.

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2.5. DNA extraction, amplification and identification of 16S rRNA gene libraries

Total DNA was extracted from the marine sediment samples (1–2 g), taken from the centre of the WRC, by the method of Rochelle et al. [16] with several modifications [17]. The molecular mass of extracted DNA was determined indirectly by agarose gel electrophoresis [18]. PCR of the methanogen and bacterial 16S ribosomal DNA (rDNA) from Cascadia Margin DNA samples was performed using primers described in Section 2.4 and the primers of Marchesi et al. [17], respectively. Pooled (2–3) 16S rDNA PCR were isolated by cloning in *E. coli* XL1-Blue using the pGEM-T Easy Vector System (Promega, Mannheim, Germany) according to the manufacturer's instructions. This cloning step produced separate bacterial and methanogen 16S rRNA gene libraries from each of the sediment samples. Approximately 100 clones from each library were analysed by restriction fragment length polymorphism (RFLP) analysis (*Hin*fI and *Hae*III) of the plasmid and insert.

2.6. DNA sequencing and phylogenetic analysis

16S rRNA clones were sequenced using unlabelled Universal M13 forward primers and labelled dye terminators, following the manufacturer's instructions, on a Prism 377 automated laser fluorescence sequencer (PE Applied Biosystems, Warrington, UK). Sequencing of ca. 500 bp from the 5′ end of the 16S rRNA gene (containing the V1–V3 variable regions and sufficient conserved regions for accurate aligning of the 16S rRNA gene sequences) was performed at least twice to ensure accuracy. The phylogenies were constructed by analysis of partial 16S rRNA gene sequences (500 bases) as this has been found to be satisfactory for comparing the phylogenetic positions with respect to cultured taxa [19]. The partial 16S rRNA gene sequences of each clone or pure culture methanogen were compared with those in the EMBL database [20] using Fasta3 [21,22] at the European Bioinformatics Institute (EBI) (URL http://www.ebi.ac.uk; Hinxton Hall, Cambridge, UK). In addition, sequences were analysed via the Ribosomal Database Project (RDP) using Similarity Rank [23] to identify most closely related database sequences. Sequence alignments were done using CLUSTAL W [24]. Evolutionary distances were calculated by the Kimura-2-parameter algorithm [25] and the phylogenetic tree determined by the neighbor-joining method [26] using Treecon for Windows [27]. Tree topologies were also compared between trees constructed by the method of maximum likelihood and maximum parsimony using Phylip version 3.5 [28] and the neighbor-joining method. Bootstrap analyses [29] of up to 500 replicates were performed on all phylogenetic trees to estimate the reproducibilities of the tree topologies. The percentage coverage of the clone libraries was calculated by the method of Good [30,31] using the equation

$$C = [1-(n_1/N)]\times 100.$$

Where n_1 represents the number of clones that occurred only once (RFLP analysis was used to determine unique clones) and N is the total number of clones examined ($N = 100$).

2.7. Nucleotide sequence accession numbers

The EMBL accession numbers or DSMZ (DSM numbers) plus RDP short identifier (RDP Sids, these are the RDP's own unique identifiers for 16S rRNA sequences) for the sequences used in the phylogenetic analysis are as follows: *Methanosarcina mazeii* (U20151); *Methanosarcina acetivorans* (M59137); *Methanosarcina siciliae* (U20153); *Methanosarcina barkeri* (M59144); *Methanolobus oregonensis* (U20152); *Methanococcoides burtoni* (X65537); *Methanothermus fervidus* (M59145); *Methanobacterium thermoformicicum* (DSM 3664 RDP Sids: Mb.tautfor); *Methanobacterium thermoautotrophicum* (X15364); *Methanobacterium formicicum* (M36508); *Methanobacterium bryantii* (M59124); *Methanobrevibacter ruminantium* (DSM 1093 RDP Sids: Mbb.rumina); *Methanobrevibacter arboriphilicus* (DSM 1536 RDP Sids: Mbb.arbori); *Halobacterium halobium* (X03407).

Accession numbers of the partial sequences of the 21 methanogen 16S rRNA gene sequences used to generate the phylogenetic tree are as follows: Cas14#1 (AJ290554); Cas14#2 (AJ290555); Cas14#3 (AJ290556); Cas14#4 (AJ290557); Cas14#5 (AJ290558); Cas14#6 (AJ290559); Cas18#1 (AJ290560); Cas18#2 (AJ290561); Cas18#3 (AJ290562); Cas18#4 (AJ290563); Cas19#1 (AJ290564); Cas19#2 (AJ290565); Cas19#3 (AJ290566); Cas19#4 (AJ290567); Cas19#5 (AJ290568); Cas19#6 (AJ290569); Cas20#1 (AJ290570); Cas20#2 (AJ290571); Cas20#3 (AJ290572); Cas20#4 (AJ290573); Cas20#5 (AJ290574).

3. Results

3.1. Construction and screening of 16S rRNA gene libraries from Cascadia Margin sediments

The specificity of the methanogen specific PCR primers was evaluated by theoretical analyses and test PCR with DNA isolated from pure cultures of representative bacteria and *Archaea*. The experimental results confirmed the theoretical expectations, in that only methanogen specific 16S rDNA gene sequences were obtained (Table 1).

High molecular mass DNA was extracted from all the Cascadia Margin sediment samples studied. The molecular mass (Da) of the DNA was consistently above 9.3×10^6. Total DNA from each sample was subsequently used separately to construct rRNA gene libraries. In total, eight gene libraries were constructed, four from bacteria specific

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PCR (one from each of the four samples, Cas14–20) and four using methanogen specific PCR from the same Cascadia Margin samples. Approximately 100 clones from each gene library were screened for the Cascadia Margin samples and grouped on the basis of RFLP profiles. Of the 800 clones analysed by RFLP, 61 bacteria and 21 methanogen clones from Cas14, Cas18, Cas19 and Cas20 had unique RFLP patterns and so were sequenced.

3.2. Determination of the bacterial diversity in the Cascadia Margin samples

In total, 61 unique clones were sequenced from four libraries constructed ($N = 400$) using PCR primers which amplified the bacterial 16S rRNA gene. The percentage coverage for these clones in the four libraries (Cas14, Cas18, Cas19, Cas20) was 90%, 92%, 92% and 92%, respectively. Thus for Cas14 the data presented here would account for 90% of clones in a similar clone library of infinite size. This is a conservative method to determine how well a small clone library represents the population from which it was generated, since related genera are clustered together rather than treated separately. However, it does give an indication of how diverse the initial population may be.

Affiliation of the bacterial 16S rRNA clones from the Cascadia Margin libraries is shown in Fig. 1 and the most abundant sequences in Table 2. Of the 61 sequences from the four libraries, 58 (96% of clones; $N = 400$) were members of the *Proteobacteria* and the remaining three sequences were related to the *Bacteroides-Cytophaga-Flexibacter* group (CFB group). Of the proteobacterial sequences, 27% were most closely related ($> 95\%$) to *Caulobacter subvibrioides* str. CB81 (accession number M83797; [32]). However, along with the identical 16S rRNA gene sequence from *C. leidyia* [33], they are not members of the *Caulobacter* genus sensu stricto [33]. There is growing evidence [33] that these isolates are members of the sphingomonads and most probably members of the *Zymomonas* group, which contains several deep subsurface aromatic-degrading isolates [34]. A further 16% were affiliated to the *Ralstonia pickettii* sub-group which are members of the β-*Proteobacteria*. The next most prevalent group, with five representatives, was related to the *Pseudomonas tolaasii* sub-group (γ-*Proteobacteria*), part of the *Pseudomonas* and relatives group. Clones from this sub-group were found in all the Cascadia Margin sediment samples. The next most prevalent sub-group with three clones was related to a *Comamonas* strain (accession no. AJ002803) and an unidentified denitrifying Fe-oxidising bacterium (accession no. U51105), but these clones were only present in Cas14 and Cas19 samples. Fifty three sequences were $\geq 95\%$ similar to 16S rDNA sequences of cultured organisms in the RDP while four sequences were between 90% and 95% similar to 16S rDNA sequences of cultured organisms. The three sequences which showed affiliation to the CFB division all came from Cas20 samples and were affiliated with the *Prevotella* group (subdivision I: *Bacteriodes* group of the CFB division also known as the *Cytophagales*). The intra-group percentage sequence similarity for clones from Cas14, Cas18, Cas19 and Cas20 sediment samples were 70–99%, 67–99%, 59–100% and 59–100%, respectively.

3.3. Phylogenetic analysis of methanogen 16S rDNA clones from Cascadia Margin

Twenty one unique clones were sequenced from four libraries constructed ($N = 400$) using PCR primers which amplified the methanogen 16S rRNA gene. The percentage coverage for the four libraries (Cas14, Cas18, Cas19, Cas20) was 94%, 97%, 94% and 97%, respectively. All the 16S rRNA clones clustered as phylotypes within the *Euryarchaeota* sub-domain of the *Archaea* (Fig. 2) and formed three methanogen groups. The fact that only sequences related to known methanogens were obtained

Table 2
Proportions of the most abundant bacterial 16S rRNA clones found in the gene libraries as determined by RFLP analysis

Sediment sample	Proportion of library (%)[a]	Closest sequence in RDP	S_{ab} range[c]
Cas14	38	*R. pickettii*	0.796–0.971
	15	*Pseudomonas putida*	0.796–1.000
	15	*C. subvibrioides*[b]	0.986–1.000
Cas18	36	*C. subvibrioides*[b]	0.907–0.983
	18	*P. putida*	0.736–0.973
Cas19	27	*C. subvibrioides*[b]	0.963–1.000
	27	*R. pickettii*	0.760–1.000
	13	*Bradyrhizobium* sp.	0.969–0.974
Cas20	30	*C. subvibrioides*[b]	0.969–1.000
	25	*Azospirillum* sp.	0.890–1.000
	15	*Prevotella oris*	0.915–0.933

[a]These are the percentages of that clone type in the library ($N = 100$ for all four samples) for the sediment sample named (i.e. clones with *R. pickettii* as a closest match made up 38% of the Cas14 clones). Clones which constituted less than 10% of the original gene library are not shown.
[b]This organism is not a member of the *Caulobacter* sensu stricto [34] and resides in the *Zymomonas* group of the sphingomonads [48].
[c]The S_{ab} scores [49] in this column show the range into which the clones fell. The lowest value 0.736 is equivalent to a percentage sequence similarity of approximately 95%.

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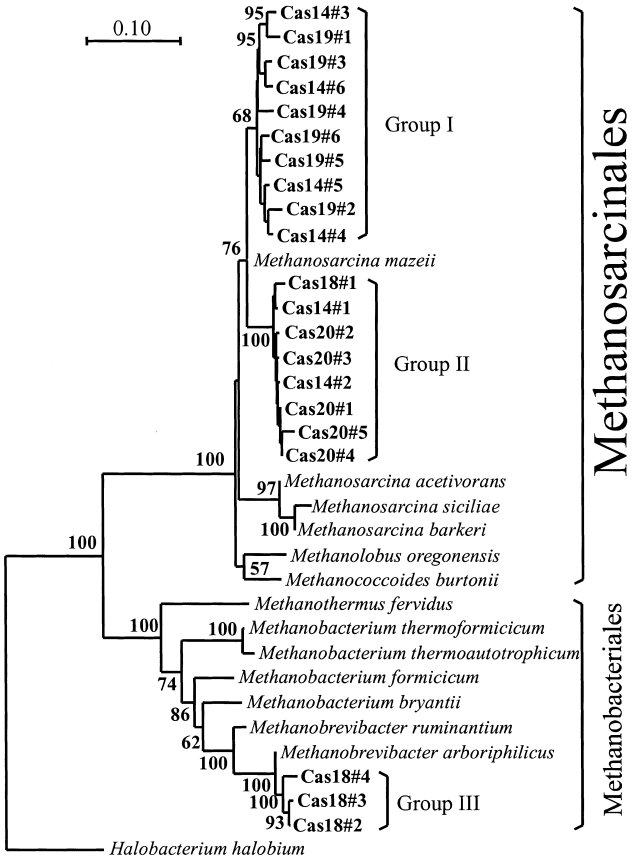

Fig. 2. A phylogenetic tree based on 16S rDNA analysis, illustrating the relationships of clones isolated from Cascadia Margin sediment with respect to other methanogens. The clones are designated CasXX#N, where Cas refers to the Cascadia Margin (i.e. source of the sediment) XX is the sample number (see Fig. 1) and N is the clone number. The tree was constructed by the neighbor-joining method from a similarity matrix based on the Kimura-2-parameter algorithm and rooted using *H. halobium* as an outgroup. The scale bar represents 10% sequence similarity. Bootstrap values were derived from 500 analyses.

confirms the specificity of the primers. The majority of the sequences (18/21; 61% of clones, $N = 400$), however, were clustered in just two groups within the *Methanomicrobacteria* and associated with the *Methanosarcina* sub-group. These clusters of methanogen sequences were clearly related to, but distinct from *M. mazeii*. The remaining three sequences, all from Cas18, were within the *Methanobacterium* group which itself is split into two further divisions and the sequences were affiliated to the *M. formicicum* group. Of the 21 sequences, 20 were $\geq 95\%$ similar to 16S rRNA sequences of cultured organisms in the RDP. Group I showed intra-group similarity values of $\geq 90\%$ (nucleotide sequence similarity), group II values of $\geq 92\%$ and group III values of $\geq 83\%$. Inter-group variation was $\leq 83\%$ between groups I and II, $\leq 68\%$ between groups I and III and $\leq 75\%$ between groups II and III. All Cas20 clones were affiliated with group II while Cas19 clones were all clustered in group I.

4. Discussion

The presence of high molecular mass DNA in all samples indicates that the DNA very likely originated from an active microbial population in these deep marine sediments, since sheared and degraded DNA was not apparent [16]. Degradation of naked DNA can start to occur within 5 min of exposure to the environment [35] and thus high molecular mass DNA indicates that it was extracted from active cells. This observation is consistent with substantial total and viable bacterial populations and active bacterial processes, including methanogenesis, throughout the core at this site [4]. Both sulfate-reducing bacteria and sulfate-reducing activity were present, but no phylotypes related to sulfate-reducing bacteria were isolated here. This result contrasts with similar studies on deep sediments from the Japan Sea where 16S rRNA gene sequences related to sulfate-reducing bacteria were obtained along with viable sulfate-reducing bacteria and active sulfate reduction [36]. In addition, all sequences from the Cascadia Margin were related to cultured organisms, compared to only 72% for the Japan Sea sequences. However, in both studies proteobacterial sequences dominated, 95% in the Cascadia Margin and 80% in the Japan Sea, although no identical sequences were found in both sediments. This high proportion of proteobacterial sequences is in agreement with other deep environment studies [37,38].

The bacterial sequences were predominantly represented by a few phylotypes with 65% being found in six phylotypes (see Table 2) which represents less diversity than has been found in previous deep sediment studies [16]. In the four libraries, the percentage coverage was $\geq 90\%$. This value indicates there was low population diversity, since a high percentage coverage was obtained from a relatively small clone library. Such low diversity may be due to the stressful or specialised conditions in these deep sea sediments. A similar observation was made for populations exposed to xenobiotic stress [39]. Low diversity was also observed for the methanogen libraries and is not unusual as low diversity has been reported for *Archaea* in another methane rich habitat [9].

Methanogens have proved difficult to cultivate from deep sediments [40] and other environments [41]. Hence, the information from gene sequence analyses is particularly important. The PCR-amplified 16S rDNA sequences from Cascadia Margin sediments were between 90 and 100% similar to those of cultured methanogen sequences deposited at the RDP, with most (18/21) clustering in the sub-order *Methanosarcinales* which contains acetate utilising methanogens [42]. The acetoclastic *M. mazeii* [42] was the most closely related culture to the sequences isolated here (98% sequence similarity). Based on carbon and hydrogen isotopic analysis, most biogenic methane in marine sediments originates from H_2/CO_2 [43]. At another deep gas hydrate site, acetate has been shown to be the major substrate for methanogenesis [2]. Furthermore, Bidle et al.

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[44] did not obtain Archaeal 16S rRNA genes from Cascadia Margin near surface sediment, but did identify methyl coenzyme M reductase gene sequences from the *Methanosarcinales* family. All these results suggest that acetate is an important methanogenic substrate in the Cascadia Margin.

The remaining sequences, all from just above the hydrate zone, were from the *Methanobacteriales* (group III, Fig. 2). These methanogens use H_2/CO_2 and sometimes formate or alcohols as substrates for methanogenesis [45]. These sequences were from the site where there was a small stimulation in rates of H_2/CO_2 methanogenesis [4] associated with a broad subsurface increase in methane concentrations [46]. Hence, conditions above the hydrate zone may be conducive to H_2/CO_2 methanogenesis. Rates of H_2/CO_2 methanogenesis also increased below the base of the hydrate zone (234 mbsf, Cas20 [4]), however, *Methanobacteriales* were not detected in this region. Consequently, except for just above the hydrate zone (Cas18) the methanogen sequences were very similar from near surface sediment (Cas14) to 234 mbsf (Cas20), with a rather limited diversity.

The methanogen sequences reported here are the first to be described for the deep sub-sea floor sediments and also deep gas hydrate deposits. The occurrence of methanogens is consistent with the presence of biogenic methane [47] and active methanogenesis within the sediment [4]. The presence of both bacterial and Archaeal gene sequences further demonstrates that deep sediments are an effective bacterial habitat [3]. However, low diversity demonstrates that at least the gas hydrate deposits are a highly selective environment.

Acknowledgements

The authors wish to express their gratitude to the Natural and Environmental Research Council for supporting this research, the Ocean Drilling Programme for providing sampling facilities and Dr. A. Schnürer for the kind gift of *Methanoculleus bourgensis* DNA.

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