Detection of novel marine methanotrophs using phylogenetic and functional gene probes after methane enrichment

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A major limitation of rRNA-targeted group-specific probes is that they may cross-react with organisms of other physiological, or even phylogenetic groups when applied to environmental samples containing unknown sequences. We have exploited the restricted physiology of methane-oxidizing bacteria to assess the specificity and efficiency of probes for this physiological type which target the 16S rRNA or genes involved in methanotroph physiology. Seawater samples were enriched for methanotrophs by addition of methane and essential nutrients. The changes in composition of the bacterial population were monitored by analysis of 16S rRNA gene libraries. Methanotroph group-specific probes failed to give a signal with samples from these enrichments even though a methanol dehydrogenase structural gene was detected. A 16S rDNA sequence that was abundant only after methane addition was recovered and found to show a close phylogenetic relationship to *Methylobacter*. Organisms containing this sequence were observed in enrichments by *in situ* hybridization. The combination of enrichment on methane and screening with the broad specificity methanol dehydrogenase probe allowed detection of novel methanotrophs that were not detected with the original suite of methanotroph group-specific probes.

Keywords: 16S ribosomal RNA, marine methane-oxidizing bacteria, oligonucleotide probe

INTRODUCTION

Methanotrophs are a group of bacteria physiologically defined by their ability to use methane as sole source of carbon and energy for growth. The methanotrophs have attracted the attention of microbiologists for two principal reasons: their biotechnological potential (Oldenhuis & Janssen, 1993) and their importance in the global methane cycle (Reeburgh *et al.*, 1993). The contribution of marine systems to the global methane cycle is generally poorly understood. Methane concentrations and oxidation rates have been determined from many oceanic sites. These data have shown the upper ocean is usually methane supersaturated with respect to the atmosphere. Both methane fluxes and oxidation rates in stratified water columns (Ward *et al.*, 1987) and at the air–sea interface (Conrad & Seiler, 1988) suggest that biological oxidation

is occurring in the sea although the methane turnover time may be slow (reviewed by Kiene, 1991). As in the terrestrial environment, methane-oxidizing bacteria are believed to be responsible for this oxidation process.

Although a large number of different methanotrophs have been isolated from soil and freshwater environments (Bowman *et al.*, 1993; Hanson & Wattenberg, 1991) there have been relatively few reports on the isolation of methanotrophs from the marine environment (Sieburth *et al.*, 1987; Lidstrom, 1988; Lees *et al.*, 1991; Sieburth *et al.*, 1993). Originally described as *Methylobacter* species, these marine strains have now all been reclassified as members of the genus *Methylobacter* (Bowman *et al.*, 1993). The lack of diversity of marine methanotrophs in culture may be due to the difficulties in isolation of these organisms. These cultural problems may have led to members of other methanotroph genera being overlooked in marine habitats.

Recent advances in microbial ecology (Ward *et al.*, 1992) make it possible to construct nucleic acid probes for

Abbreviations: ANMS, ammonium nitrate mineral salts; NMS, nitrate mineral salts; OTU, operational taxonomic unit; sMMO, soluble (cytoplasmic) methane monooxygenase.

phylogenetic, and in some cases physiological groups of bacteria which can be directly applied to the environment (Manz *et al.*, 1992; Wagner *et al.*, 1994; Brusseau *et al.*, 1994). The most widely used strategies involve detection or retrieval of 16S rRNA sequences which enable characterization of communities in terms of the phylogenetic position of their component taxa (Britschgi & Giovannoni, 1991; Schmidt *et al.*, 1991). Application of a 16S rRNA-oriented molecular ecology approach to study a physiologically defined group of bacteria requires the pre-existence of a representative culture collection. The isolation, or identification, of a greater diversity of marine methanotrophs is therefore necessary if these kinds of techniques are to be used to assess the role of methanotrophs in the marine methane cycle. We are attempting to achieve this by exploiting the existing knowledge of methanotroph physiology and phylogeny to facilitate the retrieval of new DNA sequences from the environment.

Recent studies on the phylogeny of extant methanotrophs derived from 16S rRNA sequence analysis (Bowman, 1992; Tsuji *et al.*, 1990; Bratina *et al.*, 1992; Brusseau *et al.*, 1994) have shown that they are of two types. Type I methanotrophs contain bundles of intracytoplasmic membranes, assimilate carbon via the ribulose monophosphate pathway and are γ -*Proteobacteria*. Type II methanotrophs, which possess intracytoplasmic membranes arranged around the periphery of the cell and use the serine pathway for carbon assimilation, cluster in the α -*Proteobacteria*. Within the α - and γ -subdivisions, neither group of methanotrophs has any close relatives which are not methanotrophic. This, coupled with the restricted metabolic potential of these organisms, has led us to speculate that phylogenetic position may be a reliable indicator of physiology and that we could exploit this knowledge for the detection of novel strains of methanotrophs.

The obligate nature of methanotrophs allows highly selective enrichment conditions to be established. In this study, bacterial populations in seawater were characterized by analysis of 16S rDNA genes from gene libraries constructed via PCR from total DNA. We report the detection of changes in community structure after methane addition under various enrichment regimes and the enrichment of organisms which show a close phylogenetic relationship, on the basis of 16S rRNA signature sequences, to previously isolated terrestrial methanotrophs. Despite their identification using molecular microbial techniques, it was not possible to grow these novel methanotrophs in pure cultures in the laboratory.

METHODS

Seawater sampling and enrichment cultures. Surface seawater (1–3 m depth) was collected in sterile large glass jars from several locations in the North Sea, the English Channel (Plymouth; February 1993) and the Bristol Channel (Penarth; August 1993). Samples were transported to the laboratory at ambient temperature and enrichments set up as described below.

Large-scale enrichment cultures. A batch culture was established in a 20 l working-volume fermentation vessel (LH100 series). The basal medium was the ammonium nitrate mineral salts (ANMS) of Whittenbury *et al.* (1970) at 0.4 × concentration

supplemented with 3.4% (w/v) NaCl. Ten litres of this basal medium was sterilized by autoclaving and inoculated with 10 l of seawater to give a final strength of 0.2 × ANMS. CH₄/CO₂ mixture (95:5, v/v; BOC) and air were supplied constantly at 250 and 500 ml min⁻¹, respectively. Temperature and pH were maintained at 20 °C and 7.6, respectively. The culture was stirred at 250 r.p.m. and sampled after 1, 3, 5, 7, 10, 14, 21 and 28 d.

Small-scale enrichment cultures. Cultures were established in 250 ml Quick fit flasks sealed with rubber Suba seals. Forty millilitres of basal medium was inoculated with 10 ml seawater. Multiple enrichments were set up for each sample by varying the basal medium. This consisted of ammonium mineral salts, nitrate mineral salts (NMS) or ANMS at 1 × concentration supplemented with 2.5 or 3.4% NaCl. The pH was 6.8 or 7.8 and flasks were incubated at either 20 or 30 °C. The Penarth enrichment utilized NMS containing 2% (w/v) NaCl, pH 6.8 and was incubated at 30 °C. All flasks were supplied with methane by withdrawal of 50 ml air and injection of 60 ml CH₄/CO₂ (95:5) mixture.

DNA extraction. Nucleic acids were extracted from seawater and the enrichment cultures by the method of Somerville *et al.* (1989). This involved harvesting cells from up to 250 ml of sample by filtration through 0.22 µm Sterivex filters (Millipore) and lysis *in situ* with lysozyme, proteinase K and SDS treatments. This protocol had been previously demonstrated to lyse all methanotrophs in the University of Warwick culture collection (A. J. Holmes & J. C. Murrell, unpublished). Nucleic acids were purified from the lysate by ammonium acetate precipitation of proteins, followed by ethanol precipitation to recover nucleic acids. This crude purification procedure yielded high molecular mass DNA of sufficient purity for amplification by PCR from all samples.

Phylogenetic group-specific primers. 16S rRNA sequences of methanotrophs were obtained from GenBank and from Bowman (1992). Regions diagnostic for each of the available representatives of the five genera were selected for probe design. Probe sequences and control organisms are shown in Table 1. The probes were tested for their ability to detect methanotroph genera specifically in both PCR and colony hybridizations against cloned 16S rRNA genes from representative methanotrophs in the University of Warwick culture collection. Reaction conditions for hybridizations and PCR testing were as described below. All probes gave a strong signal with their target genus and showed no cross-reactivity with methanotrophs from other genera. Results of PCR and hybridizations are summarized in Table 1.

Colony hybridization. Host *Escherichia coli* containing cloned methanotroph ribosomal RNA genes were grown on nylon membranes placed on LB agar plates containing kanamycin (50 µg ml⁻¹) and ampicillin (50 µg ml⁻¹). The colonies were lysed and their DNA fixed to the membrane by UV light using standard techniques (Sambrook *et al.*, 1989). Oligonucleotide probes were end-labelled with [γ -³²P]ATP (> 100 TBq mmol⁻¹) using T4 polynucleotide kinase and hybridized overnight to the filters at 50 °C in hybridization buffer [6 × SSC (Sambrook *et al.*, 1989), 0.5% SDS, 1 mM EDTA, 10 mM sodium phosphate, pH 6.8, 200 µg denatured Herring DNA ml⁻¹, 1 × Denhardt's solution]. The filters were washed in two changes of 2 × SSC for 30 min each at 45 °C and the temperature was raised in 2–5 °C increments in subsequent washes to determine conditions for the specificity of each probe.

PCR amplification. Bacterial 16S rRNA genes were amplified from all the DNA samples using the bacteria-specific primers f27 and r1492 (Giovannoni, 1991). Amplification reactions were

Table 1. Methanotroph functional and phylogenetic group-specific probes

Primer	Sequence (5'-3')	Target genus	Control organism*	Temperature (°C) for specificity in:	
				Colony hybridization	PCR†
Mb1007	CACTCTACGATCTCTCACAG	<i>Methylobacter</i>	<i>Mb. albus</i> BG8	60	58
Mc1005	CCGCATCTCTGCAGGAT	<i>Methylococcus</i>	<i>Mc. capsulatus</i> Bath	55	54
Mm1007	CACTCCGCTATCTCTAACAG	<i>Methylomonas</i>	<i>Mm. methanica</i> S1	55	58
Ms1020	CCCTTGCGGAAGGAAGTC	<i>Methylosinus</i>	<i>Ms. trichosporium</i> OB3b	55	54
Mm850	TACGTTAGCTCCACCACTAA	<i>Methylomonas</i>	Clone Pen 2	NT	58
<i>mx</i> aF1003	GCGGCACCAACTGGGGCTGGT	All Gram-negative methylotrophs	<i>Ms. trichosporium</i> OB3b	NT	59
<i>mx</i> aF1561	GGGCAGCATGAAGGGCTCCC	All Gram-negative methylotrophs	<i>Ms. trichosporium</i> OB3b	NT	59
<i>m</i> moC542	GGTTCTGCTGTGCCGACC	All sMMO-containing methanotrophs	<i>Ms. trichosporium</i> OB3b	NT	55
<i>m</i> moC986	ATCCCGTGCCGCCGGCGACG	All sMMO-containing methanotrophs	<i>Ms. trichosporium</i> OB3b	NT	55

NT, not tested.

*Phylogenetic group-specific probes were also tested by a search of the ribosomal database project (Maidak *et al.*, 1994). No non-methanotroph with two or less mismatches was found for all probes, except Mc1005 which shows homology to some *Vibrio* spp.

†Primers were also tested against the following strains in PCR, *Mb. pelagicus* IR-1, *Mb. whittenburyi*, *Mb. luteus*, *Methylocystis parvus* OBBP and *Ms. sporium* strain 5.

performed using the reagents supplied with Promega *Taq* polymerase kits at 1.5 mM Mg²⁺, with 20 ng template DNA and 100 pmol of each primer added. The reactions were carried out in a Hybaid TR2 thermocycler with 30 cycles of 92 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min, with a final extension at 72 °C for 5 min. Reaction products were checked for size and purity on 1% agarose gels (Sambrook *et al.*, 1989) and then ligated into the pCRII vector supplied with the T/A cloning kit (Invitrogen) according to the manufacturer's instructions. Amplifications were also carried out with primers specific for methylotroph genes (*mx*aF, which encodes the large subunit of methanol dehydrogenase) (Lidstrom *et al.*, 1994), methanotroph genes [*m*moC, which encodes Protein C of soluble methane monooxygenase (sMMO)] (MacDonald *et al.*, 1995) and phylogenetic group-specific primers targeting the 16S rDNA of methanotrophs (Mm1007, Mb1007, Mc1005 and Ms1018). The group-specific primers were used in conjunction with the f27 primer. Primer sequences and the annealing temperatures used in PCR are shown in Table 1.

DNA sequencing. Small-scale preparations of plasmids were made by the method of Saunders & Burke (1990). All sequencing reactions were carried out using the Sequenase 2.0 sequencing kit (USB) according to the manufacturer's instructions. Single-track (T-track) sequencing reactions were performed as above except that only the ddTTP termination reaction was carried out. Primers used for the sequencing reactions were complementary to conserved regions of the 16S rRNA. Their sequences can be found in Lane (1991) and Giovannoni (1991).

Phylogenetic analysis. Sequences were aligned manually to representative proteobacterial sequences obtained from the Genbank database and dendrograms constructed using the programs DNADIST, FITCH and DNAPARS from the PHYLIP version

3.4 package (Felsenstein, 1988). Only regions which could be unambiguously aligned were included in the analyses (bases 220–450, 482–836, 850–1133, 1140–1284 and 1287–1430 according to the *E. coli* numbering system). Analyses were also performed with partial sequences to check for the presence of chimaeric sequences. Secondary structure predictions for methanotroph 16S rRNAs were constructed manually, based on the published models of Neefs *et al.* (1991) and Gutell *et al.* (1994).

In situ hybridization experiments. The oligonucleotides Mm650 (5' CCTCTACTCAACTCTAGT) and Mm850 (5' TACGTTAGCTCCACCACTAA) were obtained from Genosys Europe with rhodamine groups attached via a C₆ linker at the 5' terminus. These probes are specific for the *Methylomonas* lineage (*sensu* Bowman *et al.*, 1993) and bind to bases 650–677 and 850–869 of the small-subunit rRNA (*E. coli* numbering). Binding of the probes to clone Pen 2 was tested in PCR using the amplification conditions described above.

Cultures were sampled after 1–2 d incubation (late exponential phase of growth) and fixed as described by Amann *et al.* (1990). A 100 µl aliquot of fixed cells was mixed with 10 µl 1% (v/v) Triton X-100 and collected by centrifugation. The cells were resuspended in 0.1% Triton X-100 to a concentration of approximately 10⁹ cells ml⁻¹. Three microlitres of this suspension were applied to the surface of gelatin-treated slides (Amann *et al.*, 1990) and allowed to dry. The cells were then dehydrated by immersion for 3 min each in a graded ethanol series (50, 80, 96%) and thoroughly air-dried.

Ten microlitres of hybridization solution [900 mM NaCl, 20% (v/v) formamide, 0.01% SDS, 20 mM Tris/HCl, pH 7.2, 30 ng oligonucleotide probe (Manz *et al.*, 1992)]

was added and the slides incubated in a humidified chamber at 42 °C for 2 h. The slides were then rinsed in 1 ml wash buffer (180 mM NaCl, 5 mM EDTA, 0.01 % SDS, 20 mM Tris/HCl, pH 7.2) to remove the hybridization solution and then immersed in 10 ml wash buffer at 37 °C for 30 min. Slides were dipped in distilled water before air-drying and mounting in Citifluor AF3 (Citifluor). Slides were examined with a Zeiss Axioskop microscope fitted with filter sets 09 and 15. The eubacteria-specific probe Eub338 (Amann *et al.*, 1992) labelled with fluorescein (Genosys Europe) was used as a control for all hybridizations.

RESULTS

Enrichment for bacteria which could not be obtained in pure culture

The 20 l scale fermenter batch enrichment culture was established to model a marine ecosystem with an excess supply of methane. ANMS (Whittenbury *et al.*, 1970) at 0.2 × concentration, supplemented with 3.4 % NaCl, was used to supply essential trace elements, nitrogen and phosphate for growth. The lower concentration was used to limit potential problems of selection for copiotrophs. The large volume allowed periodic sampling with minimal disturbance of the system. The smaller flask enrichments were designed to rapidly screen samples under a range of growth conditions for cultivable, or at least enrichable methanotrophs.

In the fermenter enrichment of Plymouth seawater, cell numbers remained essentially constant throughout 4 weeks incubation with CH₄/CO₂ (95:5) mixture as the sole supplied carbon source. Microscopic examination of the culture showed a mixed bacterial population of approximately 10⁶ cells ml⁻¹, consisting mainly of small (1–2 µm) rod-shaped bacteria, many of which were motile.

The small-scale flask enrichments were established to obtain samples from a wider geographical range. An enrichment established from the Penarth seawater sample (hereafter referred to as the Penarth culture) was of particular interest. This culture was characterized by its strong pink–orange pigmentation, the growth of cells as aggregated clumps rather than a dispersed culture and the presence of a pellicle in standing cultures. These features are characteristic of the genus *Methylobacter* (*sensu* Bowman *et al.*, 1993), representatives of which have not been reported from the marine environment. Numerous attempts to plate out samples from these enrichments were unsuccessful, as had previously been experienced in this laboratory (Lees *et al.*, 1991). Samples from the Penarth enrichment plated on 1.2 % (w/v) Bacto agar NMS plates incubated in methane/air (50:50, v/v) did show orange and pink colonies after a minimum of 1 week incubation at 30 °C. These colonies consisted of tightly bound aggregates of at least three cell types. We were unable to isolate a methanotroph in pure culture from this enrichment.

Construction of 16S rRNA gene libraries

High molecular mass DNA was readily extracted from all fermenter and batch culture samples. Yields of DNA were approximately 10 ng per 100 ml culture for all fermenter samples, but significantly higher DNA concentrations of 5 µg per 100 ml were obtained from the Penarth culture. Two gene libraries, termed Plym and Month, were constructed from 16S rDNA PCR products amplified from the Plymouth seawater sample and the 28-d sample of the fermenter culture. To ensure comparability of the resulting libraries, special attention was paid to ensure DNA extraction, amplification conditions and cloning procedures for these two libraries were reproduced as closely as possible. A third library was also constructed from the Penarth enrichment culture. All three libraries initially contained over 1000 clones of which approximately 80 % contained an insert of the desired size.

Characterization of Plymouth samples

PCR. The group-specific probes were used in PCR to screen DNA samples for the presence of putative methanotrophs. All probes gave a signal with the relevant control organism [Mm1007 (*Methylobacter methanica*), Mb1007 (*Methylobacter albus*), Mc1005 (*Methylococcus capsulatus*) and Ms1020 (*Methylosinus trichosporium*)] when used at their specific annealing temperature (Table 1). No PCR amplification product was obtained when total DNA samples isolated from the Plymouth or fermenter samples were used as template (data not shown). The *mxoF* primer set amplified a specific product of the expected size from the 28-d fermenter sample but not from the original Plymouth seawater sample (Fig. 1). This product was confirmed as *mxoF* by dot-blot hybridization to a probe generated from the *mxoF* gene from *Ms. trichosporium* using the methods of McDonald *et al.* (1995). None of the samples tested positive in PCR for sMMO genes.

Analysis of gene library. The 16S rDNA libraries constructed from Plymouth seawater samples were screened by single-track (T) sequencing to look for a shift in composition of the 16S rDNA library after enrichment on methane. It was expected that methanotrophic organisms, if present, would form a major component of the Month library. One hundred clones from the Plym library and 50 clones from the Month library were selected as a suitable number to allow recognition of a change in the community structure of these two samples with respect to dominant organisms. Seventy-nine clones from the Plym library and 41 clones from the Month library were found to contain the desired 16S rDNA insert. Approximately 300 bases of sequence information were read from each clone, including the variable region 1110–1160 (*E. coli* numbering). A full (four-track) sequence was included on each gel as a control, to facilitate alignment and interpretation of the T-track sequences. The T-track sequences could be readily aligned to the control sequence and clones were sorted into operational taxonomic units (OTUs) on the basis of unique sequences across this short stretch of the 16S rDNA. The majority of OTUs identified (including each of the major clusters) differed by greater

Fig. 1. PCR detection of methylotroph-specific sequences from marine samples after enrichment culture on methane. (a) Amplification of *Methylobacter* 16S rDNA (860 bp) using the genus-specific primer Mm850 and universal primer f27. Lanes: M, molecular mass markers (BRL); 1, *Mm. methanica*; 2, Penarth enrichment DNA; 3, North Sea Station 1 DNA; 4, North Sea Station 5 DNA; 5, North Sea Station 9 DNA; 6, *Ms. trichosporium*; 7, *Mc. capsulatus*. (b) Amplification of *mxoF* sequences (558 bp) from enrichment cultures. Lanes: 1, Penarth enrichment DNA; 2, Plymouth seawater DNA; 3, Plymouth enrichment (Month) DNA; 4, *Mc. capsulatus*; 5, negative control; M, molecular mass markers (BRL). Sizes of markers at 1636, 1018 and 506 bp are indicated by arrows on both gels.

than 4 bases. The results of this analysis are summarized in Fig. 2. A total of 36 different OTUs were identified from the two libraries. The majority of these were only represented by a single clone precluding any comment on their relative abundance in the two libraries and only three OTUs were common to both libraries. Nevertheless, a clear difference in community structure, as determined by the 16S rDNA gene libraries, between the natural seawater sample and the sample supplied with methane for 1 month was evident, despite the relatively small sample analysed here.

Fig. 2. Proportional representation of OTUs or sequence types, retrieved from the Plym and Month gene libraries, demonstrating the population shift which occurred after enrichment on methane. OTUs represented by less than three clones are grouped in the category 'others' (□). ▨, Plym 1; ■, Plym 5; ▩, Mth 1; ▤, Mth 17; ▥, Mth 47.

One OTU, the Plym 5 series, was found in both libraries in significant numbers. This group comprised 39% (31 out of 79) of the clones analysed from the Plym library and 20% (8 out of 41) of those from the Month library. The most significant difference between the two libraries was that they both contained a second major OTU which was not found in the other library. These were the Plym 1 series which comprised 24% (19 out of 79) of the Plym library and the Mth 17 series which comprised 24% (10 out of 41) of the Month library. The Month library also contained two other OTUs with more than two representatives which were not found in the Plym library; these were the Mth 1 (4 clones) and Mth 47 (3 clones) series. These OTUs may also represent taxa which exhibited a significant enrichment between the two seawater samples (natural and enriched).

Phylogenetic analysis. Selected clones from the Mth 17, Mth 1 and Mth 47 series, all of which showed some evidence of enrichment in the methane-supplied seawater, were analysed further. 16S rDNA was completely sequenced and examined for any possible relationship to methanotrophs. None of these clones showed any of the methanotroph signature sequences which we had identi-

Fig. 3. Unrooted dendrogram showing the phylogenetic position of clones representative of the dominant sequence types retrieved from cultures enriched on methane. Only clone Mth 13 was also found in natural seawater. The position of clone Pen 2 within the radiation of Type I methanotrophs is clearly seen. Clones Pen 10, Mth 17 and Mth 13 also branched within the γ -Proteobacteria but showed no relationship to the methanotrophs. The tree was constructed using the programs DNADIST and FITCH from the PHYLIP version 3.4 package (Felsenstein, 1988). Reference sequences were obtained from GenBank and from Bowman (1992).

fied from analysis of available methanotroph 16S rRNA sequences. Phylogenetic trees constructed by distance matrix and parsimony methods showed all these clones to be members of the γ -Proteobacteria, but to be distinct from the Type I methanotrophs which are also found in this subclass (Fig. 3). The phylogenetic positions of clones Mth 17 and Mth 13 (a member of the Plym 5 series), examples of the two dominant OTUs in the Month library, are shown in Fig. 3.

Characterization of Penarth samples

PCR. The Penarth enrichment established in NMS and supplied with methane showed strong bacterial growth, indicating the presence of methanotrophs. Surprisingly, DNA from this sample did not yield a PCR product with any of the methanotroph group-specific primers or sMMO primers under specific (stringent) amplification conditions. A specific PCR product was obtained with the *mxoF* primers, confirming the presence of methylotrophs (Fig. 1). Under lower stringency amplification conditions (50 °C annealing) a faint product was found using the primers f27 and Mm1007 from enrichment culture DNA samples but not from the original Penarth seawater sample.

Analysis of 16S rDNA library. Using colony hybridization, 153 colonies were screened for putative methanotroph sequences. Under low stringency conditions (50 °C) the *Methylomonas* probe Mm1007 bound to 49% (75 out of 153) of colonies and no colonies bound the *Methylobacter* probe Mb1007. Under more stringent hybridization conditions (55 °C) both probes bound only to the controls. These results suggested that a 16S rDNA sequence (or sequences) showing significant homology to the Mm1007 probe constituted about half of this gene library. A further 24 clones were selected from this 16S rDNA library and screened by single-track (T-track) sequencing. Two 16S rDNA sequence types were found to dominate this small sample. Clones Pen 2 and Pen 10 were selected as representatives of these OTUs and fully sequenced to determine their possible relationship to methanotrophs. Clone Pen 2 showed only 2 mismatches to the Mm1007 probe and was found to give a positive signal to this probe at 50 °C in PCR but not at the specific annealing temperature for this probe of 58 °C (see Table 1).

Phylogenetic analysis. The phylogenetic analysis of clone Pen 2 and clone Pen 10 16S rDNA sequences was carried out as described above. All methods of analysis showed a

Fig. 4. The predicted 16S rRNA secondary structures of *Mm. methanica* and clone Pen 2 for (a) helix 26/27 (bases 821–879) and (b) helix 43/44 (bases 1236–1302). Pen 2 is thought to represent an intact rRNA gene cloned from a mixed culture as the sequence shows a close fit to the bacterial secondary structural model (Neefs *et al.*, 1991). All divergences from its closest relative *Mm. methanica* (bases boxed in Pen 2) maintain the predicted helix structures by either covariation (both bases of a pair changed) or formation of non-canonical G–U pairs.

close relationship between clone Pen 2 and the Type I methanotroph cluster. Pen 10 also branched within the *γ-Proteobacteria* but did not show a close relationship to any of the organisms included in this analysis. Pen 2 showed 95% similarity to *Mm. methanica* and consistently branched with this organism, within the radiation of Type I methanotrophs (Fig. 3).

Secondary structure. The derived 16S rRNA sequence for Pen 2 showed an excellent correlation to the secondary structure model for small-subunit rRNAs of Gutell *et al.* (1994). All divergences from the *Mm. methanica* primary structure could be accommodated within the secondary structural model through compensatory base changes or by formation of non-canonical G–U base pairs. All non-canonical pairs formed in our model for Pen 2 lay in conserved helix motifs. Fig. 4 shows the predicted secondary structures for two regions of the Pen 2 and *Mm. methanica* 16S rRNA, Helix 27 and Helix 43/44, which are diagnostic for this clone.

In situ hybridization. To confirm the presence of an organism represented by this Pen 2 clone in the enrichment culture, two 5'-rhodamine-labelled oligonucleotides specific for Pen 2 (Mm650 and Mm850) were synthesized, which targeted a different domain (II) of the rRNA than our original set of probes (Mm1007, Mb1007, Mc1005 and Ms1020). These probes were found to be specific for *Methylomonas* spp. in PCR and yielded a PCR product from the Penarth enrichment DNA (Fig. 1). Initial experiments using the fluorescein-labelled probe

Eub338 confirmed that both *Mm. methanica* and *Mc. capsulatus*, harvested from 2-d cultures, were permeable to the oligonucleotide probe and could be easily visualized by fluorescence microscopy. *In situ* hybridizations using the specific probes Mm650 and Mm850 and the control probe Eub338 were performed on cells from the Penarth enrichments. All cell morphotypes stained with the control bacteria-specific probe Eub338 (Amann *et al.*, 1992) but only a single cell morphotype was stained with the rhodamine-labelled specific probes (data not shown). The 'Pen 2' cells comprised 45% (averaged over five fields of view) of the Eub338-staining cells in subcultures grown under identical conditions to the primary enrichment culture. This is a similar proportion of the total population to that found with colony blots of the original Penarth culture 16S rDNA clone bank using the Mm1007 probe under low stringency conditions and the incidence of the Pen 2 T-track sequence type found in this clone bank.

DISCUSSION

We have exploited molecular biological methods for the detection of uncultured methanotrophs in marine samples. We have previously noted that despite great difficulties in isolation, methanotrophs are readily enriched from marine habitats. 16S rDNA libraries were used to monitor the shift in bacterial populations that occurred under enrichment conditions highly selective for this physiological type. The use of specific growth conditions limits the number of physiological modes which can be supported in the system, simplifying the task of interpreting physiology from phylogenetic position and facilitating the evaluation of group-specific probes. We established both low and high cell density cultures by varying concentrations of essential nutrients and incubation temperature. These conditions were expected to select for different species of methanotrophs. The enrichments did not generally support the growth of other marine bacteria. The only cultures showing a significant increase in biomass were those from which methanotrophs or methanotroph-related DNA sequences were detected (see also Lees *et al.*, 1991).

The set of phylogenetic group-specific probes had the necessary range to detect all methanotroph strains in our culture collection. To complement these probes, marine samples were also examined for the presence of *mxaF* and *mmoC* (McDonald *et al.*, 1995). Cultures which gave a signal with either of these functional gene probes, but not the group-specific probes, may represent new methanotroph species unrelated to extant strains. We retrieved 16S rDNA sequences from these cultures to test our hypothesis that methanotrophs are indeed a phylogenetically coherent group.

The community analysis approach taken (T-track screening of gene libraries from PCR products) showed sufficient sensitivity and resolution to reveal a population shift in the low cell density enrichment (fermenter containing Plymouth seawater). Some workers have expressed concern as to bias which may be introduced

into community composition by cell lysis, DNA purification and PCR amplification procedures during the construction of gene libraries (Reysenbach *et al.*, 1992; Ward *et al.*, 1992). We were careful to reproduce experimental conditions as closely as possible for the construction of the two 16S rRNA gene libraries to minimize differential bias. It is difficult to attribute the increase in abundance of Mth 17 type clones from less than 1% (0 out of 79) to over 20% (10 out of 41) solely to experimental bias. This change is unlikely to be entirely due to death of some of the original population as this would have required at least a 20-fold decrease in total bacterial numbers. The most likely explanation is the limited growth of bacteria represented by the Mth 17 sequence type. Under the culture conditions employed, such growth could only have been via efficient scavenging of organic material, methanotrophy, chemoautotrophy or oxygenic phototrophy. The methylotroph-specific gene *mxoF* was present in the Month DNA sample but not the Plym DNA sample. It is likely therefore that a component of the enriched bacterial community has the capacity for methylotrophy. However, using the methanotroph group-specific primers and *mmoC*-specific primers, we could not find direct evidence for methanotrophs in the Month sample. The phylogenetic analysis of representatives of Mth 17, Mth 1 and Mth 47, those OTUs which showed evidence of enrichment, revealed them to be *γ-Proteobacteria*. No close relatives from which to infer their physiology were identified. If any bacteria represented by these sequences can grow on methane, then they represent a novel lineage of methanotrophs.

The Penarth enrichment culture showed an increase in biomass. As the only carbon source available to support growth to such high biomass (approx. 5×10^8 cells ml⁻¹) was methane, methanotrophs must have been present in this system. Such organisms are clearly novel strains as they do not react, under stringent conditions, with any of the group-specific probes for described methanotroph species, but only with the *mxoF*-specific primers. Dendrograms constructed from phylogenetic analysis of 16S rDNA sequences of clone Pen 2 all showed a stable branching position within the radiation of Type I methanotrophs, with its closest relatives being *Methylobacter* spp. The relationship to *Methylobacter* is also supported by phenotypic similarities, particularly the presence of a pink–orange pigment and pellicle formation in enrichment cultures.

The formation of chimaeric sequences (Liesack *et al.*, 1991; Kopczynski *et al.*, 1994) and misincorporation of bases during PCR (Keohavong & Thilly, 1989) is a possible source of error in molecular phylogenetic analysis. However, the stable branching position for Pen 2 in all analyses and the excellent correlation of its inferred 16S rRNA secondary structure to the bacterial model provide strong evidence that it represents an intact 16S rRNA gene cloned from an organism from the enrichment culture. This was confirmed by identification of only one cell morphotype in subcultures of this enrichment using fluorescently labelled oligonucleotide probes specific for the Pen 2 clone. Probes Mm650 and Mm850 target

separate structural motifs in the 16S rRNA. The abundance of Pen 2 cells in subcultures of the enrichment and of the Pen 2 sequence type in the gene library constructed from the original enrichment was found to be similar by several methods. Clearly the Pen 2 clone is representative of the 16S rRNA of a dominant organism in these marine enrichment cultures. The data presented here indicate that Pen 2 is a Type I methanotroph affiliated to the genus *Methylobacter* (as classified by Bowman *et al.*, 1993). It can grow at NaCl concentrations of up to 3.4% and does not appear to contain sMMO. This supports our hypothesis that for methanotrophs, phylogenetic position may well be an accurate indicator of physiology. Using the 'population shift' approach, we identified a novel methanotroph which could not be detected by our original set of probes or cultivated using current methods. This strategy will be most useful for those organisms which can be enriched under very specific conditions, and could be extended to any group for which both functional probes (i.e. those targeting genes involved in the expression of the phenotype) and phylogenetic probes are available.

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