NOTE**Rapid identification of hyperthermophilic methanococci isolated from deep-sea hydrothermal vents**

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16S rDNAs amplified by PCR from 22 hyperthermophilic methanococci isolated from deep-sea hydrothermal vents were compared with those of the six type strains of the genus *Methanococcus* by RFLP analysis. Restriction fragments obtained with *HaeIII* enabled four of the type species to be distinguished. Restrictions with *HhaI*, *BstUI* and *MspI* were necessary to differentiate *Methanococcus jannaschii* and *Methanococcus fervens*. The results indicate that the 16S rDNA PCR–RFLP method provides a rapid and reliable tool for the identification of newly isolated hyperthermophilic *Methanococcus* spp.

Keywords: *Methanococcus*, RFLP, 16S rDNA, *Archaea*, hydrothermal vents

Marine extremely thermophilic methanogens belonging to the genus *Methanococcus* have been isolated from shallow water systems, deep-sea hydrothermal vents and sea-water-flooded oilfields (Nilsen & Torsvik, 1996; Stetter, 1996; Jeanthon *et al.*, 1998, 1999). Thermophilic methanococci may represent the predominant methanogenic *Archaea* occurring at deep-sea hydrothermal vents (Jones *et al.*, 1989; Harmsen *et al.*, 1997), but little is known about their distribution and diversity. One of the reasons for this is the difficulty of rapidly and reliably identifying *Methanococcus* strains (Boone & Whitman, 1988; Keswani *et al.*, 1996). The paucity of readily identifiable phenotypic characteristics useful for distinguishing methanogens has led to a classification system based largely on phylogenetic assessments (Keswani *et al.*, 1996). 16S rDNA sequence analysis and DNA–DNA hybridization have become standard methods for the investigation of their phylogenetic relationships and for the differentiation among species, respectively (Woese, 1987; Boone & Whitman, 1988; Keswani *et al.*, 1996). However, these laborious and time-consuming methods cannot currently be considered as appropriate tools for routine identification of multiple isolates from the environment. Recently, a simple and rapid approach combining PCR and RFLP analysis has been used successfully for rapid identification at the species level of a variety of organisms including mesophilic methanogens (Vanechoutte *et al.*, 1992; Laguerre *et al.*, 1994; Hiraishi *et al.*, 1995; Blanc *et al.*, 1997; Sato *et al.*, 1997; Urakawa *et al.*, 1997).

The aim of the present study was to investigate the feasibility of using restriction fragment analysis of PCR-amplified rDNA for the differentiation of the hyperthermophilic species belonging to the genus *Methanococcus* and for the identification of new isolates from deep-sea hydrothermal vents.

Samples of hydrothermal chimneys or sediments were collected in the Guaymas Basin (27° 01' N, 111° 24' W) at a depth of 2000 m, on the East Pacific Rise (12° 49' N, 103° 56' W) at a depth of 2600 m and on the Mid-Atlantic Ridge (23° 22' N, 44° 57' W) at a depth of 3500 m. Serial dilutions of hydrothermal samples were carried out in 50 ml vials containing (per litre distilled water): 30 g sea salts (Sigma), 1 g NH₄Cl, 0.35 g KH₂PO₄, 3.46 g PIPES, 1 g NaHCO₃, 2 g Difco yeast extract, 2 g Difco peptone, 1 g sodium acetate, 0.5 g cysteine·HCl, 1 ml trace element mixture (Widdel & Bak, 1992), 30 mg tungstate, 0.5 mg selenate, 1 ml vitamin mixture (Widdel & Bak, 1992), 1 ml thiamin solution (Widdel & Bak, 1992), 0.05 mg vitamin B₁₂, 1 ml growth-stimulating factors (Pfennig *et al.*, 1981) and 1 mg resazurin. The pH was adjusted to 6.5 using 1 M HCl before autoclaving and the medium was reduced by adding appropriate amounts of sodium sulphide (Na₂S·9H₂O). H₂/CO₂ [80/20; 200 kPa (above atmospheric pressure)] was used as the gas phase. Unless indicated otherwise, cultures were incubated at 80 °C for 1–6 d. Pure colonies were isolated by repeated streaking on the same medium that was reduced with a titanium(III) citrate solution (Zehnder

& Wuhrman, 1976) and solidified with 0.7% (w/v) Phytagel (a gellan gum from Sigma).

The type strains listed below were used in this study. *Methanococcus infernus* strain ME^T (DSM 11812^T) and *Methanococcus vulcanius* M7^T (DSM 12094^T) were isolated in our laboratory (Jeanthon *et al.*, 1998, 1999). *Methanococcus jannaschii* strain JAL-1^T (DSM 2661^T), *Methanococcus fervens* strain AG86^T (DSM 4213^T), *Methanococcus igneus* strain Kol 5^T (DSM 5666^T) and *Methanococcus thermolithotrophicus* strain SN-1^T (DSM 2095^T) (Huber *et al.*, 1982; Jones *et al.*, 1983; Zhao *et al.*, 1988; Burggraf *et al.*, 1990; Jeanthon *et al.*, 1999) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany).

Genomic DNA extraction and purification were carried by using the procedure described by Charbonnier & Forterre (1994). Amplified 16S rDNA was obtained from each strain by PCR with the following specific archaeal primers: forward 5' TTCCGGTTGATC-CYGCCGGA 3', reverse 5' GGTTACCTTGTT-ACGACTT 3', corresponding to the positions 2–21 and to 1510–1492 of *Escherichia coli* 16S rRNA, respectively (Brosius *et al.*, 1978). PCR was performed on a Robocycler Gradient 96 (Stratagene). The initial denaturation step consisted of heating the reaction mixture at 95 °C for 4 min, and the thermal profile then consisted of 25 cycles of denaturation at 94 °C for 1 min 45 s, annealing at 50 °C for 1 min 45 s and extension at 72 °C for 2 min 45 s. A final extension step was carried out at 72 °C for 4 min. The volume of PCR product used in the restriction mixture was adjusted arbitrarily, based on the visually observed strength of the amplified rDNA fragments in the control gel (0.8% agarose). 16S rDNA genes were digested singly with restriction endonucleases, i.e. *Hae*III, *Hha*I and *Msp*I (Promega), or with *Bst*UI (New England Biolabs), according to the manufacturer's instructions. Digestion products were separated by 2–3% agarose (NuSieve 3:1 agarose; FMC BioProducts) gel electrophoresis in Tris/acetate/EDTA buffer. The gels were stained with ethidium bromide and photographed under UV light.

Isolation of hyperthermophilic methanococci

After incubation of enrichment cultures at 80 °C, 22 strains of hyperthermophilic methanogens were isolated from samples collected from four geographically separated hydrothermal vent sites. Five strains (isolates M1, M2, M3, M4 and M5) were isolated from sediments collected at Guaymas Basin, one (isolate M6) from chimney fragments collected at 13° N (East Pacific Rise) and 16 (isolates M8, M9, M10, MB, MH, MI, MJ, MK, MP, MR, MS, MT, MU, MV, MY and MZ) from chimneys sampled at 23° N on the Mid-Atlantic Ridge. All strains consisted of motile or non-motile coccoid cells that formed pale-yellow round colonies on solid medium, exhibited bright-green fluorescence at 420 nm, and produced methane from

Fig. 1. Restriction patterns of 16S rDNA genes from some reference *Methanococcus* strains digested with *Hha*I, *Hae*III and *Msp*I. Marker, 100 bp ladder (Promega).

CO₂ and H₂. On the basis of these characteristics and their thermophilic mode of life, they were presumptively assigned to the genus *Methanococcus* (Whitman *et al.*, 1992).

PCR restriction analysis of 16S rDNAs

Five to six distinct RFLP patterns were detected with each of the four endonucleases and strains used (Figs 1 and 2). Restriction fragments shorter than 42 bp produced by the endonucleases were not well resolved by electrophoresis in 3% NuSieve 3:1 agarose and were disregarded. Distinctive patterns were obtained after restriction of the amplified 16S rDNA of the six type strains of the genus *Methanococcus*, namely, *M. fervens*, *M. jannaschii*, *M. infernus*, *M. vulcanius*, *M. igneus* and *M. thermolithotrophicus*, with *Hha*I, *Bst*UI and *Msp*I (Figs 1 and 2). Restriction patterns of *M. jannaschii* and *M. fervens* digested with *Msp*I showed strong similarities. However, for *M. jannaschii* bands of 161 and 181 kb were separated on the gel whereas for *M. fervens* bands of 161 and 171 kb were not, and appeared as a unique band of higher intensity (Fig. 1). The profiles obtained with *Hae*III showed distinctive patterns for all the type strains used except for *M. fervens* and *M. jannaschii*. Since six different type strains were involved in this study, we can assume that the combination of the four restriction enzymes generated a number of restriction fragments which were sufficient and appropriate to allow the analysis of this group of related organisms (Heyndrickx *et al.*, 1996; Moyer *et al.*, 1996).

Fig. 2. Restriction patterns of 16S rDNA genes of the six *Methanococcus* type strains and of four isolates from deep-sea hydrothermal vents selectively amplified and digested with *Bst*UI. Marker, 100 bp ladder.

This combination of four tetracutter restriction enzymes was also applied to identify the 22 newly isolated methanogens. As an example, a gel of amplified 16S rDNA genes from the six type species of the genus *Methanococcus* and four new isolates digested with *Bst*UI is shown in Fig. 2. On the basis of restriction site similarities with the four enzymes, the new isolates could be arranged in four genotypes. Isolates M3 and M4, isolated from Guaymas Basin, and isolate MP, isolated from the Mid-Atlantic Ridge (23° N), displayed the same four patterns as those observed for *M. jannaschii*. Patterns produced by isolates M2 and M5 from Guaymas Basin and isolate MY from the 23° N site on the Mid-Atlantic Ridge were identical to those of *M. fervens*. The set of patterns observed for *M. igneus* was the most frequently observed within the isolates (15 out of 22). One of these strains (M1) was isolated from sediments collected at Guaymas Basin, and all the others (isolates M8, M9, M10, MB, MH, MI, MJ, MK, MR, MS, MT, MU, MV and MZ) came from chimneys taken at 23° N on the Mid-Atlantic Ridge. Isolate M6 and *M. vulcanius*, which originated from the 13° N site on the East Pacific Rise, showed similar patterns with the four enzymes, indicating that these strains may correspond to the same species.

The 16S rDNA gene sequences of the six reference strains of *Methanococcus* from GenBank were assessed to compare the theoretical restriction profiles with those obtained experimentally. Particularly for the 16S rDNA sequences of *M. igneus* and *M. thermolithotrophicus* (accession nos M59125 and M59128, respectively) where undetermined nucleotide positions frequently occurred, the number and size of theoretically simulated restriction fragments were different

from the experimentally observed fragments. Some ambiguous results were also caused by fragments of unexpected size, most commonly those including end points of sequences. However, the RFLP patterns obtained in this study for the other type strains generally matched the expected profiles.

The PCR-RFLP profile analysis appears to be a promising technique to obtain rapid indicative taxonomic information on hyperthermophilic methanogens. Its reliability and accuracy allowed us to select strains for detailed polyphasic taxonomic studies (Jeanthon *et al.*, 1998, 1999) and to estimate rapidly their diversity and distribution. However, because of the conserved nature of the 16S rDNA sequences, this method may be limited in the differentiation of closely related species. Additional discriminatory methods (DNA-DNA hybridization, extensive phenotypic characterization) would therefore be required for a rigorous species assignment.

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