Cah. Biol. Mar. (2002) 43 : 421-424

Archaeal diversity in a deep-sea hydrothermal sample from the East Pacific Rise (13°N) investigated by cultivation and molecular methods: preliminary results

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Introduction

Since their discovery in the late 1970's, deep-sea hydrothermal ecosystems have been subjected to numerous research activities including intensive microbiological analyses. The first efforts of microbiologists aimed at cultivating and isolating microorganisms associated with several ecological niches, including (i) epibionts and endosymbionts associated with hydrothermal macrofauna (ii) microbial consortia exposed to relatively low temperature hydrothermal fluids on the rocks and chimney surfaces (iii) free-living mesophiles from hydrothermal plumes and (iv) free-living hyperthermophiles thriving within the chimneys and discharged in the hydrothermal plumes (Karl, 1995). Despite the application of a diversity of culture techniques, many hydrothermal microorganisms such as the epi- and endosymbionts and those forming microbial mats still escaped to cultivation. During the last decade, molecular techniques mostly based on analyses of the 16S rRNA molecule have been proven effective for characterizing complex microbial assemblages in the hydrothermal environment (see Jeanthon, 2000 for review). In particular, recent molecular phylogenetic analyses based on 16S rRNA sequencing have revealed that thermophilic assemblages associated with the hydrothermal vents were composed of a surprising diversity of uncultivated bacterial and archaeal populations (Moyer et al., 1995; Takai & Horikoshi, 1999; Reysenbach et al., 2000; Takai et al., 2001). However, the diversity and ecological significance of Archaea are still poorly known. The aim of this preliminary study was to characterize the archaeal communities in a sample collected at the East Pacific Rise (13°N, 104°W), by culture-dependent and culture-independent methods. The first results presented here support the existence of a wide archaeal diversity at this site.

Materials and methods

Study site and sampling procedures
The cruise AMISTAD (Advanced Microbiological Study on Thermophiles: Adaptation and Diversity) was organized in May 1999 at 13°N (12°48'N, 103°57'W) on the East Pacific Rise. The hydrothermal sites located at 2500 m depth were sampled using the submersible *Nautile* and its mothership *Atalante*. The 13°N site is composed of both active and inactive hydrothermal areas. Active black smokers and diffuse flow vents are generally colonized by dense macrofaunal assemblages including vestimentiferans and polychaetes. An active hydrothermal area, named Pulsar PP55, was selected to deploy an in situ collector designed to concentrate microorganisms discharged by the emitted fluids. The collector consisted of a stainless steel wire mesh bag (15 cm x 15 cm x 2 cm) filled with aquarium filtering wool (Vitakraft, Bremen, Germany). The temperature of fluids recorded in the first centimetres above the vent, before and after the 11 days of deployment ranged from 50 to 100 °C. The collector was brought to the surface in an insulated box to minimize its exposition to pelagic microbial populations. On board, portions of the aquarium filtering wool used for molecular analyses were immediately frozen at -20 °C. For further enrichments, the remaining was transferred in 50 ml glass vials and flooded with a sterile solution of 3% (w/v) Sea Salts (Sigma Chemical CO., St. Louis, Mo). The vials were then closed tightly with butyl rubber stoppers (Bellco, Vineland, NJ, USA), pressurized with N_2 (100 kPa), reduced with sodium sulphide, and stored at 4 °C until processed.

DNA extraction, PCR reactions and cloning
Nucleic acids were extracted according to Porteous *et al.* (1994). Briefly, DNA extraction was performed in quadruplicate (0.5 g wet sample in 350 µl of 250 mM NaCl,

100 mM Na_2-EDTA, pH 8). Cells lysis was achieved by sonication and successive additions of lysozyme (100 µg), SDS (2%, final concentration) and lauryl-sarkosyl (2%, final concentration). After extraction, nucleic acids were precipitated overnight in isopropanol at -20 °C and recovered by centrifugation. Nucleic acids from each replicate were pooled in a 200 µl of sterile water. In order to generate the archaeal clone library, the universal reverse primer 1407R (5'- GACGGGCGGTGWGTRCAA-3') in combination with the forward primer 4F (5'-TCCGGTTGATCCTGCCRG-3') were used in initial attempts. PCR reaction mixtures containing 1 µl of DNA extract consisted of (as final concentrations) 50 mM KCl, 10 mM Tris-HCl (pH 9 at 25 °C), 0.1% Triton X100, 1.5 mM $MgCl_2$; 0.2 mM of each dATP, dCTP, dGTP, and dTTP, 0.2 µM of each amplification primer, 1.25 U of *Taq* DNA polymerase (Promega Corp., Charbonnières, France) in a final volume of 25 µl. Reaction mixtures were incubated in a thermal cycler (model Robocycler 96; Stratagene, La Jolla, Calif.) at 95 °C for 5 min followed by 35 cycles at 95 °C for 1.5 min, 53 °C for 1.5 min, and at 72 °C for 2.5 min and then by a final extension period of 8 min at 72 °C. Since no PCR products were obtained under these conditions, a semi-nested PCR approach was used. The reactions and PCR conditions described before were used except that only 15 cycles were performed. Aliquots (1 µl) of these amplifications were used as templates for secondary amplifications with the 1407R primer and the forward primers 341F (5'-CCTAYGGGGYGCASCAGGCG-3'). These reaction mixtures were incubated at 95 °C for 5 min followed by 30 cycles at 95 °C for 1.25 min, 56 °C for 1.25 min, and at 72 °C for 2.25 min and then by a final extension period of 7.5 min at 72 °C. PCR products were cloned with a TOPO TA Cloning Kit in accordance with the manufacturer's instructions (Invitrogen, Groningen, The Netherlands).

A DNA extraction control was also performed on a sterilized chimney sample amended or not with a pelleted cells of *Thermococcus barophilus* Marteinsson, 1999, previously isolated in our lab (Marteinsson et al., 1999). As PCR controls, no template DNA or DNA from *Archaeoglobus fulgidus* Stetter, 1988 (DSMZ 4304) were added to the PCR mixtures.

The quality and the quantity of extracted and amplified DNAs were evaluated by electrophoreses in a 0.6% agarose gel containing 0.5 µg ml^{-1} of ethidium bromide. Nucleic acids were visualised on a UV-transilluminator.

16S rDNA sequencing and data analyses

Archaeal clones were sequenced by cycle sequencing using the ABI Prism Big Dye Terminator Cycle sequencing kit (PE Applied Biosystems Inc.) and the primer M13 Forward (5'-GTAAAACGACGGCCAG-3'). The sequences were determined on an automated sequencer (models ABI PRISM 373XL and 377 DNA sequencers; PE Applied Biosystems Inc.).

Partial sequences (about 550-600 bp) from clones were submitted to the GenBank (http://www.ncbi.nih.gov) and RDP databases (http://www.cme.msu.edu/RDP) to identify putative close phylogenetic relatives. A cutoff value of 97% similarity was used to categorized partial sequences into different phylogenetic groups.

Enrichment cultures and microscopic observations

Enrichments were performed anaerobically in 50 ml vials containing 10 ml of medium. The medium designed by Jones et al. (1989) was used to enrich for hydrogenotrophic methanogens. The pH was adjusted to 6.5 and a H_2/CO_2 mixture (80/20; 200 kPa) was used as the gas phase. Heterotrophic sulphur-reducers were cultivated in the medium described by Erauso et al. (1993) at pH 7, with N_2 (100 kPa) as a gas phase. Sulphate-reducers were grown in the basal medium recommended by Widdel & Bak (1992) supplemented with acetate (15 mM) as carbon source. The pH was adjusted to 7 and N_2/CO_2 (80:20; 100 kPa) was used as the gas phase. Media were inoculated using 0.3 ml of filter suspension and incubated at 65, 80 and 90 °C. An Olympus BH-2 microscope equipped with an Olympus OM-2 camera was used routinely to observe the cells.

Results and Discussion

Although significant amounts of partially sheared nucleic acids were extracted from the sample (Fig. 1a), no PCR products were obtained when the primers 4F and 1407R and routine amplification conditions were used to generate the archaeal library. This could be attributed to the extremely low quantity of archaeal DNA in the extract. However, amplicons of the expected size (around 1 kb) (Fig 1b) were obtained when the semi-nested PCR approach described in Materials and methods was utilized. The 180 clones that composed the archaeal library generated after cloning were partially sequenced (550 to 600 bp). Preliminary results revealed that 49% of the clones were very closely related to members of the family *Thermococcaceae* (99% similarity with species from the genera *Pyrococcus* and *Thermococcus*) known so far as hyperthermophilic heterotrophic sulphur-reducers. Almost 20% of the library consisted in sequences affiliated with the families *Methanopyraceae* (98% similarity with *Methanopyrus kandleri* Kurr, 1992, the single species of this genus) and

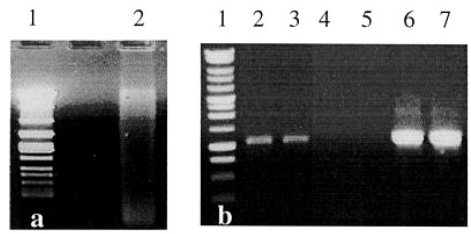

Figure 1. Gel electrophoreses of DNA extracted from the studied sample (a) and PCR products obtained with primers 341F and 1407R (b). (a) Lane 1, DNA from phage λ digested with *Hind*III, *Bam H*I and *Eco R*I; lane 2, DNA extract of the studied sample. (b) PCR results obtained with: (2 and 3), DNA extract as template; (4) Template obtained after extraction from a sterilized chimney sample; (5) No template DNA; (6) Template obtained after extraction from a sterilized chimney sample amended with cells of *Thermococcus barophilus;* (7) DNA from *Archaeoglobus fulgidus*. (1) 1 kb DNA ladder (Promega Corp., Charbonnières, France).

Methanocaldococcaceae (99% similarity with *Methanocaldococcus vulcanius* Jeanthon, 1999). Both these families are composed of hyperthermophilic strictly autotrophic methanogens. Sequences closely related to the *Archaeoglobaceae* (98% similarity with environmental clone VC2.1Arc8), described as mainly hyperthermophilic sulphate-reducers were also detected. They represented 12% of the clone library. Few sequences (4% of the library) were affiliated with strictly or facultatively autotrophic hyperthermophilic sulphur-reducing members of the family *Desulfurococcaceae* (98% similar to *Ignicoccus pacificus* Huber & Stetter, 2000 or *Staphylothermus marinus* Stetter & Fiala, 1986). As a whole, more than 80% of the clones were closely related to cultivated organisms. Few sequences (2%) were closely related to uncultivated clones from the phyla *Euryarchaeota* and *Crenarchaeota* (98% similarity with environmental clones pMC2A10 or pIVWA5) (Takai & Horikoshi, 1999), the remaining clones (15%) were distantly related to *Methanocaldococcus jannashii* Jones, 1984 (84-87%).

Table 1. Comparison of archaeal communities retrieved in deep-sea and coastal hydrothermal environments[a]

[a] (+) indicates that members of the phylogenetic group were cultivated or detected, (-) indicates that members of the phylogenetic group were not detected in the hydrothermal areas.

Bold symbols indicate that the phylogenetic groups were detected in this study. Data related to deep-sea and coastal marine vents are from (1) Huber & Stetter (2001a, b); (2), Reysenbach et al. (2000); (3), Takai & Horikoshi (1999) (4), Marteinsson et al., (2001); (5) and Takai & Sako (1999).

	Deep-sea hydrothermal sites			Coastal hydrothermal sites		
Phylogenetic groups	EPR[1]	MAR[1;2]	Okinawa Trough[3]	Vulcano island[1]	Iceland[1;4]	Tashibana Bay[3]
Thermococcaceae	+	+	+	+	+	+
Archaeoglobaceae	+	+	+	+	+	
Methanopyraceae	+	+	-	-	+	-
Methanocaldococcaceae	+	+	-	-	+	+
Pyrodictiaceae	+	+	-	+	+	+
Desulfurococcaceae	+	-	-	+	+	+
Thermoproteales	-	-	-	-	-	+
Uncultivated *Euryarchaeota*	+	+	+	-	-	-
Uncultivated *Crenarchaeota*	+	+	+	-	-	+
"*Korarchaeota*"	-	-	-	-	+	+

In addition to the molecular survey, we examined the metabolic diversity of anaerobic cultures cultivated at 60, 80, and 90 °C. Microscopic observations of positive cultures demonstrated the presence of regular motile heterotrophic cocci growing best at 80 and 90 °C in the presence of sulphur (presumably *Thermococcaceae*). In the medium favoring the growth of hydrogenotrophic methanogens, regular and irregular motile cocci (about 1 µm in diameter) and non motile rods (about 3 µm in length) were enriched at 80 and 90 °C, respectively. Both cell types showed a blue-green fluorescence when exposed to UV. According to their morphologies, temperatures of growth and metabolism, methanogenic cocci and rods could presumptively be assigned to members of the families *Methanocaldococcaceae* and *Methanopyraceae*, respectively. Irregular cocci growing on acetate as carbon source in medium containing sulphate as electron acceptor were also enriched at 80 and 90 °C. These organisms that also fluoresced under UV shared typical characteristics with members of the family *Archaeoglobaceae*.

Several bias associated with enzymatic amplification of the nucleic acids have been reported recently (Head et al., 1998). Since phylogenetic grouping of most 16S rRNA gene sequences is consistent with the presumptive identification of the cultivated organisms, we can assume that the sequences retrieved in this study likely correspond to organisms present in the collected sample. Evaluation of the ecological significance of the phylogenetic groups in the collected sample would require in situ hybridization experiments. The archaeal diversity demonstrated in a single sample collected at 13°N appears to be higher than that found so far in similar studies (Takai & Horikoshi, 1999; Reysenbach et al., 2000; Takai et al., 2001) (Table 1). Whether these observations are unique to the studied sample or are specific to the 13°N hydrothermal field is now under investigation. Our current efforts aim at examining higher numbers of samples collected during the same cruise, in order to more completly document the extent and the nature of archaeal diversity at 13°N.

Acknowledgements

The authors are grateful to Dr. Alexander Lebedinskii, Jean-Louis Birrien, Claire Geslin, Stéphane L'Haridon and Edmond Jolivet for their help and discussions. The AMISTAD cruise was organized by CNRS (C. Jeanthon, chief scientist) with the N.O. '*Atalante*' and the D.S.V. '*Nautile*' operated by IFREMER. We thank the captain and the crew of the N.O. '*Atalante*' and the D.S.V. '*Nautile*' pilots for skillful operations and support crew. This work was supported by Dorsales, CNRS/Rhône-Poulenc and Intas 99-1250 programs. O. Nercessian is supported by the Communauté Urbaine de Brest.

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