

# Continuous Enrichment Culture and Molecular Monitoring to Investigate the Microbial Diversity of Thermophiles Inhabiting Deep-Sea Hydrothermal Ecosystems

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**Abstract.** The microflora developing during a continuous enrichment culture from a hydrothermal chimney sample was investigated by molecular methods. The culture was performed in a gas-lift bioreactor under anaerobic conditions, at 90°C and pH 6.5, on a complex medium containing sulfur as the terminal electron acceptor. Archaeal and bacterial diversity was studied. Microorganisms affiliated with the genera *Pyrococcus*, *Marinitoga*, and *Bacillus* were detected through DGGE analysis of 16S rDNA. Additional sequences phylogenetically related to *Thermococcus* and  $\epsilon$ -*Proteobacteria* were detected by cloning and sequencing of 16S rDNA from two samples of the enrichment culture. In comparison, the sequences retrieved from cloning analysis from an enrichment culture performed in a flask (batch condition) using the same culture medium showed that only members of the genus *Thermococcus* were cultivated. Therefore, continuous enrichment culture using the gas-lift bioreactor can be considered as an efficient and improved method for investigating microbial communities originating from deep-sea hydrothermal vents.

## Introduction

Identifying the populations constitutive of microbial communities is still a major challenge. It was estimated that 90 to 99% of the organisms remained uncultivated [6], due to difficulties in reproducing their physiological niche in the laboratory. The results of molecular approaches dedicated to deep-sea hydrothermal systems were mainly obtained by 16S rDNA analysis. They showed the wealth of microbial diversity [21, 28] and the limitations of traditional cultivation techniques in improving our knowledge of the biodiversity inhabiting deep-sea hydrothermal vents. Nevertheless, *in fine*, cultivation remains necessary to describe new microbial representatives thriving in hydrothermal deep-sea vents [15, 23] and allows the study of *in situ* living microorganisms that express their physiological properties as members of the natural microflora. Since current culture

techniques do not always satisfy the need for providing a good balanced picture of the microflora composition, future developments in the study of bacterial diversity should include improvements in the culture methods to approach the physicochemical conditions of natural habitats [22]. Traditional culture attempts from hydrothermal samples were usually performed by enrichment cultures in flask. In this study, an enrichment culture was performed in a 2-liter gas-lift bioreactor. This equipment was previously developed to grow anaerobic hyperthermophilic microorganisms [24], to study the metabolism of members of the order *Thermococcales* [14], and to develop a minimal medium for growth of these hyperthermophiles [25]. Enrichment culture in the gas-lift bioreactor was expected to allow the growth of microorganisms (i) poorly represented in the ecosystem, (ii) exhibiting a long latency phase, or (iii) not previously cultivated. This could be induced by a continuous substrate supply, gas elimination by N<sub>2</sub> sparging, pH regulation, and a long incubation time (during several

weeks). It has already been reported that bacterial and archaeal population dynamics can be monitored in an anaerobic digester according to 16S rRNA, and 16S rDNA sequences, and changes in the composition of the microflora were highlighted [9, 17]. In this paper, we describe the use of a gas-lift bioreactor to perform an enrichment culture under controlled conditions. Among the molecular tools available, denaturing gradient gel electrophoresis (DGGE) and 16S rDNA cloning analysis of two culture samples were chosen to investigate microbial dynamics and diversity. In comparison, enrichment cultures in flasks were performed in the same culture medium and the microflora cultivated was investigated through cloning and sequencing analysis.

## Materials and Methods

**Samples.** During the ATOS cruise (European project VENTOX) located in the Mid-Atlantic Ridge (MAR), in the Rainbow field (36°13'N 33°54'W) at a 2275-m depth, an active black smoker was collected by the Remotely Operated Vehicle (ROV) *Victor* and brought to the surface in a decontaminated insulated box. Onboard, subsamples of the chimney were crushed into an anaerobic chamber and stored in sterile serum vials filled with sterile seawater containing 0.5 mg L<sup>-1</sup> Na<sub>2</sub>S. The subsamples were pooled to represent the whole chimney and used as an inoculum at 2%.

### Enrichment conditions in the bioreactor and culture monitoring

**Medium.** The growth medium was modified SME medium [29] in which yeast and peptone were replaced by 1 g L<sup>-1</sup> yeast extract, 0.5 g L<sup>-1</sup> casamino acids, 0.4 g L<sup>-1</sup> glucose, 0.4 g L<sup>-1</sup> dextrin (from corn), 0.2 g L<sup>-1</sup> D(+)-galactose, 0.2 g L<sup>-1</sup> dextran 0.1 g L<sup>-1</sup> glycogen, 0.2 g L<sup>-1</sup> pyruvate, 0.1 g L<sup>-1</sup> acetate, and 3 g L<sup>-1</sup> colloidal sulfur.

**Culture conditions.** The medium was sterilized by filtration (Sartoban; 0.22 μm) in a 20-L Nalgene bottle containing colloidal sulfur previously sterilized by heating twice at 100°C for 30 min on 2 successive days. The culture was performed at 90°C and pH 6.5. The temperature was regulated and the pH was controlled by the addition of either 1 N NaOH or 1 N HCl solutions. Moreover, the culture was sparged with N<sub>2</sub> (0.1 v·v<sup>-1</sup>·min<sup>-1</sup>) to maintain anaerobic conditions and to eliminate possible volatile fermentation end products that might inhibit the growth of fermentative microorganisms.

**Culture monitoring.** The culture was maintained during 50 days. Culture samples from the bioreactor were first collected every 3 h during the 34-h batch culture, and then daily during the continuous culture, representing a total of 51 samples. Cell concentration of each sample was determined by direct cell counting, using a Thoma chamber (0.02 mm deep) with an Olympus BH2 phase-contrast microscope (×400).

**Sample preservation.** For each sampling, eight 1.8-mL cryotubes of culture were frozen at -20°C in the presence of 5% DMSO. Cells were also recovered from 15 mL of culture by centrifugation (30 min at 8000 g), washed with 23 g L<sup>-1</sup> sterile NaCl, and the cell pellets were stored at -20°C.

**Enrichment conditions in flask and culture monitoring.** Enrichment cultures were performed in 100-mL serum vials as previously described [13], with the same medium, temperature, and pH as in the

bioreactor. Cell pellets for molecular analysis were recovered from 15 mL of culture in flask, after 45 and 65 h of incubation and, also, after a 20-h subculture from the first 45-h culture.

**DNA extraction.** Extractions were performed on frozen pellets following the extraction protocol of Alain *et al.* [2].

**16S rDNA PCR-DGGE analysis.** The variable v3 region of the 16S rRNA gene from extracted DNA was amplified using specific primers corresponding to both archaeal and bacterial domains. Archaeal 16S rRNA genes were amplified using primers 344F-GC and 915R [7]. Bacterial 16S rRNA genes were amplified using primers 341F-GC and 907R [19, 20]. The PCR procedure and analysis of the fragments by DGGE using a Bio-Rad Dcode apparatus were previously described by Muyzer *et al.* [19]. Samples were loaded onto 8% (w/v) polyacrylamide gels, in 1× TAE with a denaturing gradient ranging from 50 to 80% urea-formamide (UF) for archaeal DNA analysis and from 35 to 38% UF for bacterial DNA analysis (100% corresponding to 7 M urea and 40% [v/v] formamide). Electrophoresis was run for 16 h at 70 V and stained with ethidium bromide. DGGE bands were cut out with a sterile blade. The DNA of each fragment was eluted overnight at 4°C in 50 μl sterile and purified water. One microliter of the eluted DNA of each DGGE band was reamplified, using the same conditions as above. Running an aliquot of the PCR product, in DGGE gels as described above, checked the success of this operation. The PCR products, which yielded a single band comigrating with the original band, were then purified on a QIAquick silica-gel spin column (Qiagen) and sequenced.

**16S rDNA PCR-cloning analysis.** Archaeal and bacterial DNAs were amplified using universal primers and the PCR procedure described previously [31]. PCR products were then checked on a 0.8% (w/v) agarose gel and directly cloned using the TOPO TA Cloning kit (Invitrogen) according to the manufacturer's instructions. Clone libraries were constructed by transforming *E. Coli* TOP10F cells. An archaeal and a bacterial library were constructed for two culture samples collected after 7 days (T7) and 28 days (T28) of culture. Three archaeal libraries were constructed from the enrichment cultures in flask.

**16S rDNA sequencing and phylogenetic analysis.** DNA fragments obtained by DGGE were sequenced by Genome Express S.A. (Grenoble, France). Clones were cultivated overnight at 37°C on Deepwell microplates, in 2× LB medium. Plasmids were extracted using Plasmid Miniprep Kits (Qiagen) and partially sequenced. Sequences were compared to those available in databases, using the BLAST [5] network service to determine phylogenetic affiliations, aligned with the rDNA sequences from the RDP (Ribosomal Database Project) using the GCG CLUSTALW program [30], and refined manually using the SEAVIEW program [12]. Trees were constructed using the PHYLO\_WIN program, on the basis of evolutionary distance [27] and maximum likelihood methods [11]. The robustness of inferred topologies was tested by the bootstrap resampling of trees [10] calculated on the basis of the evolutionary distance, neighbor-joining algorithm with Jukes-Cantor correction [16], and maximum likelihood. If related sequences displayed more than 97% sequence similarity, only one of the sequences was retained for phylogenetic analysis. One representative of each unique phylotype was completely sequenced.

## Results

**Monitoring of the enrichment culture in the bioreactor.** The bioreactor was inoculated at 2% (v/v) with a chimney sample suspension. Very low cell

densities were measured during the first 34 h of the batch. Then a dilution rate of  $0.04 \text{ h}^{-1}$  ( $80 \text{ mL h}^{-1}$ ) was applied. The period of batch culture was fixed arbitrarily in order to avoid washing-out of the cells before they had become adapted to the growth conditions. Cell density stabilized at around  $5 \times 10^5 \text{ cells mL}^{-1}$  until 105 h of culture. By 124 h, the cell number had increased to  $1.8 \times 10^8 \text{ cells mL}^{-1}$ . This was associated with  $\text{H}_2\text{S}$  production (detected using Zn acetate strips). Among the observed morphologies, coccoid cells, single or in pairs, were dominant throughout the enrichment culture. Short rod-shaped cells appeared singly or in chains within an outer sheath-like structure, comparable to the 'toga' specific to *Thermotogales* [1]. From 350 h until the end of the culture, only single or paired coccoid cells were observed.

**DGGE monitoring of the culture in the bioreactor.** Total DNA was successfully extracted from samples between the 4th and the 50th days of culture. From the earlier samples, difficulties in DNA extraction were encountered, probably due to the very low cell density. The DGGE procedure was tested with 16S rDNA fragments from reference strains. Conditions were optimized to analyze separately archaeal and bacterial fragments. Fragments from the two *Pyrococcus* species, *P. abyssi* and *P. glycovorans*, could not be separated (data not shown). Archaeal 16S rDNA genes were amplified with the DGGE primers from 29 distinct culture samples covering all the culture duration. One band was obtained for each sample, at the same height in all cases. Twelve bands were excised, amplified by PCR, checked on a DGGE gel, and directly sequenced. A similarity of more than 97% was shared between those sequences on about 320 bp of the variable v3 region of the 16S rRNA gene. The type sequence Apa10 (Table 1) presented 100% identity on 417 bp with seven matching organisms from the BLAST analysis, all belonging to the *Pyrococcus* genera. As a result only sequences related to *Pyrococcus* could be detected throughout the culture. Using the same procedure, the v3 region of bacterial 16S rDNAs was amplified with specific DGGE primers (Table 1). Bacterial 16S rDNA genes were amplified successfully from nine distinct culture samples, from day 5 to day 35. Several dense bands were obtained at different heights. Among the 14 sequences obtained from excised bands, 5 of them represented by the sequence Apb7 (Table 1), matched by a BLAST analysis with sequences of the *Bacillus* genus. These sequences were retrieved from samples between day 5 and day 9. Nine sequences obtained from excised bands were closely related to the genus *Marinitoga* and were retrieved between day 7 and day 35. DGGE analysis revealed the phylogenetic

Table 1. Identification of bands obtained from DGGE after BLAST Analysis

DGGE Band	Number of bands associated <sup>d</sup>	Closest relative	% similarity
Apa10 <sup>b</sup>	12	<i>Pyrococcus abyssi</i> L19921	100
Apb7 <sup>c</sup>	5	<i>Bacillus</i> sp. USC14 AF346495	99
Apb9 <sup>d</sup>	9	<i>Marinitoga piezophila</i> AF326121	99

<sup>a</sup>More than 97% similarity between the sequences in the same group.

<sup>b</sup>417 bp used for BLAST analysis.

<sup>c</sup>553 bp used for BLAST analysis.

<sup>d</sup>555 bp used for BLAST analysis.

affiliation of the dominant cultivated strains and showed a larger diversity among *Bacteria* than *Archaea*.

**Cloning–sequencing analysis of the culture in the bioreactor.** In the two archaeal libraries corresponding to the two culture samples T7 and T28, all the sequences belonged to the order *Thermococcales* (Fig. 1). Within 59 clones partially sequenced from the earlier culture sample T7, two phylotypes were identified and were closely related to the genus *Pyrococcus*. The type sequences T7a-44 and T7a-60 shared 99% similarity with *Pyrococcus horikoshii* as well as with *Pyrococcus abyssi* and *Pyrococcus furiosus* based on about 1500 pb. However, the complete sequences of these two representative clones shared only 96% similarity, so that two distinct *Pyrococcus* species could have been cultivated. Within 49 clones partially sequenced from the second culture sample T28, 4% represented by the sequence type T28a-17 were related to the genus *Pyrococcus*, while 96% were closely related to the genus *Thermococcus*, represented by the sequence T28a-18 (Fig. 1). The nearest strain according to the BLAST analysis was "*Thermococcus sulfurophilus*" (AF394925). Among the T7 bacterial library, six phylotypes were defined: one within the  $\gamma$ -*Proteobacteria* (3% of the sequences), two within the  $\varepsilon$ -*Proteobacteria* (38% of the sequences), two within the *Bacillus/Clostridiales* group (36% of the sequences), and one within the *Thermotogales* order (23% of the sequences) (Fig. 2). Interestingly, all the  $\varepsilon$ -*Proteobacteria* sequences were affiliated with uncultured bacteria, all originated from deep-sea hydrothermal environments. Within the *Bacillus/Clostridiales* group and the *Thermotogales* order, the retrieved sequences were related (with 99% of identity) to *Caminicella sporogenes* and *Marinitoga piezophila*, respectively. Within the T28 bacterial library, 17% of

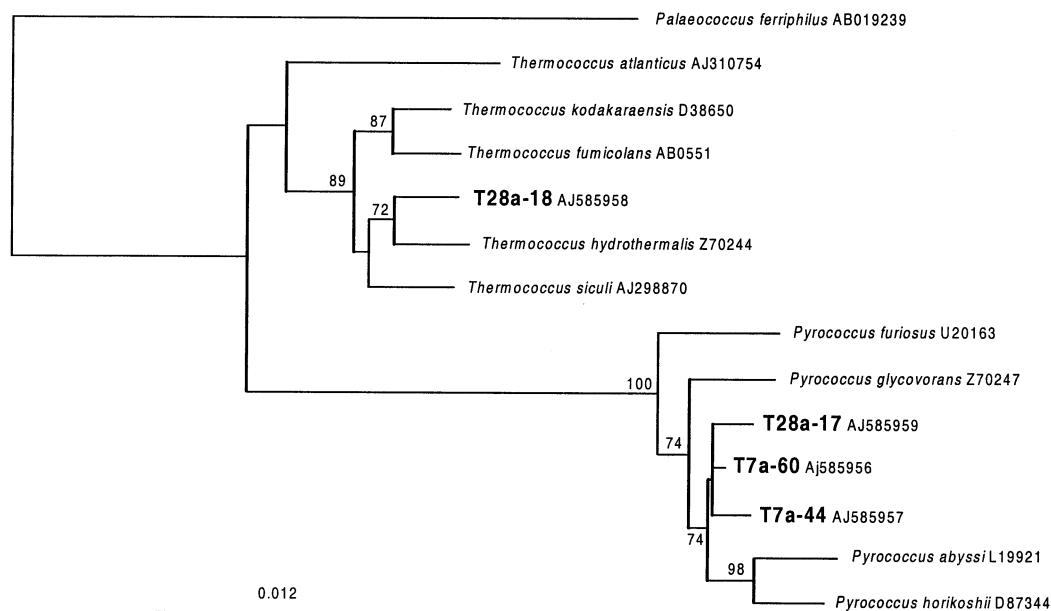


Fig. 1. Phylogenetic relationship of archaeal 16S rDNA sequences as determined by neighbor-joining analysis. *Palaeococcus ferriphilus* was used as the outgroup. The numbers at the nodes are the bootstrap values (as percentages). Bootstrap values above 70% are displayed. For the analysis, 1402 sites were used, with 500 bootstrap replicates. Scale bar indicates the expected number of changes per sequence position.

the sequences were affiliated with uncultured  $\varepsilon$ -*Proteobacteria*. Nine percent of the sequences were closely related to the genus *Caminiella*, and 61% to the genus *Marinitoga*. No sequences related to  $\gamma$ -*Proteobacteria* were retrieved in the T28 bacterial library and 13% of the sequences were assigned to deep-sea hydrothermal sequences of the *Aquificales* order, which were not retrieved in the earlier culture sample.

**Cloning–sequencing analysis of the culture in a flask.** The medium was inoculated at 2% (v/v) with the same suspension of chimney sample. Total DNA was successfully extracted from the enrichment cultures in a flask, after 45 and 65 h of incubation at 90°C, and from a 20-h subculture from the 45-h culture. Only the archaeal 16S rRNA gene could be amplified. Three archaeal clone libraries were constructed and 64 clones of each were sequenced. As a result, the sequences obtained were all affiliated with the *Thermococcus* genus. The nearest described species according to the BLAST analysis was *Thermococcus barophilus* (AY099172). No *Pyrococcus* or bacteria could be detected among the strains cultivated in batch.

## Discussion

Compared to conventional enrichment culture in batch, a novel approach was used to investigate the microbial

diversity of a hydrothermal vent chimney sample. The use of the gas-lift bioreactor technology to perform a continuous culture provides a controlled laboratory environment for the enrichment and cultivation of different microbial groups under hyperthermophilic conditions (90°C). pH and temperature were continuously monitored and adjusted. In addition, the culture was continuously sparged with N<sub>2</sub>, thus homogenizing the medium and also eliminating possible toxic products from fermentation processes. Since the medium was continually renewed, the enrichment culture could be maintained for a long time, during which the analysis of the microbial population was performed. The dynamics in the cultivated microflora could be highlighted all through the enrichment culture in the bioreactor, as it was already observed in anaerobic digestors [8, 17]. Actually, the evolution of the cell morphologies including rods and cocci could be observed, and modifications of the microbial diversity were ascertained through 16S rDNA sequence analysis.

The diversity of microbial population was first assessed using the DGGE technique. Here we demonstrate the efficiency of the DGGE technique for studying thermophilic *Archaea* and *Bacteria* diversity and monitoring the population dynamics. Using this technique, we demonstrated that 16S rDNA sequences, retrieved from the continuous culture, were closely related to members of the genera *Bacillus*, *Marinitoga*, and *Pyrococcus*. Cloning analysis of the microbial population

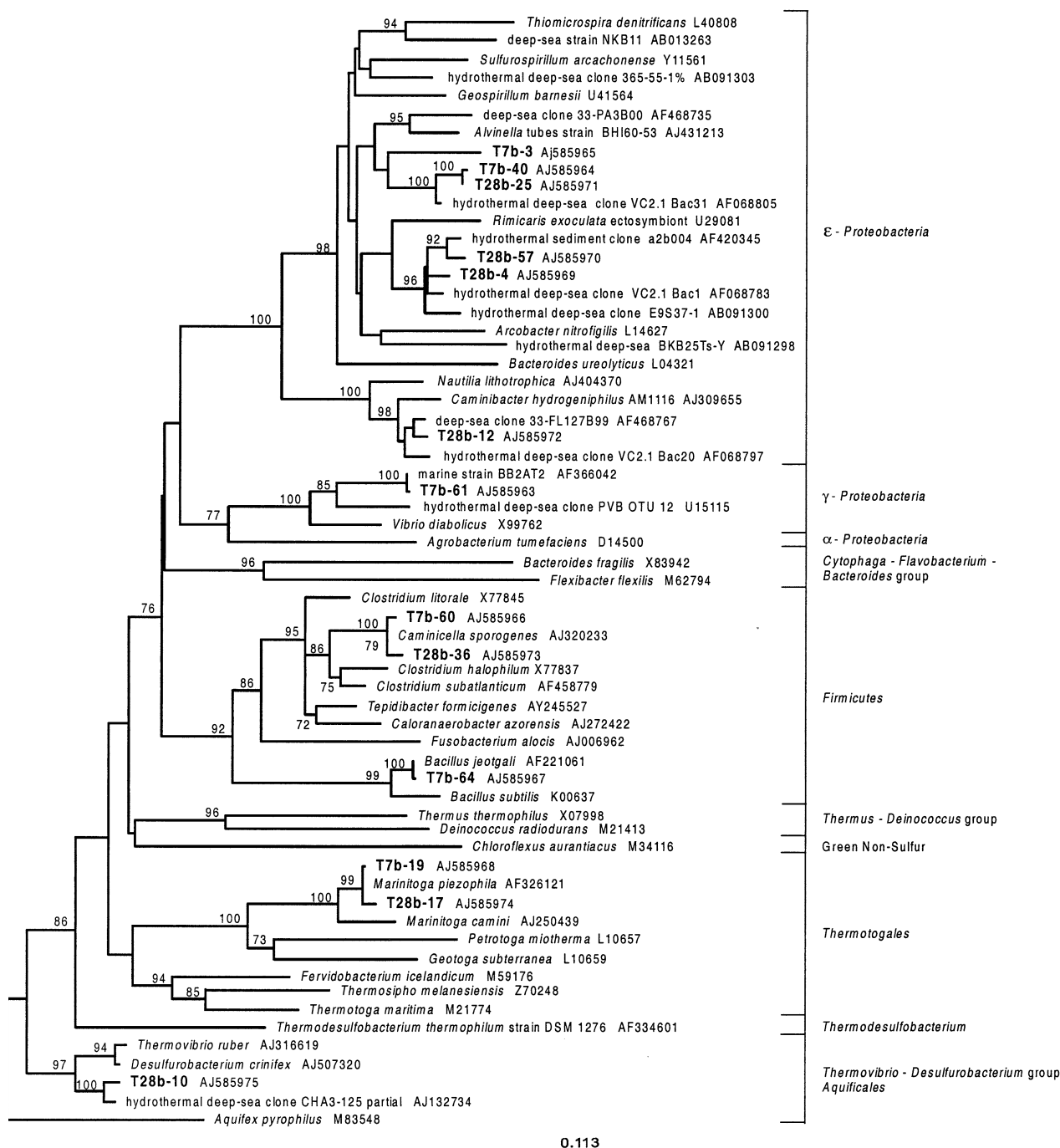


Fig. 2. Phylogenetic relationship of bacterial 16S rDNA sequences as determined by neighbor-joining analysis. The outgroup used was *Aquifex pyrophilus*. The numbers at the nodes are the bootstrap values (as percentages). Bootstrap values above 70% are displayed. For the analysis, 404 sites were used, with 500 bootstrap replicates. Scale bar indicates the expected number of changes per sequence position.

was performed on two culture samples at day 7 (T7) and at day 28 (T28). Similarly to the DGGE technique, *Pyrococcus*, *Caminicella*, and *Marinitoga* related sequences were detected at day 7, whereas *Pyrococcus* and

*Marinitoga* were detected at day 28. Cloning analysis revealed a larger biodiversity than DGGE, since members of *Thermococcus*,  $\gamma$ - and  $\epsilon$ -*Proteobacteria*, and *Aquificales* were only detected by cloning. Therefore

our results confirm the need to combine several molecular methods to avoid the bias inherent in each and to obtain a better knowledge of the microbial diversity.

All the archaeal sequences, detected by DGGE and by cloning, were phylogenetically related to the order *Thermococcales* and, more specifically, to the genera *Pyrococcus* and *Thermococcus*, known as heterotrophic and hyperthermophilic microorganisms. Interestingly, 16S rDNA sequences analysis showed that the *Thermococcus* strain enriched in a flask was different from the one detected in the bioreactor. Actually, the closest relative of retrieved *Thermococcus* 16S rDNA sequences from cultures in flasks was *Thermococcus barophilus* [18], whereas sequences retrieved from the bioreactor were close to "*Thermococcus sulfurophilus*," a sulfur-reducing archaeon also originating from the Rainbow site. These results emphasize the interest of using a continuous bioreactor to enrich cultures since providing evidence of a different diversity of *Thermococcus* strains compared to that obtained under batch conditions. Besides their detection in the continuous bioreactor by molecular techniques, the two novel archaeal strains belonging to the genera *Pyrococcus* and *Thermococcus* have also been isolated by the dilution-to-extinction technique [1].

Experiments conducted to look for the occurrence of microorganisms of the domain *Bacteria* indicated that no bacterial sequences could be obtained from the batch cultures in flasks. In contrast, studies within the continuous bioreactor revealed a large bacterial diversity through the cloning–sequencing analysis of 16S rDNA (Fig. 2). This again confirms the interest in using a different methodological approach to investigate the biodiversity of microorganisms inhabiting deep-sea hydrothermal vents. Among the 16S rDNA sequences detected in this study, close relatives of *Caminiella sporogenes* [3] and *Marinitoga piezophila* [1] were found. These species are known to grow at temperatures much lower than 90°C (maximal growth temperature of 65 and 75°C, respectively). The presence of these bacteria at 90°C may indicate (i) the existence of unknown species belonging to both genera and thriving under these conditions, (ii) the possible growth of close representatives of previously isolated *Caminiella* and *Marinitoga* spp. under hyperthermophilic conditions, due to thermoprotective molecules released by the other members of the enrichment culture, or (iii) unknown interactions between bacteria and archaeons. Many sequences retrieved during this study were affiliated with uncultivated microorganisms belonging to the *ε-Proteobacteria*. They were, in particular, closely related to the hydrothermal sequences VC2.1 Bac31 and VC2.1 Bac1, from the Snake Pit deep-sea vent site, on the Mid-

Atlantic Ridge [26]. In all the deep-sea vent microbial communities studied to date, *ε-Proteobacteria* phylogenotypes were shown to be dominant, accounting for 40–98% of the bacterial clone libraries. The *ε-Proteobacteria* detected in our enrichment culture at 90°C were adapted at higher temperatures than those described for all *ε-Proteobacteria* characterized so far, which do not grow over 70°C in pure culture [4]. These results confirm the importance of pursuing efforts for isolating new populations of hyperthermophiles from their natural environments. With regard to numerous reports, it is now more and more obvious that the combination of both a molecular approach and cultivation is the best way found so far to assess the microbial diversity of hydrothermal vent chimney. Molecular studies suffer from the drawback that metabolic properties can only rarely be inferred from the 16S rRNA sequence data alone. Likewise, limitations of molecular tools must be kept in mind [2]. In this respect, the use of both cultivation and molecular methods may help to answer these questions.

The gas-lift bioreactor technology therefore appears to be an improved method for performing enrichment cultures. During the enrichment culture in the continuous bioreactor, different microorganisms were cultivated and a much larger diversity was exhibited compared to cultures performed in flasks (batch conditions). Even new microorganisms inhabiting the deep-sea hydrothermal vent could be evidenced, and isolation of two strains by subcultures in flasks has already been successful. The use of the bioreactor for further enrichment experiments should contribute to better simulating hydrothermal physiological niches in the laboratory and to reproducing environmental perturbations *in vitro* (temperature, pH, O<sub>2</sub>, nature and concentration of the substrates etc.). This should improve our understanding of the key ecological roles played by microorganisms in the hydrothermal ecosystems.

**Nucleotide sequence accession numbers.** The EMBL accession numbers of the sequences used in this study are AJ585953 to AJ585977.

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