Extremophiles (2009) 13:595–608
DOI 10.1007/s00792-009-0242-6

ORIGINAL PAPER

# Effect of variation of environmental conditions on the microbial communities of deep-sea vent chimneys, cultured in a bioreactor

**Nathalie Byrne · Françoise Lesongeur · Nadège Bienvenu · Claire Geslin · Karine Alain · Daniel Prieur · Anne Godfroy**

Received: 30 October 2008 / Accepted: 30 March 2009 / Published online: 19 April 2009
© Springer 2009

**Abstract** Both cultivation and molecular techniques were used to investigate the microbial diversity and dynamic of a deep-sea vent chimney. The enrichment cultures performed in a gas-lift bioreactor were inoculated with a black smoker chimney sample collected on TAG site on the mid-Atlantic ridge. To mimic as close as possible environmental conditions, the cultures were performed in oligotrophic medium with nitrogen, hydrogen and carbon dioxide ($N_2/H_2/CO_2$) gas sweeping. Also, the temperature was first settled at a temperature of 85°C and colloidal sulphur was added. Then, the temperature was lowered to 60°C and sulphur was omitted. Archaeal and bacterial diversity was studied in both culture and natural samples. Through 16S rRNA gene sequences analysis of the enrichment cultures microorganisms affiliated to Archeoglobales, Thermococcales were detected in both conditions while, Deferribacterales and Thermales were detected only at 65°C in the absence of sulphur. Single-stranded conformational polymorphism and quantitative PCR permit to study the microbial community dynamic during the two enrichment cultures. The effect of environmental changes (modification of culture conditions), i.e. temperature, medium composition, electron donors and acceptors availability were shown to affect the microbial community in culture, as this would happen in their environment. The effect of environmental changes, i.e. temperature and medium composition was shown to affect the microbial community in culture, as this could happen in their environment. The modification of culture conditions, such as temperature, organic matter concentration, electron donors and acceptors availability allowed to enrich different population of prokaryotes inhabiting hydrothermal chimneys.

**Keywords** Deep-sea hydrothermal vent · Microbial diversity · Continuous enrichment culture · Bioreactor · Environmental condition · Oligotrophic condition · 16S rRNA genes

Communicated by A. Driessen.

N. Byrne (✉) · F. Lesongeur · N. Bienvenu · C. Geslin · K. Alain · D. Prieur · A. Godfroy
Laboratoire de Microbiologie des Environnements Extrêmes, UMR6197 IFREMER, Centre de Brest, BP70/IUEM-Place Nicolas Copernic, 29280 Plouzané, France
e-mail: nathalie.byrne@gmail.com

## Introduction

Hydrothermal chimneys are porous mineral structures that precipitate as a result of mixing of the high temperature anoxic fluids with the cold oxygenated sea water. In hydrothermal chimneys, environmental chemical and physical conditions can change quickly on both time and spatial scale. They, hence, represent diverse habitats for a wide range of physiologically and phylogenetically diverse microbial communities. Numerous cultural approaches led to the isolation and description of a great diversity of novel species belonging to the domains archaea and bacteria, the metabolic properties of which are likely to cover all biogeochemical cycles (Miroshnichenko and Bonch-Osmolovskaya 2006; Takai et al. 2008). Otherwise, molecular diversity studies provided an overview of hydrothermal chimneys microbial communities. They revealed the presence of a large and in many cases unexpected microbial diversity (Nercessian et al. 2005; Takai and Horikoshi 1999). Cultural approaches represent a promising way to address the questions of the ecosystem functioning and to link a metabolism to a phylogenetic group. Besides, they remain necessary to describe new microbial representatives thriving in

hydrothermal deep-sea vents. They allow the study of in situ living prokaryotes that express their physiological properties as members of the natural microflora. Amongst the diverse cultural techniques developed during the past decade, continuous cultures in bioreactors have proven their efficiency in the cultivation and physiological study of hyperthermophilic prokaryotes from deep-sea vent (Godfroy et al. 2000; Postec et al. 2005a, b; Raven et al. 1992). The use of bioreactors that might allow to better simulate environmental conditions appears to have a great potential to assess how modification of these conditions may affect the structure of microbial communities in hydrothermal chimneys. As a kind of example, Houghton et al. (2007) used a special device to enrich nitrate-reducing or sulphate-reducing thermophiles under high pressure and at high temperature. They used their equipment with the success to study the effect of fluid chemistry on microbial communities.

In our laboratory, a gas-lift bioreactor allowing the long-term cultivation of prokaryotes by enabling a continuous substrate supply and an elimination of volatile metabolic end products (by gas sparging), was used to perform enrichment cultures with deep-sea vent chimney samples. Previous continuous cultures performed with this equipment in parallel to batch cultures demonstrated the efficiency of this system over classical batch cultures by enabling the cultivation of a larger fraction of the natural community (Postec et al. 2005a, b).

In previous experiments with this bioreactor, enrichment cultures from hydrothermal chimney samples were performed under anaerobic conditions, at 90 and 60°C, pH 6.5 and using a complex and rich medium containing carbohydrates, peptides, sulphate and sulphur. Archaeal and bacterial diversity was studied using molecular tools (i.e. DGGE and cloning/sequencing of 16S rRNA genes). Not only these studies showed the efficiency of such system to obtain greater diversity in culture, but also led to the isolation of new species and enabled to hypothesise metabolic interactions between species (Postec et al. 2005a, b, 2007).

Today, one promising way to grow, as-yet, uncultivated microorganisms is to permit inter-species interactions and to examine microbial metabolisms inside communities rather than to extrapolate them from pure cultures (Tor et al. 2003). The bioreactor represents a powerful tool to investigate in vitro interactions between populations that may occur in situ. In addition, the gas-lift bioreactor permits the maintaining of community culture for a long period. Thus, it represents a suitable device to investigate in vitro the effect of physicochemical perturbations on the microbial community structure from deep-sea hydrothermal vents. This system is particularly suitable for the study of the communities inhabiting such a disturbed system.

So far, chemoorganotrophic enrichment cultures with hydrothermal chimney samples were mainly performed with media containing high concentration of organic matter. Few data are available about the organic matter concentration retrieved in deep-sea vent chimneys, but it can be assumed that it might vary from high concentration to oligotrophic conditions.

In the present study, we used the gas-lift bioreactor to study the microbial diversity and dynamics of thermophilic communities from a deep-sea hydrothermal vent. A black smoker chimney sample was used to inoculate the gas-lift bioreactor using the medium described in Postec et al. (2005a, b) with a 100-fold diluted organic matter concentration, the enrichment was performed (1) in the presence of hydrogen and carbon dioxide (nearer to the environmental conditions) at 85°C with colloidal sulphur, then (2) after reinoculation, temperature was decreased to 60°C and the sulphur was removed from the medium in order to investigate the effect of a temperature shift and a change of the chemical environment on the microbial community structure. This change mimicked an environmental change such as those encountered commonly at deep-sea vents. The microbial community structure and composition within the enrichment culture was analysed using the molecular techniques based on 16S rRNA genes including cloning/sequencing, single-stranded conformation polymorphism, and quantitative PCR, in order to asses both the diversity and dynamics.

## Materials and methods

### Samples

In 2005, an active chimney sample was recovered from the hydrothermal site "TAG" (26°08′N, 44°49′W) located on the mid-Atlantic ridge (MAR) during the EXOMAR cruise. This sample (EXO15E1) was collected with the remotely operated vehicle (ROV) Victor 6000, and brought to the surface into an insulated box under aseptic conditions (Fig. 1). Temperature and pH measured in situ were, respectively, 345°C and 3.31. On board, chimney fragments were crushed into an anaerobic chamber under $N_2/H_2/CO_2$, 90:5:5 gas atmosphere (La Calhene, France) and was immediately transferred into a flask, filled with sterile seawater and preserved under anaerobic conditions ($N_2/H_2/CO_2$, 90:5:5 gas phase) at 4°C. This suspension was used to inoculate the enrichment cultures. An aliquot was preserved at −80°C for DNA extraction.

### DNA extraction and ribosomal 16S rRNA gene amplification

DNA extractions from both culture samples and chimneys were performed using the FastDNA® SPIN Kit for Soil

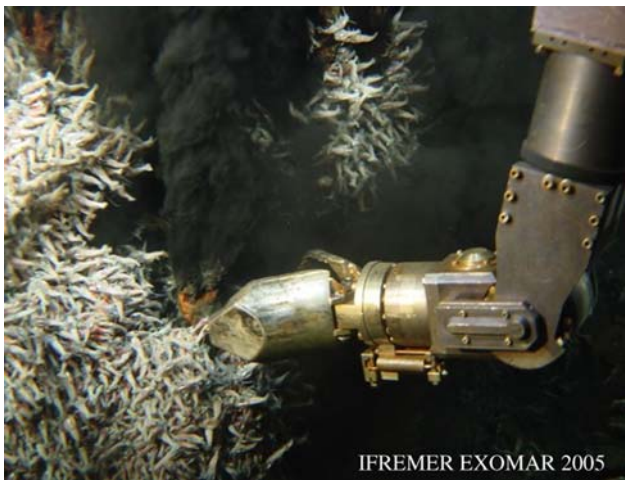


**Fig. 1** The black smoker EXO15E1 recovered by the arm "Maestro" of the Remotely Operated Vehicle (ROV) Victor 6000. Presence of shrimps swarm around the chimney

(MPBiomedical) (Webster et al. 2003). For the chimney sample, four parallel extractions were performed and the DNA was then pooled and concentrated. The 16S rRNA genes from the extracted DNA were then amplified using specific primers corresponding to both archaeal and bacterial domains; archaeal 16S rRNA genes were amplified using 344F and 915R or 4F and 1492R. Bacterial 16S rRNA genes were amplified using 8F and 1492R (Muyzer and Smalla 1998; Muyzer et al. 1995). PCR products were subsequently cloned with the pGEM®-T Vector cloning kit (Promega) according to the manufacturer instructions.

## Continuous enrichment culture in a gas-lift bioreactor

### *Medium*

The growth medium was the modified SME medium modified by Postec et al. (2005a, b, 2007) in which organic matter concentrations were as follows: 0.01 g $L^{-1}$ yeast extract (Difco), 0.005 g $L^{-1}$ casaminoacids (Difco), 0.004 g $L^{-1}$ glucose, 0.004 g $L^{-1}$ dextrin (from corn), 0.002 g $L^{-1}$ galactose, 0.002 g $L^{-1}$ dextran, 0.001 g $L^{-1}$ glycogen, 0.002 g $L^{-1}$ pyruvate and 0.001 g $L^{-1}$ acetate (all purchased from Sigma) corresponding to a total organic substrates concentration of 0.031 g $L^{-1}$. The medium was supplemented with 2 g $L^{-1}$ of colloidal sulphur during the first part of the enrichment culture (F1) while no sulphur was added for the second part (F2).

The medium was sterilised by filtration (Sartroban, 0.22 μm) into a sterile 20 L Nalgene bottle. When sulphur was added to the medium, the colloidal sulphur solution was sterilised directly in the Nalgene bottle by heating twice at 100°C for 30 min on two successive days.

### *Culture conditions*

The culture was performed in a 2 L glass gas-lift bioreactor (Raven et al. 1992), at 85 and 60°C (respectively for F1 and F2) and the pH was regulated at 6.5. Temperature and pH were controlled by a 4–20 mA Controller and AFS Bio Command system from New Brunswick (Nijmegen, the Netherlands) connected to a pH transmitter (Broadley James) associated with a gel pH electrode (Mettler Toledo) and a PTFE-covered PT100 temperature probe associated with a temperature controlled heated circulated bath (Huber) filled with water. Acid (1 N HCl) and base (1 N NaOH) were added using peristaltic pumps (Masterflex). The bioreactor was inoculated with 40 mL of the chimney sample (EXO15E1) suspension (2%). Fresh medium feeding and product draw-off were performed using peristaltic pumps (Masterflex). The culture was continuously flushed with $N_2/H_2/CO_2$ (75/20/5) (0.2 v v $min^{-1}$) to maintain anaerobic conditions. The bioreactor was maintained as a batch culture for the first 24 h to prevent washout of the cells before they growth. After 24 h, fresh medium was provided by applying a dilution rate of 0.04 $h^{-1}$. The first part of the enrichment culture (F1) was performed in the presence of elemental sulphur at 85°C. After 26 days (624 h), the sample was reinoculated, the sulphur containing medium was replaced by a medium without sulphur and the temperature was lowered to 60°C. After a batch period of 30 h, a dilution rate of 0.04 $h^{-1}$ was applied and this second culture (F2) was maintained for 22 days (528 h).

### *Culture monitoring and sample preservation*

Cell concentration was determined every day by direct cell counting, using a Thoma chamber (0.02 mm depth) viewed with an Olympus BX60 phase-contrast microscope (×400×). Culture samples from the bioreactor were collected every 48 h, from T4 (day 4) to T20 (day 20) for F1 and from T5 (day 5) to T14 (day 14) for F2. For each sampling, two 15 mL aliquot of culture were preserved anaerobically [under $N_2/H_2/CO_2$ (75/20/5)] at 4°C in serum vial. For DNA extraction, cells were recovered from 15 mL of culture by centrifugation (20 min at 8,000*g*). Cell pellets were then re-suspended in 5 mL of lysis buffer TE–Na 1·(Tris–HCl pH 8, 100 mM; NaCl 100 mM, EDTA pH 8, 50 mM), and stored at −80°C until DNA extraction.

## Amplification of the 16S rRNA gene and SSCP (single-stranded conformational polymorphism) analysis

Amplification of the V3 region of SSU rRNA genes from culture DNA samples was performed with primers W49F

(5′-ACGGTCCAGACTCCTACGGG-3′) for bacterial domain, W116F (5′-TET-TTACCGCGGCTGCTGGCAC-3′) for archaeal domain, and W104R (5′-6FAM-TTA CCGCGGCTGCTGGCAC-3′) as the universal reverse primers. An initial denaturation step at 94°C for 2 min, was followed by 25 cycles of a three-stage program with 30 min at 94°C, 30 min at 51°C for archaea and 61°C for bacteria, and 30 min at 72°C, and a final elongation for 10 min at 72°C. DNA polymerase was *pfu* Turbo (Stratagene) that generated blunt-ended PCR fragments. For electrophoresis, PCR SSCP products were diluted ten times in water before mixing with 18.8 µL formamide Hi-Di (Applied Biosystems, Courtaboeuf, France) and 0.2 µL internal standard internal DNA molecular weight marker Genescan-400 HD ROX (Applied Biosystems, Courtaboeuf, France) (Delbes et al. 2000). In all, 1 µL of template was added to the mixture. The sample mixture was denatured at 95°C for 5 min and immediately cooled on ice before loading on the instrument. SSCP analyses were performed on an automatic sequencer abi310 (Applied Biosystem). The non-denaturing polymer consisted of 5% CAP polymer, 10% glycerol and 3100 buffer (Applied Biosystems, Courtaboeuf, France). The migration time was set at 30 min at 32°C and 12 kV. The results obtained were analysed using SAFUM 2.0 Statistical Analysis of Fingerprints Using Matlab (Loisel et al. 2006; Zemb et al. 2007). To identify SSCP peaks of interest, 16S rRNA gene clone from the clone library were amplified by SSCP–PCR and analysed by SSCP under the same conditions and the resulting peaks were compared with those of the communities' pattern.

## 16S rRNA gene sequencing and phylogenetic analysis

DNA fragments obtained were sequenced at OUEST-Genopole (Roscoff, France). Sequences were compared with those available in databases using the BLAST network service to determine phylogenetic affiliations. Sequences were aligned using the ClustalW program with the BioEdit software version *7.0.5* (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Trees were constructed using the PHYLO_WIN program on the basis of evolutionary distance and maximum likelihood methods (Galtier et al. 1996). The robustness of inferred topologies was tested by the bootstrap resampling of trees calculated on the basis of the evolutionary distance, neighbour-joining algorithm with Jukes–Cantor correction.

## Quantification of archaeal and bacterial community by quantitative PCR

All quantitative-PCR experiments were run using an Applied Biosystems 7300 Real-Time PCR thermocycler equipped with 7300 Real-Time PCR Software. Quantitative-PCR conditions were as follow: 12.5 µL Absolute blue Quantitative-PCR Rox mix (AbGene), 400 nM final concentration of each primers, 200 nM final concentration for bacterial and archaeal TaqMan probes, 1 µL template and water added to a final volume of 25 µL. All reactions were done in 96 well quantitative-PCR plates.

Archaeal 16S rRNA genes were detected using the primer set Arch349F (5′-GYGCASCAGKCGMGAAW-3′) and Arch806R (5′-GGACTACVSGGGTATCTAAT-3′). The TaqMan probe was Arch516F (5′-TGYCAGCCGC CGCGGTAAHACCVGC-3′) (Takai and Horikoshi 2000). *Thermococcus litoralis* DNA was used as the quantification standard in 10-fold dilutions ranging from $10^{0}$ to $10^{-7}$. Thermocycling was performed as follows: 50°C for 2 min, initial denaturation 95°C for 15 min; amplification 40 cycles, denaturation 95°C for 25 s, primer annealing 60°C for 6 min.

Bacterial 16S rRNA genes were detected using the primer set 331F (5′-TCCTACGGGAGGCAGCAGT-3′) and 797R (5′-GGACTACCAGGGTATCTAATCCTGTT-3′). The TaqMan probe was 6-FAM-5′-CGTATTACCGCGG CTGCTGGCAC-3′-TAMRA (Nadkarni et al. 2002).

Plasmid harbouring *Deferribacter* sp. DNA was used as the quantification standard in 10-fold dilutions ranging from $10^{0}$ to $10^{-7}$. Thermocycling was performed as follows: 50°C for 2 min, initial denaturation 95°C for 15 min; amplification 40 cycles, denature 95°C for 15 s, primer annealing 60°C for 1 min.

The TaqMan probes had a fluorescent reporter dye (6-carboxyfluorescein) covalently attached to the 5′-end and a fluorescent quencher dye (6-carboxytetramethylrhodamine) attached six or more bases downstream from the reporter dye.

## Nucleotide sequence accession numbers

The EMBL accession numbers of the sequences used in this study are FM242714 to FM242737.

## Virus detection

Three samples from the enrichment culture F1 (F1T14, F1T18 and F1T20) were used for virus-like particles observation. All solutions (i.e. sodium pyrophosphate, ultrapure water, etc.) were filter-sterilised on 25 mm anodisc syringe-filter membranes with pore sizes of 0.02 µm. An aliquot (0.5 mL) of culture or MilliQ water (negative control) were taken and added to 4 mL of MilliQ water and 1 mL of sodium pyrophosphate (10 mM final concentration) and incubated for 15°C at room temperature. Following incubation, samples were centrifuged at 10000*g* for 20 min. The supernatant was then filtered through a 0.8/0.2 µm filter (acrodisc 25 mm PF syringe-filter 0.8/0.2 µm support membrane). In order to eliminate uncertainties in

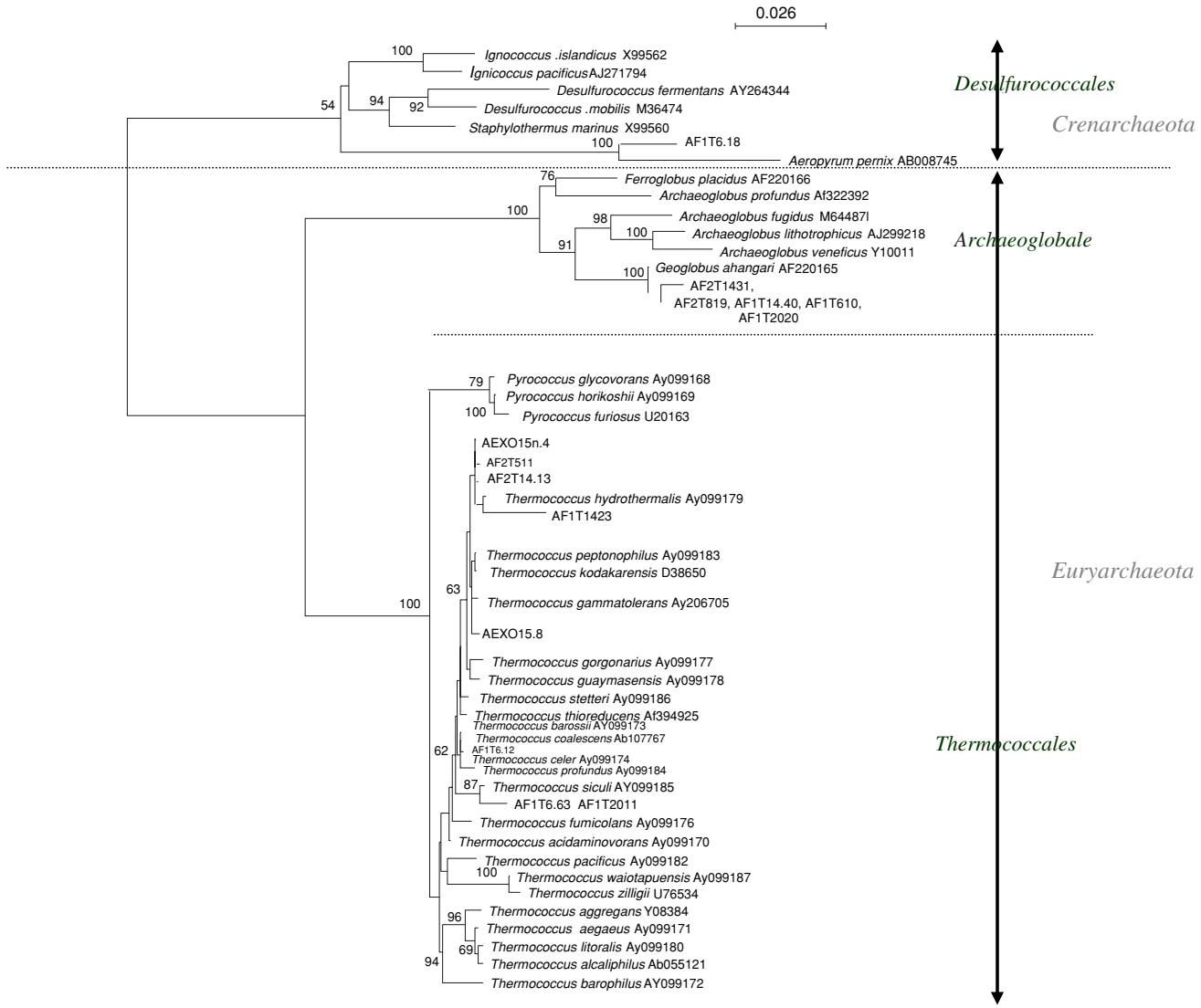


**Fig. 2** Phylogenetic analysis of archaeal 16S rRNA gene sequences obtained in the diverse study AEXO15 (chimney sample), F1 and F2 (enrichment sample) and determined by neighbour-joining analysis. The numbers at the nodes are the bootstrap values (as percentage). Bootstraps above 50% are displayed. For the analysis 824 sites were used with 500 bootstrap replicates. *Scale bar* indicates the expected number of changes per sequence position

virus counting due to extracellular interference, 2 µL of DNase (Turbo DNAse Ambion 2 U/µL) was added to each sample and incubated for 10 min at 37°C. The supernatant was finally filtered through a 25 mm anodisc membrane with pore sizes of 0.02 µm and stained with 100 µL of SYBR Green I or SYBR Gold (10×) for 15 min in the dark, 100 µL of an anti-fading solution were also added on the filter.

Samples (F1T14, F1T18 and negative control) were observed immediately after preparation. The observation of virus-like particles in each sample was made from duplicate filters and from at least 10 randomly selected fields per filter, using a LEICA QWin Pro Version 2.3 (Leica Microsystems, Wetzlar, Germany) microscope.

## Results

### Archaeal and bacterial diversity in the chimney sample

Archaeal diversity in the chimney sample was low since only sequences related to the genus *Thermococcus* were retrieved (Fig. 2). Within this genus, two sequences were detected: the first one referenced as AEXO15.8 (71 clones) was affiliated to *Thermoccoccus gammatolerans* (isolated from the Guaymas Basin) and the second one referenced as AEXO15n.4 (24 clones) was closely related to *Thermococcus hydrothermalis* (isolated from the East Pacific Rise).

Concerning 16S rRNA gene bacterial diversity, the majority of the sequences were affiliated to the *Alpha, Delta*

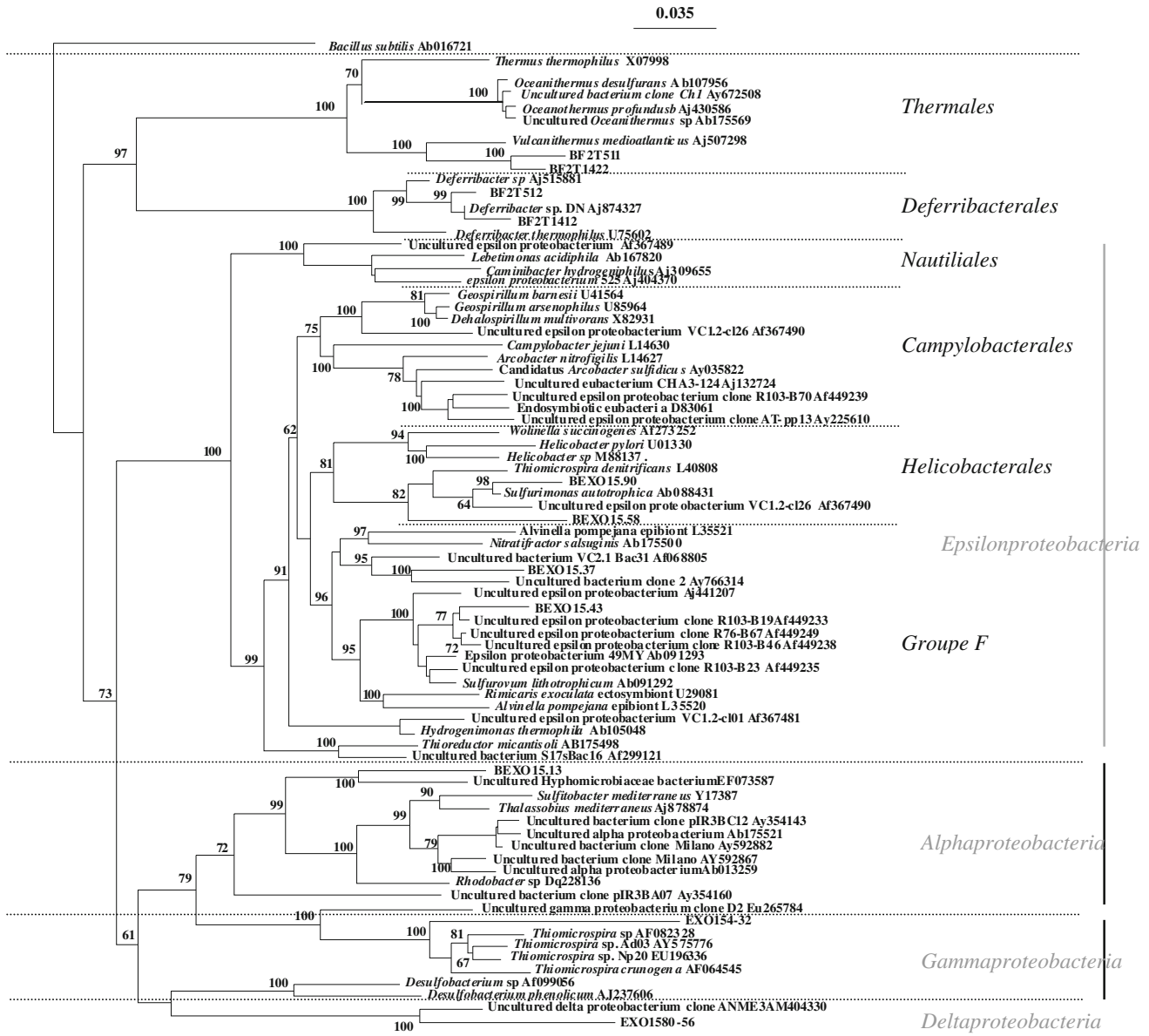


**Fig. 3** Phylogenetic analysis of bacterial 16S rRNA gene sequences obtained in the diverse study: BEXO15 (chimney sample), F1 and F2 (enrichment sample) and determined by neighbour-joining analysis. The *numbers* at the nodes are the bootstrap values (as percentage). Bootstraps above 50% are displayed. For the analysis 991 sites were used with 500 bootstrap replicates. *Scale bar* indicates the expected number of changes per sequence position

and *Epsilonproteobacteria* (Fig. 3). Within the *Epsilonproteobacteria*, numerous sequences were related to environmental sequences. Clones BEXO15.90 and BEXO15.58 (respectively 22 and 2 clones) were affiliated to *Sulfurimonas autotrophica*, a strain which was isolated from deep-sea sediments at the Hatoma Knoll in the Mid-Okinawa Trough hydrothermal field. BEXO15.43 (5 clones) (Lopez-Garcia et al. 2002) was affiliated to an uncultured bacterium clone within the group of *Sulfurovum* species (Inagaki et al. 2004). Other sequences BEXO15.37 (38 clones) within the *Epsilonproteobacteria* were close to clone 2 detected in a microbial diversity study of an in situ growth chamber at deep-sea hydrothermal vents. In the *Alphaproteobacteria* group, sequence BEXO15.13 (1 clone) was affiliated to *Sulfitobacter* and *Thalassobius* species (Pukall et al. 1999). Finally, in the *Deltaproteobacteria* group, one sequence BEXO1580.56 was related to a Mud Volcano clone (AM404330) from the Haakon Mosby Mud Volcano (Barents Sea) within the group of *Desulfobacteriales* (Losekann et al. 2007).

## Culture enrichment at 85°C with colloidal sulphur

A continuous enrichment culture was performed in a gas-lift bioreactor to study the culture fraction of the microbial

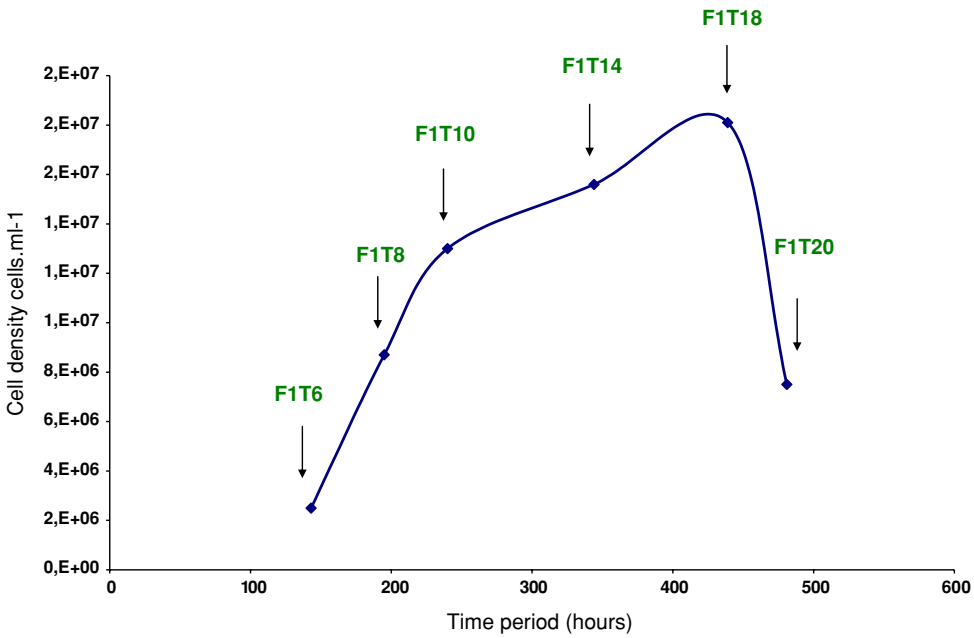


**Fig. 4** Cell densities of culture samples collected from T6 to T20 from the bioreactor. Cell densities are expressed in cell $mL^{-1}$. The time period is expressed in hours, F1T6, F1T8, F1T10, F1T14, F1T18 and F1T20 correspond to the sampling date. F1 is for "first fermentation", T6, T8, T10, T14, T18, T20 correspond to 6, 8, 10, 14, 18, and 20 days after the beginning of the culture

diversity of a chimney from the TAG MAR hydrothermal site using oligotrophic conditions and high temperature. Analyses of the compositional changes in the bacterial and archaeal communities in response to a shift in two environmental factors were also performed.

The first part of the enrichment culture in the bioreactor (F1) was performed at 85°C in the presence of colloidal sulphur. Starting from $3.1 \times 10^6$ cell $mL^{-1}$ at T4, cell density reached $1.8 \times 10^7$ cell $mL^{-1}$ at T18 and decrease at T20. Coccoid cells single or in pairs were dominant (Fig. 4).

## Quantitative PCR and 16S rRNA gene diversity results

According to quantitative-PCR results, archaea were dominant and this was confirmed by the absence of amplification using bacterial primers. 16S rRNA gene diversity analysis of enrichment culture samples showed the presence of numerous archaeal sequences related to the genus *Geoglobus* and *Thermococccus* (Table 1). *Thermococcus* retrieved sequences were affiliated to *Thermococcus coalescens* and *Thermococcus siculi* at the beginning of the culture (T6), while all retrieved sequences at T14 and T20 were affiliated to *Thermococcus hydrothermalis* and *Thermococcus siculi*, respectively. At the beginning of the enrichment, one sequence related to the genus *Aeropyrum* was present. Sequences related to *Geoglobus* were detected all along the culture.

## SSCP

In order to study the archaeal community structure dynamic by SSCP, control, i.e. clones sequenced previously and affiliated to *Thermococcales* sp. and *Geoglobus* sp. with, respectively, 98 and 97% of similarity were used. SSCP is a semi-quantitative method, and according to the results obtained, an increase in the *Thermococcales* population could be witnessed starting from T8 until T18. This can be correlated as well with the increase noted in cell number. At T20, the "*Thermococcales* peak" was smaller; this was in compliance with the diminution of the total cell density. *Geoglobus* sp. remained present during throughout the culture even when the global cell number decreased in the culture.

## Virus-like particle

The recent evidence of astonishing virus populations associated with hydrothermal species of *Thermococcales* (Geslin et al. 2003) led us to search for the presence of viruses in the culture to try to understand the decrease in cell number at T20. Using epifluorescence microscopy, virus-like particles were detected in two culture samples (T14 and T18). The presence of numerous virus-like particles as found (Fig. 5) at T14 might explain the decrease in the cell density observed at T20.

## Enrichment culture at 60°C without colloidal sulphur

Because of the decrease in cell numbers, the bioreactor was inoculated again using 40 mL of the chimney sample EXO15E1. The temperature was settled at 60°C and sulphur was omitted in the culture medium, such conditions were chosen as being less favourable for the growth of *Thermococcus* species. In this second culture (F2) both

**Table 1** Distribution and phylogenetic affiliations of archaeal and bacterial 16S rRNA gene sequences retrieved from the two enrichment culture (F1 and F2)

| Culture sample | Phylogenetic affiliation | Number of clones | Closest match organism | Identity % |
|---|---|---|---|---|
| Enrichment culture F1 | | | | |
| T6 | *Archeoglobales* | 7 | AF220165 *Geoglobus ahangari strain 234* | 97 |
| | *Thermococcales* | 14 | AB107767 *Thermococcus coalescens* | 99 |
| | | 2 | AY099185 *Thermococcus siculi* | 98 |
| | *Desulfurococcales* | 2 | AB008745 *Aeropyrum pernix* K1 | 96 |
| T14 | *Archeoglobales* | 6 | AF220165 *Geoglobus ahangari strain 234* | 97 |
| | *Thermococcales* | 13 | AY099185 *Thermococcus hydrothermalis* | 99 |
| T20 | *Archeoglobales* | 2 | AF220165 *Geoglobus ahangari strain 234* | 97 |
| | *Thermococcales* | 11 | AY099185 *Thermococcus siculi* | 99 |
| Enrichment culture F2 | | | | |
| T5 | *Archeoglobales* | 26 | AF220165 *Geoglobus ahangari strain 234* | 97 |
| | *Thermococcales* | 7 | AY099163 *Thermococcus hydrothermalis* | 99 |
| | *Thermales* | 43 | AJ507298 *Vulcanithermus medioatlanticus* | 96 |
| | *Deferribacterales* | 57 | AJ515882 *Deferribacter abyssi* | 99 |
| T14 | *Archeoglobales* | 80 | AF220165 *Geoglobus ahangari strain 234* | 97 |
| | *Thermococcales* | 30 | AY099163 *Thermococcus hydrothermalis* | 99 |
| | *Thermales* | 88 | AJ507298 *Vulcanithermus medioatlanticus* | 96 |
| | *Deferribacterales* | 12 | AJ515882 *Deferribacter* sp. | 99 |

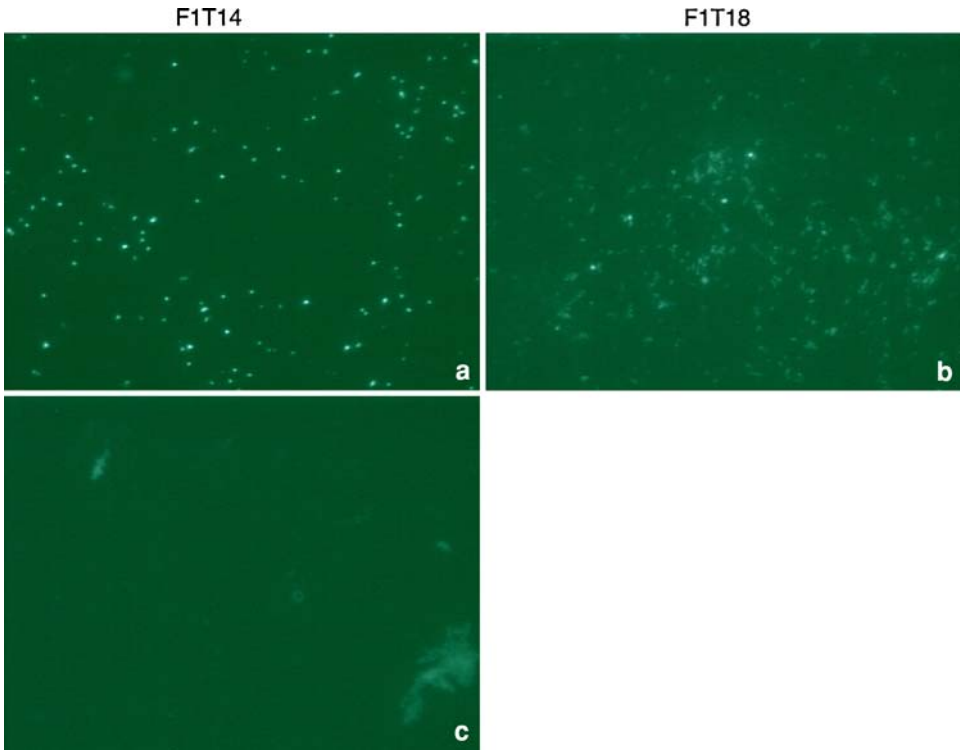


**Fig. 5** Epifluorescence pictures of two samples F1T14 (**a**) and F1T18 (**b**) prepared for virus detection. Negative control (**c**)

rods and coccoid cells were observed. Starting from $6.25 \times 10^6$ cell $mL^{-1}$ at T0, cell density reached its maximum value at T14 with $3.13 \times 10^7$ cells $mL^{-1}$ (Fig. 6).

Quantitative PCR and phylogenetic results

Both archaea and bacteria were present in the culture. Archaea were dominant at the beginning of the culture

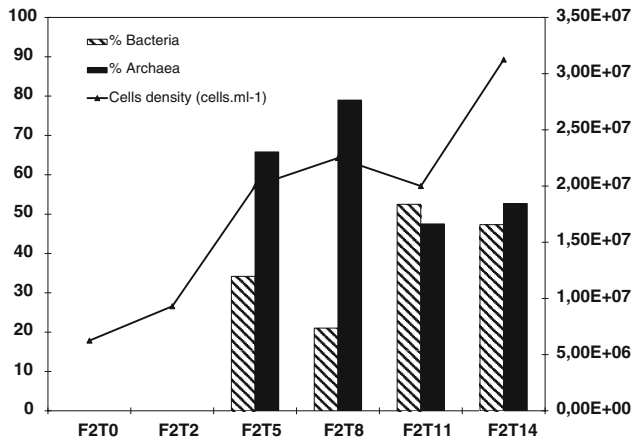


**Fig. 6** Total cell densities of the bioreactor culture F2 from T0 to T14 expressed in cells $mL^{-1}$. Archaea and bacteria ratio are expressed in percentages of total cells determined by quantitative PCR

(ratio varying from 70 to 80%). Archaeal and bacterial proportions were roughly the same at the end of the culture (Fig. 6).

The 16S rRNA gene diversity analysis of this second enrichment culture showed again the presence, within the archaeal community, of species belonging to the genera *Geoglobus* and *Thermococcus* (Table 1; Fig. 2). Within the genus *Thermococcus* only sequences related to *Thermococcus hydrothermalis* were detected. The analysis of the bacterial community showed the presence of species related to the genus *Deferribacter* and *Vulcanithermus*; sequences were, respectively, affiliated to *Deferribacter abyssi* (99% 16S rRNA gene sequence similarity and *Vulcanithermus medioatlanticus* (96% similarity) (Fig. 3).

### CE-SSCP

In culture F2 only the bacterial community dynamic was studied. As described above for culture F1 previously sequenced clones affiliated to *Deferribacter* sp. and *Vulcanithermus* sp. were used to identify peaks in the profiles. A small increase in the "*Deferribacter*" peak can be observed but no strong variations were noted all along this enrichment culture (Fig. 7).

## Discussion

### A low diversity in the chimney sample used as an inoculum

This low archaeal diversity is quite surprising compared with previous studies but can be explained by (1) the age of the chimney and/or (2) methodological biases.

The hydrothermal chimney sampled at the TAG site had a mineral matrix dominated by anhydrite (80–90%), chalcopyrite (5–7%) and pyrite (2–3%), as determined by X-ray diffraction analysis (Yves Fouquet, personal communication), forming a complex mineral assemblage. This type of mineralogical composition is assumed to correspond to young active hydrothermal chimneys. As a rule, hydrothermal chimney formation begins with the precipitation of anhydrite due to the interaction between reduced hydrothermal fluid and sea water. The sea water that can then circulate by advection current through the porous anhydrite chimney matrix leads to continuous input of sea water components including organic matter. In the present case, the chimney was surrounded by a large community of shrimps *Rimicaris exoculata* (Fig. 1). Consequently the organic matter due to the shrimp swarm was obviously present in suspension in the sea water around the chimney and could explain the dominance of chemoorganotrophic species such as *Thermococcales*.

Several studies reported that there is a relationship between the chimney mineral composition and the microbial community composition (Page et al. 2008; Takai et al. 2008). Depending on the geographical location of black smokers, distinct archaeal and bacterial communities were retrieved (McCliment et al. 2006; Page et al. 2008; Schrenk et al. 2003). These compositional differences were associated with the mineralogical and geochemical settings of the chimneys.

Bacterial diversity in this study showed the presence of sequences affiliated to *Proteobacteria* (*Epsilon-, Alpha- and Delta-*); Some sequences were related to environmental clones and other were affiliated to species involved in the sulphur metabolism including sulphate reducers (*Desulfobulbus*-related clones) and chemolithoautotrophic or chemoorganoheterotrophic sulphur compound-oxidisers (*Sulfurimonas*, and *Sulfuribacter*-related clones).

The low diversity obtained might also be explained by the method used for the 16S rRNA gene amplification. Teske and Sorensen have recently shown that the archaeal detection depends on the specificity of the archaeal PCR 16S rRNA primers; only one pair of primers was used in this study. PCR surveys with a multiple primers combination would contribute to a better view of the archaeal community within the chimney sample (Teske and Sorensen 2007).

### Diversity of the cultured microbial community: dynamics and effect of environmental conditions

At 85°C, in the presence of sulphur (Culture F1) the cultivated microbial community in the bioreactor was mainly composed of hyperthermophilic archaeal species belonging to the genera *Thermococcus* and *Geoglobus*.

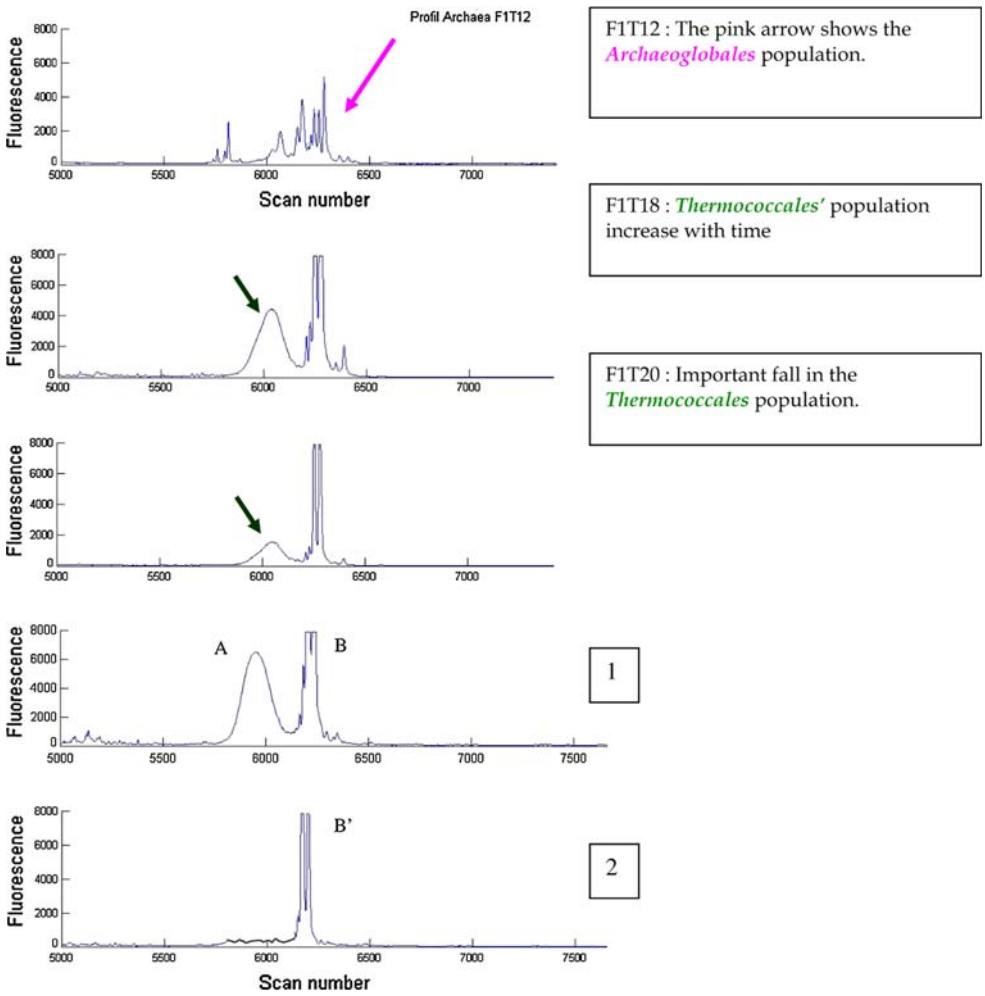


**Fig. 7** Dynamic of Archaea population in the first enrichment culture by SSCP

*Thermococcus* species are widespread within hydrothermal ecosystems. Numerous species were isolated from these environments; described species are chemoorganotrophic organisms that can use both proteinaceous and carbohydrates substrates. Some species were described as being able to use amino acids or organic acids as pyruvate, acetate as carbon sources. Most species of this genus were shown to grow better in the presence of elemental sulphur. They were also shown to be inhibited by hydrogen when grown in batch closed-culture vessels, this inhibition being alleviated by the addition of sulphur. The growth of *Thermococcus* species in our experiment was made possibly by the presence of complex organic carbon substrates, carbohydrates and organic acids. Despite the presence of hydrogen in the gas phase (Atomi et al. 2004; Godfroy et al. 1996, 1997; Marteinsson 1999), the addition of elemental sulphur had allowed their growth.

Interestingly, the presence of prokaryotes related to *Geoglobus ahangari* (Kashefi et al. 2002) was shown all along the culture F1 and F2. *Geoglobus ahangari* is an hyperthermophilic anaerobic euryarchaeon able to grow chemoorganotrophically on organic acids, amino acids and short-chain fatty acids and autotrophically on hydrogen with Fe(III) [poorly crystalline Fe(III) oxide] serving as favourite electron acceptor. Active chimneys of the hydrothermal site TAG are mainly composed of iron-containing minerals, including pyrite ($FeS_2$) and chalcopyrite ($CuFeS_2$) (Campbell et al. 1988; Charlou et al. 2002). Up to 1640 μM of Fe was measured in the hydrothermal fluid. As shown in Fig. 1, the black smoker is surrounded by a community of shrimps. Recent studies have revealed an important iron oxide deposits in the gill chambers of shrimps (Corbari et al. 2008; Schmidt et al. 2008). The presence of iron oxides in the immediate proximity of the TAG vent may have a substantial impact on microbial community inhabiting the chimney and may be a source of iron oxide by providing Fe(III) oxides. If the strain present in the bioreactor has the same requirements than *Geoglobus ahangari*, the presence of ferric iron [Fe(III)] available for its growth may have been supplied by the chimney sample, as no Fe(III) was added to the medium. The presence of both acetate and pyruvate in the culture

medium, the production of short-chain fatty acids as known metabolic products of the metabolism of proteinaceous substrates by *Thermococcus* species (Godfroy et al. 1996, 1997), and the presence of hydrogen in the gas phase may have allowed the build up of suitable conditions for the growth of this organism. *Geoglobus* species were detected in the culture from T6 to T20 and, based on SSCP results; they even dominated the beginning of the culture. Microorganisms able to perform dissimilatory Fe(III) reduction were already detected in deep-sea hydrothermal chimneys from the East Pacific Rise; these species belonged to various genera such as *Archaeoglobales, Deferribacterales* or *Thermotogales* (Kashefi et al. 2002; Slobodkin et al. 2001). Their presence on the MAR chimneys on TAG site (this study) but also in other MAR samples was demonstrated by obtaining positive enrichments on specific iron reducer medium (Kashefi et al. 2002) (Holden, personal communication). All these results together suggest that within hydrothermal habitat, Fe(III) reduction could be an important metabolism being realised by *Geoglobus*-related species at temperatures above 90°C and other species as *Deferribacter* at lower temperatures. At the beginning of the enrichment culture F1, a sequence affiliated to the genus *Aeropyrum* was detected. The two species of this genus *Aeropyrum camini* and *Aeropyrum pernix* are strictly thermophilic aerobic species (Nakagawa et al. 2004; Sako et al. 1996). Their presence can be explained by possible not strictly anaerobic conditions at the beginning of the culture as this could happen by oxygenated sea water intrusion by advection into hydrothermal chimneys.

After 20 days of culture, an important decrease in the cell density was noted in order to explain this decrease, the culture was checked for the presence of viruses. The results obtained allow us to detect the presence of virus-like particle in the culture and also to deduce their role on the microbial community. Up to date, the only hyperthermophilic marine virus described, PAV1, was shown to persist in its host strain, *Pyroccocus abyssi*, in a stable carrier state without spontaneous lysis (Geslin et al. 2003). Indeed, only one archaeal virus, isolated from a hot and acidic habitat, is known to cause lysis of its host cells (Haring et al. 2005). According to SSCP data, *Geoglobus* peak surface did not seem to change while the total cell concentration decreased in the culture suggesting that only Thermococcales were affected by virus like-particles.

Moreover, *Thermococcus*-related phylotypes retrieved from the enrichment culture in bioreactor were affiliated to *T. coalescens* and *T. siculi* at the beginning of the culture at 85°C (F1), whereas at the end and during the culture at 60°C (F2) *Thermococcus*-related phylotype were affiliated to the group of *T. hydrothermalis* indicating a decrease of the diversity of "Thermococcales". The second enrichment culture was performed at 60°C in the absence of elemental sulphur. When grown in the absence of sulphur, *Thermococcus* species are known to produce hydrogen as an end metabolic product. In addition, hydrogen was present in the gas phase (20% hydrogen). Despite those a priori unfavourable conditions, *Thermococcus* species grew. We can hypothesise that not only the cultivation of *Geoglobus* species but also *Deferribacter* species that use hydrogen as electron donor to grow autotrophically, allowed the growth of *Thermococcus* species by lowering the hydrogen concentration in the medium.

Decreasing the temperature to 60°C in the second enrichment culture (F2) allowed the growth of thermophilic *bacteria*. One species closely related to *Deferribacter abyssi* was detected, this species is a chemolithoautotrophic thermophilic bacteria and can grow using hydrogen, formate, propionate as electron donors and elemental sulphur, nitrate or Fe(III) as electron acceptors (Miroshnichenko et al. 2003a, b). Also species closely related to the hydrothermal species *Vulcanithermus medioatlanticus* was detected; this species is a thermophilic chemoorganotrophic bacteria that use oxygen or nitrate (in anaerobic conditions) as electron acceptor (Miroshnichenko et al. 2003a, b). Again the growth of both chemolithotrophic or mixotrophic and chemoorganotrophic species was made possible by the way of metabolic interactions.

With the exception of *Thermococcus* species, all the species detected in the enrichment culture were absent in the molecular inventory of the diversity performed with the chimney sample. This demonstrates that both cultural and molecular approaches are complementary to assess the microbial diversity in hydrothermal chimneys. The use of low organic matter concentrations allowed the growth of the same chemoorganotrophic species as those obtained in high organic substrates conditions but also allowed the enrichment of a greater metabolic diversity of organisms (Postec et al. 2005a, b, 2007).

## Metabolic diversity within already known species and cultivable but not isolable species

Based on the molecular data that identified sequences related to cultured microorganisms formerly described, several mediums were designed for the sub-cultivation in vials and the isolation of the detected strains. As previously described (Postec et al. 2007), media referenced as DS and DN were tested and allowed the cultivation and isolation of the *Deferribacter* strain. This species is closely related to *D. abyssi* and able to grow on acetate as carbon source and nitrate as electron acceptor. Several attempts to cultivate the strain of *Vulcanithermus* sp., including the use of the enrichment medium in aerobic conditions or anaerobic conditions in

presence of nitrate and the use of *V. medioatlanticus* type strain isolation medium (Miroshnichenko et al. 2003a, b) remained unsuccessful.

Also attempts to obtain *Geoglobus* species using *G. ahangari* medium (Kashefi et al. 2002) remained unsuccessful when enrichment culture samples were used as inoculum. Nevertheless, positive culture was obtained on *G. ahangari* medium (Kashefi et al. 2002) using pyruvate as electron donor and poorly crystalline Fe(III) oxide as electron acceptor with the chimney sample as inoculum. Sequencing of the 16S rRNA genes retrieved from the isolated species assigned it as a *Thermococcus* sp. The difficulty in obtaining detected species in pure culture could be explained by the existence of metabolic diversity within known groups.

For example, while most *Thermococcus* species are known to be sulphur-dependant, hydrogen-sensitive and chemoorganotrophs, Sokolova et al. (2004) described one species of *Thermococcus* able to grow lithotrophically by oxidising carbon monoxide. In our study, we have shown the growth of *Thermococcus* species in the absence of sulphur and the presence of hydrogen, and also the growth of *Thermococcus* species using pyruvate as carbon source, and iron oxide as electron acceptor. These observations suggest the presence of a greater metabolic diversity than commonly admitted within the *Thermococcus* genus, with species using a wide range of electron donors and acceptors. These species may have adapted their metabolism to the chemical variations of their environment. This might explain why *Thermococcus* species are present in all hydrothermal vents samples (according to both cultivation approaches and molecular survey). Their ability to grow quickly (Postec et al. 2005a, b), to thrive with instable conditions such as temperature changes (Postec et al. 2005a, b, 2007), availability of organic matter and in addition their persistence in cold oxygenated water (Huber et al. 1990) make them good candidates for early colonisation of hot areas of new vent sites. Difficulties in isolating detected microorganisms might be due to their inability in growing as a pure culture.

## Conclusion

This enrichment culture in the bioreactor (in vitro) represents a fraction of the diversity of the hyperthermophilic and thermophilic microorganisms inhabiting the TAG chimney. The effect of environmental changes, i.e. temperature and medium composition was shown to affect the microbial community in culture, as this could happen in their environment.

The availability of such a cultivation system to point out the co-existence of species using various electron donor and acceptor, carbon sources is a promising path to study the diversity of microorganisms. It offers the possibility to copy the variations of their environment and show how in hydrothermal chimneys microbial population follows one another. This was hypothesised by our precedent work (Postec et al. 2007) and confirmed in this study. The modification of culture conditions, such as temperature, organic matter concentration, electron donors and acceptors availability allowed to enrich different population of prokaryotes inhabiting hydrothermal chimneys.

Again the cultivation of microbial community instead of pure culture appears to be the unique manner to get into the structure of active microbial communities, to study how microorganisms interact in their environment and to discover the physiology and metabolic properties of microorganisms. The gas-lift bioreactor represents a promising tool to investigate in vitro the effect of physicochemical perturbations on the microbial community structure. In the future, it would be interesting to use the bioreactor to continue to mimic the environmental conditions and, for example, using hydrothermal fluid in the culture medium or investigate the culture under pressurised conditions as in the environment.

**Acknowledgments** The authors want to thank the captain and crew of the research vessels Atalante and the Victor team for their work during the EXOMAR cruise (chief scientist Anne Godfroy). Frédérique Duthoit (LMEE, UMR 6197) is thanked for his help on the quantitative-PCR technique. All people working in the department of Microbiology LMEE laboratory in Brest are thanked for the hospitality and help during various part of the work. We also thank Jim Holden, Mathilde Le Roy and Helene Ver Eecke for their contribution on the isolation of ironreducing microorganisms. We thank Yves Fouquet for the mineralogical analysis of the chimney sample. This work was supported by ANR DEEP OASES and Ministère de l'Éducation Nationale, de la Recherche et de la Technologie (grant for NB).

## References

Atomi H, Fukui T, Kanai T, Morikawa M, Imanaka T (2004) Description of *Thermococcus kodakaraensis* sp. nov., a well studied hyperthermophilic archaeon previously reported as *Pyrococcus* sp. KOD1. Archae 1:263–267

Campbell AC, Palmer MR, Klinkhammer GP, Bowers TS, Edmond JM, Lawrence JR, Casey JF, Thompson G, Humphris S, Rona P, Karson JA (1988) Chemistry of hot springs on the Mid-Atlantic Ridge. Nature 335:514–519

Charlou JL, Donval JP, Fouquet Y, Jean-Baptiste P, Holm N (2002) Geochemistry of high $H_2$ and $CH_4$ vent fluids issuing from ultramafic rocks at the Rainbow hydrothermal field (36[deg]14′N, MAR). Chem Geol 191:345–359

Corbari L, Cambon-Bonavita M-A, Long GJ, Grandjean F, Zbinden M, Gaill F, Compère P (2008) Iron oxide deposits associated with the ectosymbiotic bacteria in the hydrothermal vent shrimp *Rimicaris exoculata*. Biogeosciences 5:1295–1310

Delbes C, Moletta R, Godon JJ (2000) Monitoring of activity dynamics of an anaerobic digester bacterial community using 16S rRNA polymerase chain reaction single strand conformation polymorphism analysis. Environ Microbiol 2:506–515

Galtier N, Gouy M, Gautier C (1996) Seaview and Phylowin two graphic tools for sequence alignment and molecular phylogeny. Comput Appl Biosci 12:543–548

Geslin C, Le Romancer M, Erauso G, Gaillard M, Perrot G, Prieur D (2003) PAV1, the first virus-like particle isolated from a hyperthermophilic Euryarchaeote, *Pyrococcus abyssi*. J Bacteriol 185:3888–3894

Godfroy A, Meunier J-R, Guezennec J, Lesongeur F, Raguénès G, Rimbault A, Barbier G (1996) *Thermococcus fumicolans* sp. nov. a new hyperthermophilic archaeum isolated from deep-sea hydrothermal vent in North Fiji bassin. Int J Syst Bacteriol 46:1113–1119

Godfroy A, Lesongeur F, Raguénès G, Quérellou J, Antoine E, Meunier J-R, Guezennec J, Barbier G (1997) *Thermococcus hydrothermalis* sp. nov., a new hyperthermophilic archaeon isolated from deep-sea hydrothermal vent. Int J Syst Bacteriol 47:622–626

Godfroy A, Raven NDH, Sharp RJ (2000) Physiology and continuous culture of the hyperthermophilic deep-sea vent archaeon *Pyrococcus abyssi* ST549. FEMS Microbiol Lett 186:127–132

Haring M, Vestergaard G, Rachel R, Chen L, Garrett RA, Prangishvili D (2005) Virology: independent virus development outside a host. Nature 436:1101–1102

Houghton JL, Seyfried WE Jr, Banta A, Reysenbach A-L (2007) Continuous enrichment culturing of thermophiles under sulfate and nitrate-reducing conditions and at deep-sea hydrostatic pressures. Extremophiles 11:371–382

Huber R, Stoffers P, Cheminee JL, Richnow HH, Stetter KO (1990) Hyperthermophilic archaebacteria within the crater and open sea plume of erupting Macdonald seamount. Nature 345:179–182

Inagaki F, Takai K, Nealson KH, Horikoshi K (2004) *Sulfurovum lithotrophicum* gen. nov., sp. nov., a novel sulfur-oxidizing chemolithoautotroph within the {epsilon}-*Proteobacteria* isolated from Okinawa Trough hydrothermal sediments. Int J Syst Evol Microbiol 54:1477–1482

Kashefi K, Tor JM, Holmes DE, Gaw Van Praagh CV, Reysenbach AL, Lovley DR (2002) *Geoglobus ahangari* gen. nov., sp. nov., a novel hyperthermophilic archaeon capable of oxidizing organic acids and growing autotrophically on hydrogen with Fe(III) serving as the sole electron acceptor. Int J Syst Evol Microbiol 52:719–728

Loisel P, Harmand J, Zemb O, Latrille E, Lobry C, Delgenès J-P, Godon J-J (2006) Denaturing gradient electrophoresis (DGE) and single-strand conformation polymorphism (SSCP) molecular fingerprintings revisited by simulation and used as a tool to measure microbial diversity. Environ Microbiol 8:720–731

Lopez-Garcia P, Gaill F, Moreira D (2002) Wide bacterial diversity associated with tubes of the vent worm *Riftia pachyptila*. Environ Microbiol 4:204–215

Losekann T, Knittel K, Nadalig T, Fuchs B, Niemann H, Boetius A, Amann R (2007) Diversity and abundance of aerobic and anaerobic methane oxidizers at the Haakon Mosby Mud Volcano, Barents Sea. Appl Environ Microbiol 73:3348–3362

Marteinsson VT (1999) Isolation and characterization of *Thermus thermophilus* Gy1211 from a deep-sea hydrothermal vent. Extremophiles 3:247–251

McCliment EA, Voglesonger KM, O'Day PA, Dunn EE, Holloway JR, Cary SC (2006) Colonization of nascent, deep-sea hydrothermal vents by a novel Archaeal and Nanoarchaeal assemblage. Environ Microbiol 8:114–125

Miroshnichenko ML, Bonch-Osmolovskaya EA (2006) Recent developments in the thermophilic microbiology of deep sea hydrothermals vents. Extremophiles 10:85–96

Miroshnichenko ML, Slobodkin AI, Kostrikina NA, L'Haridon S, Nercessian O, Spring S, Stackebrandt E, Bonch-Osmolovskaya EA, Jeanthon C (2003a) *Deferribacter abyssi* sp. nov., an anaerobic thermophile from deep-sea hydrothermal vents of the Mid-Atlantic Ridge. Int J Syst Evol Microbiol 53:1637–1641

Miroshnichenko ML, L'Haridon S, Nercessian O, Antipov AN, Kostrikina NA, Tindall BJ, Schumann P, Spring S, Stackebrandt E, Bonch-Osmolovskaya EA, Jeanthon C (2003b) *Vulcanithermus mediatlanticus* gen. nov., sp. nov., a novel member of the family *Thermaceae* from a deep-sea hot vent. Int J Syst Evol Microbiol 53:1143–1148

Muyzer G, Smalla K (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Van Leeuwenhoek 73:127–141

Muyzer G, Teske A, Wirsen KO, Jannasch HW (1995) Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vents samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. Arch Microbiol 164:165–172

Nadkarni MA, Martin FE, Jacques NA, Hunter N (2002) Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiol 148:257–266

Nakagawa S, Takai K, Horikoshi K, Sako Y (2004) *Aeropyrum camini* sp. nov., a strictly aerobic, hyperthermophilic archaeon from a deep-sea hydrothermal vent chimney. Int J Syst Evol Microbiol 54:329–335

Nercessian O, Fouquet Y, Pierre C, Prieur D, Jeanthon C (2005) Diversity of *Bacteria* and *Archaea* associated with a carbonate-rich metalliferous sediment sample from the Rainbow vent field on the Mid-Atlantic Ridge. Environ Microbiol 7:698–714

Page A, Tivey MK, Stakes DS, Reysenbach A-L (2008) Temporal and spatial archaeal colonization of hydrothermal vent deposits. Environ Microbiol 10:874–884

Postec A, Pignet P, Cueff-Gauchard V, Schmitt A, Querellou J, Godfroy A (2005a) Optimisation of growth conditions for continuous culture of the hyperthermophilic archaeon *Thermococcus hydrothermalis* and development of sulphur-free defined and minimal media. Res Microbiol 156:82–87

Postec A, Urios L, Lesongeur F, Ollivier B, Quérellou J, Godfroy A (2005b) Continuous enrichment culture and molecular monitoring to investigate the microbial diversity of thermophiles inhabiting the deep-sea hydrothermal ecosystems. Curr Microbiol 50:138–144

Postec A, Lesongeur F, Pignet P, Ollivier B, Quérellou J, Godfroy A (2007) Continuous enrichment cultures: insights into prokaryotic diversity and metabolic interactions in deep-sea vent chimneys. Extremophiles 11:747–757

Pukall R, Buntefuss D, Fruhling A, Rohde M, Kroppenstedt RM, Burghardt J, Lebaron P, Bernard L, Stackebrandt E (1999) *Sulfitobacter mediterraneus* sp. nov., a new sulphite-oxidizing member of the {alpha}-proteobacteria. Int J Syst Bacteriol 49:513–519

Raven N, Ladwa N, Sharp R (1992) Continuous culture of the hyperthermophilic archaeum *Pyrococcus furiosus*. Appl Microbiol Biotechnol 38:263–267

Sako Y, Nomura N, Ushida A, Ishida Y, Morii H, Hoaki T, Maruyama T (1996) Aeropyrum pernix, gen. nov; sp. nov., a novel aerobic hyperthermophilic archaeon growing at temperatures up to 100°C. Int J Syst Bacteriol 46:1070–1077

Schmidt C, Vuillemin R, Le Gall C, Gaill F, Le Bris N (2008) Geochemical energy sources for microbial primary production in the environment of hydrothermal vent shrimps. Mar Chem 108:18–31

Schrenk MO, Kelley DS, Delaney JR, Baross JA (2003) Incidence and diversity of microorganisms within the walls of an active deep-sea sulfide chimney. Appl Environ Microbiol 69:3580–3592

Slobodkin A, Campbell B, Cary SC, Bonch-Osmolovskaya E, Jeanthon C (2001) Evidence for the presence of thermophilic

Fe(III)-reducing microorganisms in deep-sea hydrothermal vents at 13°N (East Pacific Rise). FEMS Microbiol Ecol 36:235–243

Sokolova TG, Jeanthon C, Kostrikina NA, Chernyh NA, Lebedinski AV, Stackebrandt E, Bonch-Osmolovkaya EA (2004) The first evidence of anaerobic CO oxidation coupled with $H_2$ production by a hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent. Extremophiles 8:317–323

Takai K, Horikoshi K (1999) Molecular phylogenetic analysis of archaeal intron-containing genes coding for rRNA obtained from a deep-subsurface geothermal water pool. Appl Environ Microbiol 65:5586–5589

Takai K, Horikoshi K (2000) Rapid detection and quantification of members of the archaeal community by quantitative PCR using fluorogenic probes. Appl Environ Microbiol 66:5066–5072

Takai K, Nunoura T, Ishibashi J-I, Lupton J, Suzuki R, Hamasaki H, Ueno Y, Kawagucci S, Gamo T, Suzuki Y, Hirayama H, Horikoshi K (2008) Variability in the microbial communities and hydrothermal fluid chemistry at the newly discovered Mariner hydrothermal field, southern Lau Basin. J Geophys Res 113. doi:10.1029/2007JG000521

Teske A, Sorensen KB (2007) Uncultured archaea in deep marine subsurface sediments: have we caught them all? ISME J 2:3–18

Tor JM, Amend JP, Lovley DR (2003) Metabolism of organic compounds in anaerobic, hydrothermal sulphate-reducing marine sediments. Environ Microbiol 5:583–591

Webster G, Newberry CJ, Fry JC, Weightman AJ (2003) Assessment of bacterial community structure in the deep sub-seafloor biosphere by 16 s rDNA-based techniques: a cautionary tale. J Microbiol Methods 55:155–164

Zemb O, Haegeman B, Delgenes JP, Lebaron P, Godon JJ (2007) Safum: statistical analysis of SSCP fingerprints using PCA projections, dendrograms and diversity estimators. Mol Ecol Notes 7:767–770