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

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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 (N2/H2/CO2) 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

1. 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 so 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 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 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. Among 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. 2005, 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 and colleagues (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 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. 2005).

In previous experiments with this bioreactor, enrichment cultures from hydrothermal chimney samples were performed under anaerobic conditions, at 90°C 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 hypothesize metabolic interactions between species (Postec, et al. 2005, Postec, et al. 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. It thus represents a suitable device to investigate *in vitro* the effect of physico-chemical 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. (Postec, et al. 2005) with a 100- fold diluted organic matter concentration, the enrichment was performed (i) in the presence of hydrogen and carbon dioxide (nearer to the environmental conditions) at 85°C with colloidal sulphur, then (ii) 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 Strand Conformation Polymorphism, and quantitative-PCR, in order to asses both diversity and dynamics.

2. 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 (Figure 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 (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 Smalla 1998, Muyzer, et al. 1995). PCR products were subsequently cloned with the pGEM®-T Vector cloning kit (Promega) according to the manual of the manufacturer.

Continuous enrichment culture in a gas-lift bioreactor:

Medium

The growth medium was the modified SME medium (Sharp Raven 1997) modified by Postec et a*l* (Postec, et al. 2005, Postec, et al. 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 sterilized 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 sterilized 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 (Godfroy, et al. 2006, Raven, et al. 1992), at 85°C 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 to a gel pH electrode (Mettler Toledo) and a PTFE-covered PT100 temperature probe associated to 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 40ml 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 24h to prevent wash-out 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 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 an 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 (X400 magnification). Culture samples from the bioreactor were collected every 48h, 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 resuspended 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 Strand 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-TTACCGCGGCTGCTGGCAC-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' at 94°C, 30' at 51°C for Archaea and 61°C for Bacteria, and 30' 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 10 times in water before mixing with 18.8µl formamide Hi-Di (Applied Biosystems, Courtaboeuf, France) and 0.2µl internal standard internal DNA molecular weight marker Genescan-400 HD ROX (Applied Biosystems, Courtaboeuf, France) (Delbes, et al. 2000). 1 µl of template was added to the mix. 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 by 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 to those of the communities' pattern.

16SrRNA gene sequencing and phylogenetic analysis:

DNA fragments obtained were sequenced at OUEST-Genopole (Roscoff, France). Sequences were compared to 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, neighbor-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: 12.5µl Absolute blue Quantitative-PCR Rox mix (AbGene), 400 nM final concentration of each primers, 200nM 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 16SrRNA genes were detected using the primer set Arch349F (5'-GYGCASCAGKCGMGAAW-3') and Arch806R (5'-GGACTACVSGGGTATCTAAT-3'). The TaqMan probe was Arch516F (5'-TGYCAGCCGCCGCGGTAAHACCVGC-3') (Takai 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 sec, primer annealing 60°C for 6 min.
Bacterial 16SrRNA genes were detected using the primer set 331F (5'-TCCTACGGGAGGCAGCAGT -3') and 797R (5'-GGACTACCAGGGTATCTAATCCTGTT-3'). The TaqMan probe was 6-FAM-5'- CGTATTACCGCGGCTGCTGGCAC -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 sec, 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…) were filter-sterilized on 25 mm anodisc syringe-filter membranes with pore sizes of 0.02 µm.

An aliquot (0.5ml) of culture or MilliQ water (negative control) was 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. After 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 virus counting due to extra cellular interference, 2 µl of DNase (Turbo DNAse Ambion 2U/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 X) 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.

3. 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 (Figure 2). Within this genus, 2 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* and *Epsilonproteobacteria* (Figure 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 culturable fraction of the microbial diversity of a chimney from the TAG Mid-Atlantic Ridge (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 3.1×10^6 cell ml^{-1} at T4, cell density reached 1.8×10^7 cell ml^{-1} at T18 and decrease at T20. Coccoid cells single or in pairs were dominant (Figure 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 *Geoglobu*s were detected all along the culture.

SSCP (Figure 7)

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 (Figure 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 rods and coccoid cells were observed. Starting from 6.25 $x10^6$ $cell.ml^{-1}$ at T0, cell density reached its maximum value at T14 with 3.13 $x10^7$ $cells.ml^{-1}$ (Figure 6).

Quantitative PCR and phylogenetic results:

Both Archaea and Bacteria were present in the culture. Archaea were dominant at the beginning of the culture (ratio varying from 70 to 80%). Archaeal and Bacterial proportions were roughly the same at the end of the culture (Figure 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 and Figure 2). Within the genus *Thermococcus* only sequences related to *Thermococcus hydrothermalis* were detected. 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) (Figure 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 of the "*Deferribacter*" peak can be observed but no strong variations were noted all along this enrichment culture.

4. Discussion

A low diversity in the chimney sample used as an inoculum

This low archaeal diversity is quite surprising compared to previous studies but can be explained by (i) the age of the chimney and/or (ii) 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* (Figure 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 affilated to species involved in the sulphur metabolism including sulfate-reducers (*Desulfobulbus*-related clones) and chemolithoautotrophic or chemoorganoheterotrophic sulphur compound-oxidizers (*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 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*.
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 possible 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, Godfroy, et al. 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 1640µM of Fe was measured in the hydrothermal fluid. Like shown in Figure 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, Godfroy, et al. 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 Mid Atlantic Ridge 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 pers. com). 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 hypothesize 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 *Thermoccocus* 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. 2003). 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. 2003). 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 perfomed 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. 2005, Postec, et al. 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. 2003) 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 and collaborators described one species of *Thermococcus* able to grow lithotrophically by oxidizing carbon monoxide (Sokolova, et al. 2004). 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. 2005), to thrive with instable conditions such as temperature changes (Postec, et al. 2005, Postec, et al. 2007), availability of organic matter and in addition their persistence in cold oxygenated water (Huber, et al. 1990) make them good candidates for early colonization of hot areas of new vent sites. Difficulties in isolating detected microorganisms might be due to their inability in growing as a pure culture.

5. 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 hypothesized 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 micro-organisms interact in their environment and to discover the physiology and metabolic properties of micro-organisms. The gas-lift bioreactor represents a promising tool to investigate in vitro the effect of physico-chemical 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 pressurized conditions as in the environment.

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Figures

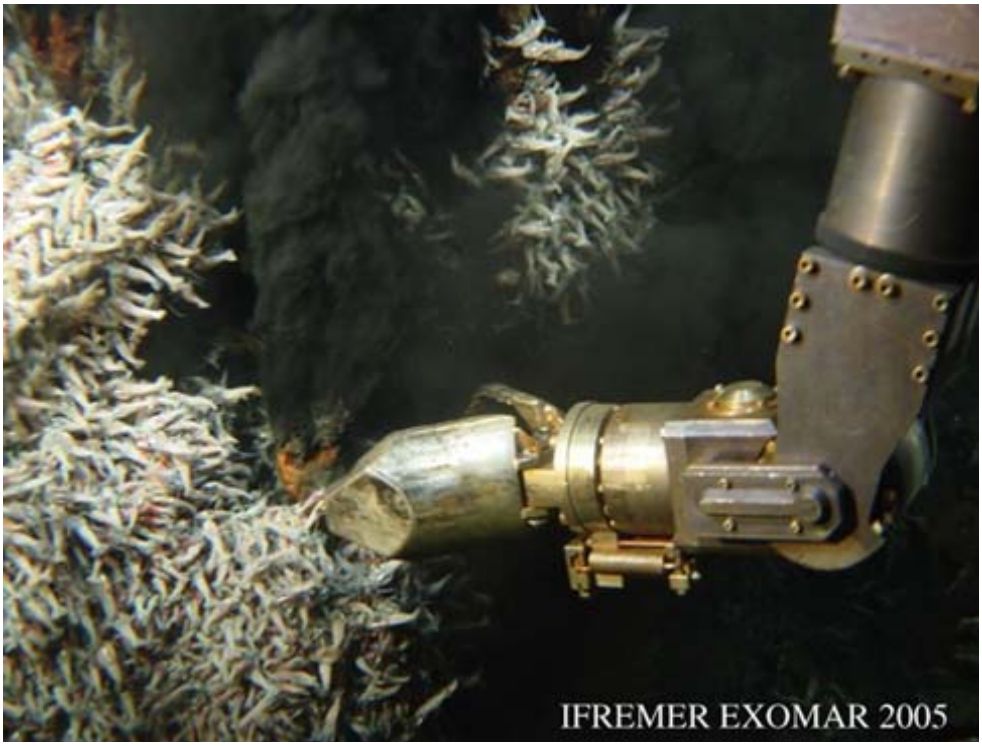


Fig. 1 The black smoker EXO15E1 recovered by the arm ‘‘Maestro’’ of the Remotely Operated Vehicle (ROV) Victor 6000. Presence of shrimps swarm around the chimney

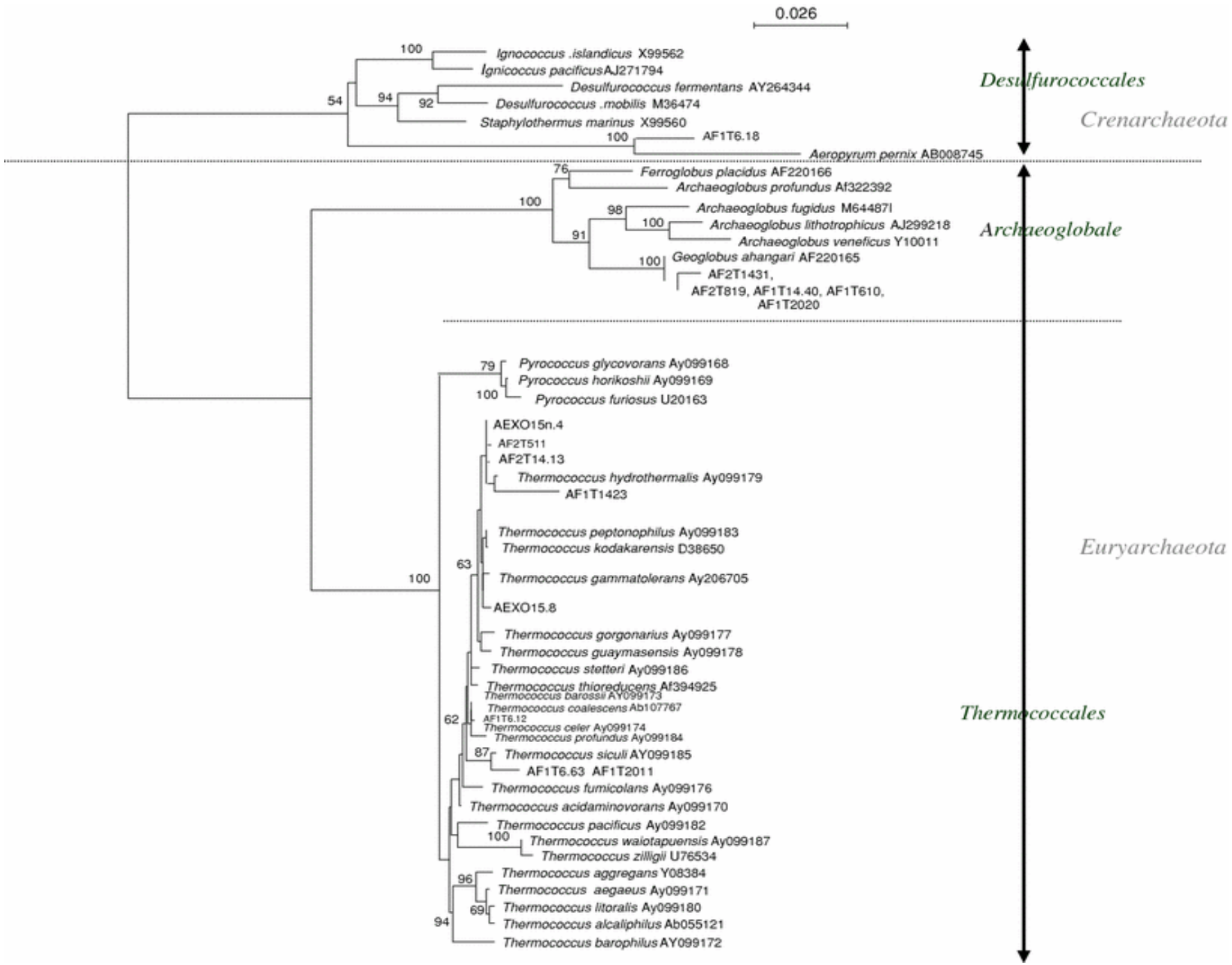
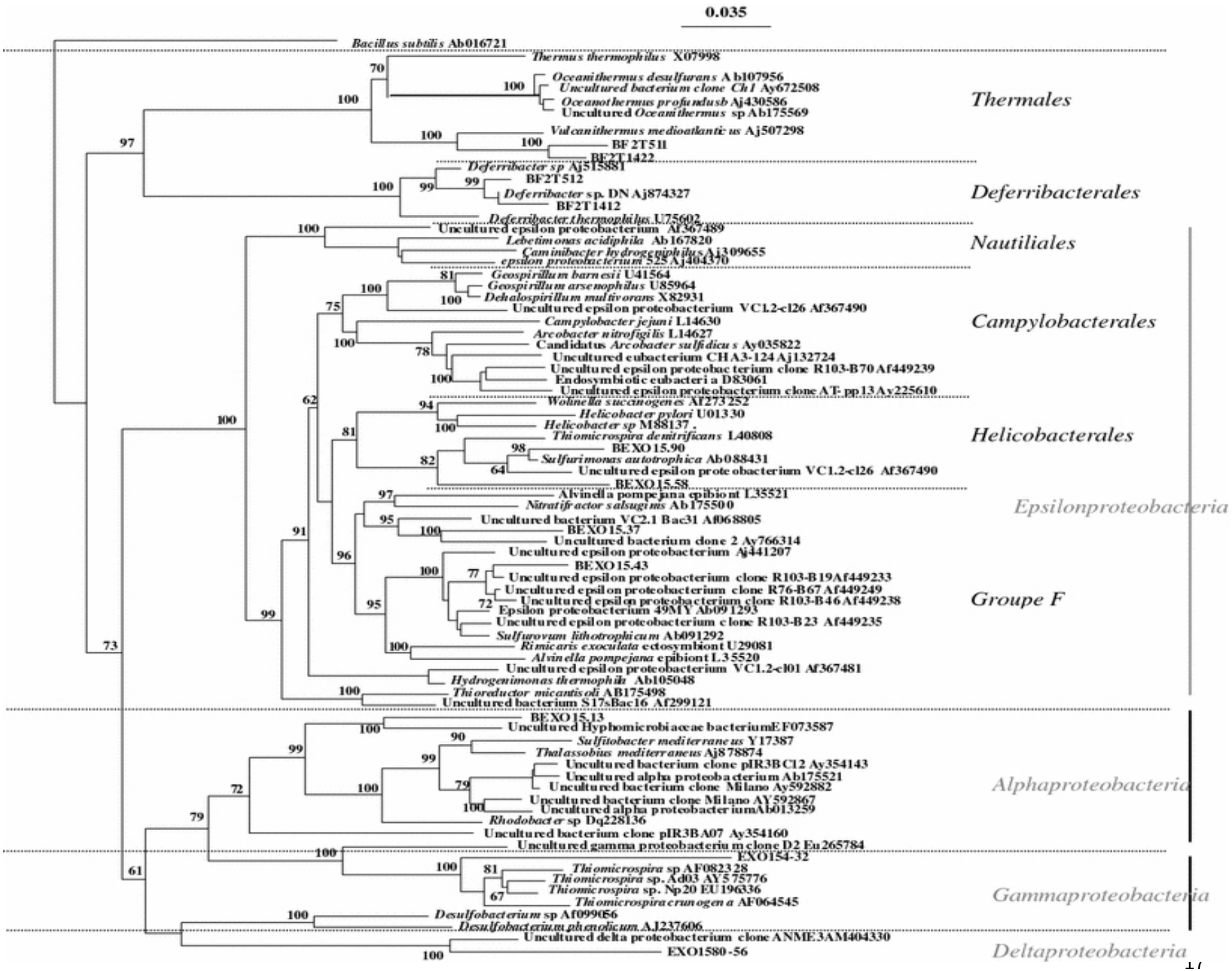


Fig. 2 Phylogenetic analysis of archaeal 16S rRNA gene sequences obtained in the diverse study AEXO15 (chimney sample), F1 and F2 (enrichment sample) and determined by neighbour-joining analysis. The numbers at the nodes are the bootstrap values (as percentage).


0.035
Bacillus subtilis Ab016721
Thermus thermophilus X07998
Oceanithermus desulfurans Ab107956
Uncultured bacterium clone Ch1 Ay672508
Oceanothermus profundusb Aj430586
Uncultured Oceanithermus sp Ab175569
Vulcanithermus medioatlanticus Aj507298
BF2T511
BF2T1422
Thermales
Deferribacter sp Aj515881
BF2T512
Deferribacter sp. DN Aj874327
BF2T1412
Deferribacter thermophilus U75602
Deferribacterales
Uncultured epsilon proteobacterium Af367489
Lebetimonas acidiphila Ab167820
Caminibacter hydrogeniphilus Aj309655
epsilon proteobacterium 525 Aj404370
Nautiliales
Geospirillum barnesii U41564
Geospirillum arsenophilus U85964
Dehalospirillum multivorans X82931
Uncultured epsilon proteobacterium VC1.2-cl26 Af367490
Campylobacter jejuni L14630
Arcobacter nitrofigilis L14627
Candidatus Arcobacter sulfidicus Ay035822
Uncultured eubacterium CHA3-124 Aj132724
Uncultured epsilon proteobacterium clone R103-B70 Af449239
Endosymbiotic eubacteria D83061
Uncultured epsilon proteobacterium clone AT-pp13 Ay225610
Campylobacterales
Wolinella succinogenes Af273252
Helicobacter pylori U01330
Helicobacter sp M88137
Thiomicrospira denitrificans L40808
BEXO15.90
Sulfurimonas autotrophica Ab088431
Uncultured epsilon proteobacterium VC1.2-cl26 Af367490
BEXO15.58
Helicobacterales
Alvinella pompejana epibiont L35521
Nitratifractor salsugines Ab175500
Uncultured bacterium VC2.1 Bac31 Af068805
BEXO15.37
Uncultured bacterium clone 2 Ay766314
Uncultured epsilon proteobacterium Aj441207
BEXO15.43
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Uncultured epsilon proteobacterium clone R103-B46 Af449238
Epsilon proteobacterium 49MY Ab091293
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Sulfurovum lithotrophicum Ab091292
Rimicaris exoculata ectosymbiont U29081
Alvinella pompejana epibiont L35520
Uncultured epsilon proteobacterium VC1.2-cl01 Af367481
Hydrogenimonas thermophila Ab105048
Thioreductor micantisoli AB175498
Uncultured bacterium S17sBac16 Af299121
Groupe F
Epsilonproteobacteria
BEXO15.13
Uncultured Hyphomicrobiaceae bacterium EF073587
Sulfitobacter mediterraneus Y17387
Thalassobius mediterraneus Aj878874
Uncultured bacterium clone pIR3BC12 Ay354143
Uncultured alpha proteobacterium Ab175521
Uncultured bacterium clone Milano Ay592882
Uncultured bacterium clone Milano AY592867
Uncultured alpha proteobacterium Ab013259
Rhodobacter sp Dq228136
Uncultured bacterium clone pIR3BA07 Ay354160
Alphaproteobacteria
Uncultured gamma proteobacterium clone D2 Eu265784
EXO154-32
Thiomicrospira sp AF082328
Thiomicrospira sp. Ad03 AY575776
Thiomicrospira sp. Np20 EU196336
Thiomicrospira crunogena AF064545
Gammaproteobacteria
Desulfobacterium sp Af099056
Desulfobacterium phenolicum AJ237606
Uncultured delta proteobacterium clone ANME3AM404330
EXO1580-56
Deltaproteobacteria

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

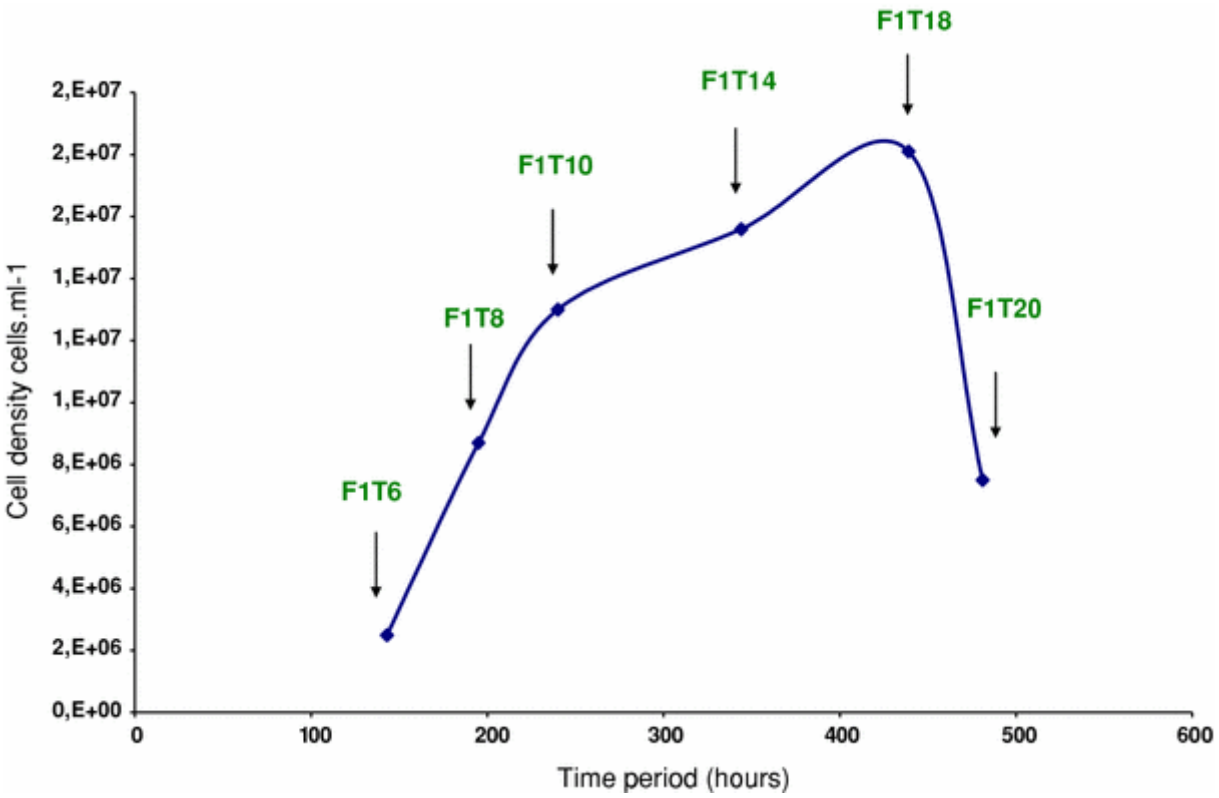

Fig. 4 Cell densities of culture samples collected from T6 to T20 from the bioreactor. Cell densities are expressed in cell mL^{-1}. The time period is expressed in hours, F1T6, F1T8, F1T10, F1T14, F1T18 and F1T20 correspond to the sampling date. F1 is for “first fermentation”, T6, T8, T10, T14, T18,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

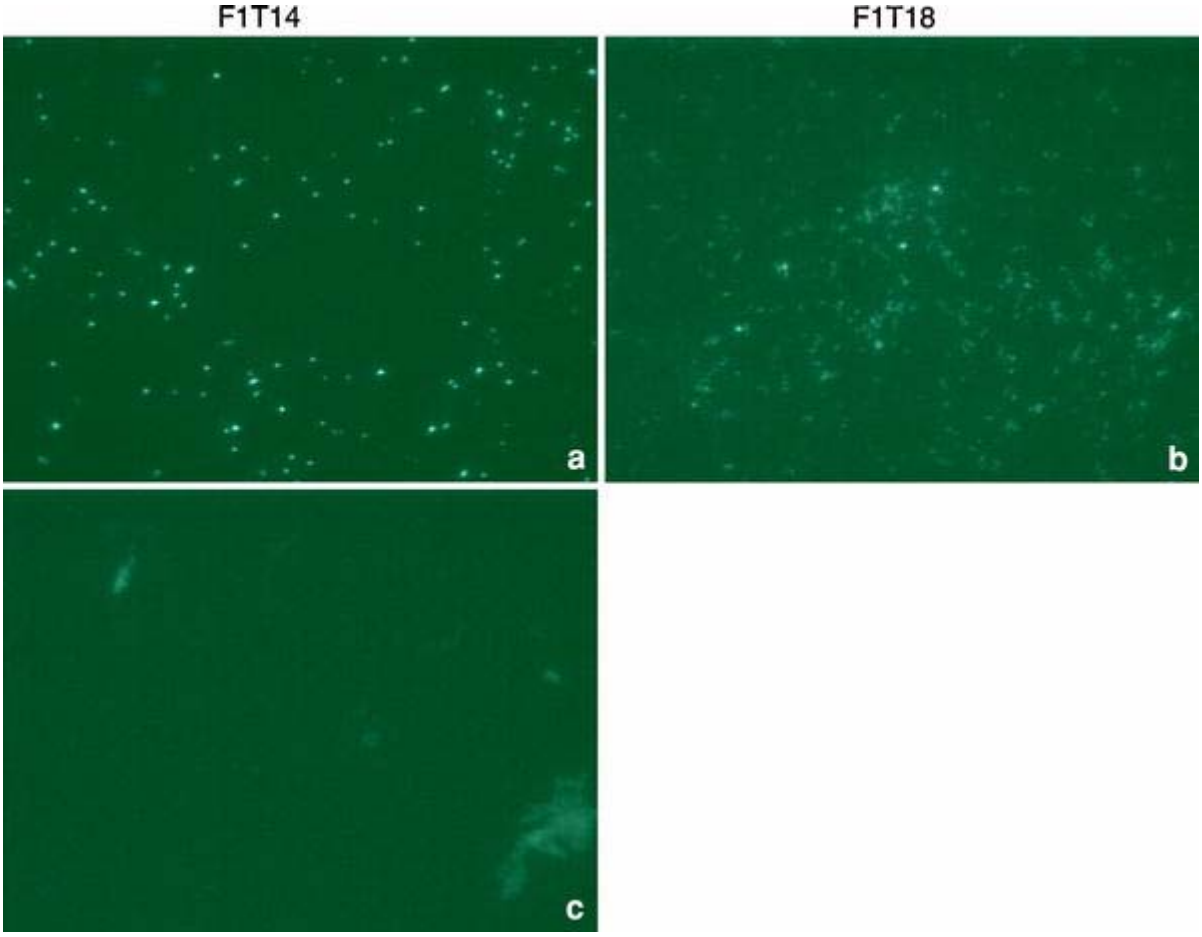

Fig. 5 Epifluorescence pictures of two samples F1T14 (**a**) and F1T18 (**b**) prepared for virus detection. Negative control (**c**)

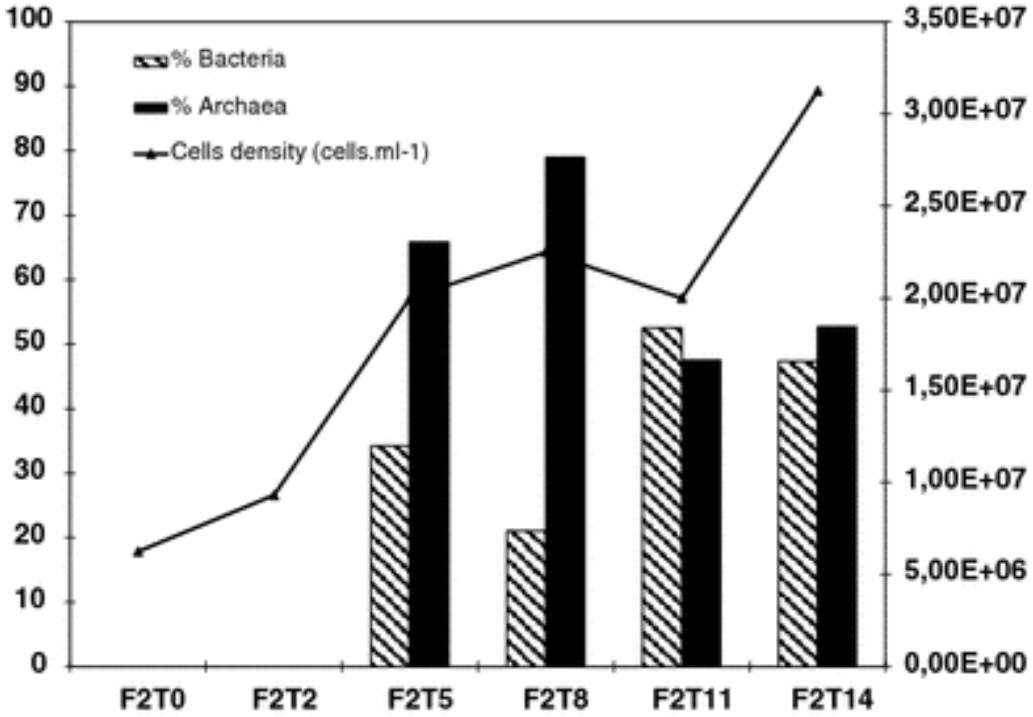

Fig. 6 Total cell densities of the bioreactor culture F2 from T0 to T14 expressed in cells mL^{-1}. Archaea and bacteria ratio are expressed in percentages of total cells determined by quantitative PCR

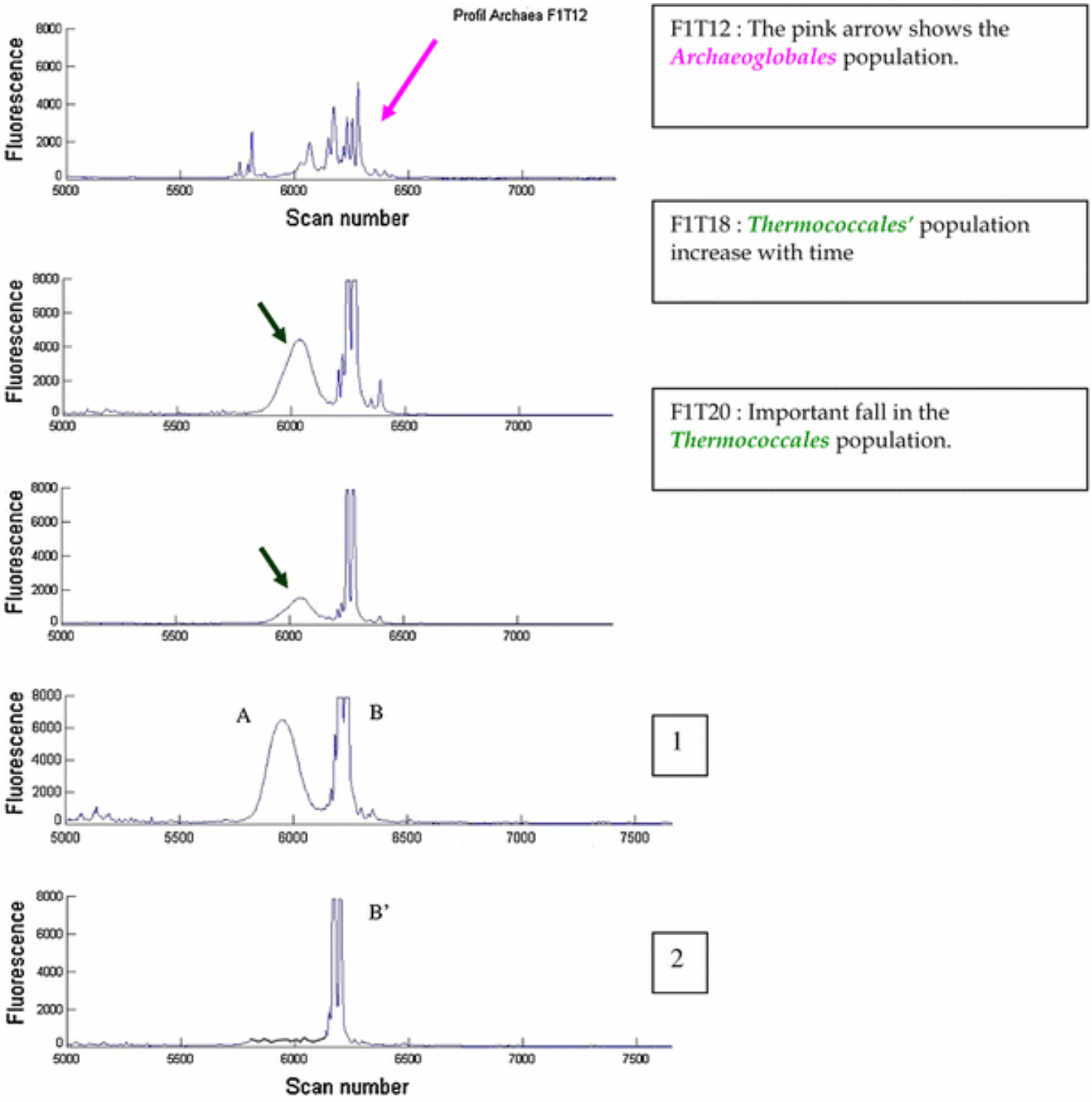

Fig. 7 Dynamic of Archaea population in the first enrichment culture by SSCP

Tables

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