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# Continuous enrichment cultures: insights into prokaryotic diversity and metabolic interactions in deep-sea vent chimneys

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**Abstract:**

The prokaryotic diversity of culturable thermophilic communities of deep-sea hydrothermal chimneys was analysed using a continuous enrichment culture performed in a gas-lift bioreactor, and compared to classical batch enrichment cultures in vials. Cultures were conducted at 60°C and pH 6.5 using a complex medium containing carbohydrates, peptides and sulphur, and inoculated with a sample of a hydrothermal black chimney collected at the Rainbow field, Mid-Atlantic Ridge, at 2,275 m depth. To assess the relevance of both culture methods, bacterial and archaeal diversity was studied using cloning and sequencing, DGGE, and whole-cell hybridisation of 16S rRNA genes. Sequences of heterotrophic microorganisms belonging to the genera Marinitoga, Thermosipho, Caminicella (Bacteria) and Thermococcus (Archaea) were obtained from both batch and continuous enrichment cultures while sequences of the autotrophic bacterial genera Deferribacter and Thermodesulfatator were only detected in the continuous bioreactor culture. It is presumed that over time constant metabolite exchanges will have occurred in the continuous enrichment culture enabling the development of a more diverse prokaryotic community. In particular, CO2 and H2 produced by the heterotrophic population would support the growth of autotrophic populations. Therefore, continuous enrichment culture is a useful technique to grow over time environmentally representative microbial communities and obtain insights into prokaryotic species interactions that play a crucial role in deep hydrothermal environments.

**Keywords:** Microbial diversity - Deep-sea hydrothermal vent - Continuous enrichment cultures - Bioreactor - Thermophiles - 16S rRNA gene - Metabolic interactions

## Introduction

The widespread application of 16S rRNA gene based molecular methods to identify microorganisms in natural samples has revealed an extensive and, in many cases, unexpected microbial diversity. Within deep-sea hydrothermal environments, the diversity of microbial communities associated with *in situ* colonizers (McCliment et al. 2006), mats (Moussard et al. 2006), animals (DeChaine et al. 2006), sediments (Inagaki et al. 2006) and chimneys (Kormas et al. 2006) have been reported in recent molecular surveys. The rise of molecular microbial ecology has resulted in the detection of many microorganisms that have as yet not been cultivated, for example the widespread Marine *Crenarchaeota* Group I (MGI) and the *Korarchaeota*. To understand the physiology and ecological significance of these uncultivated microorganisms, an effort has to be made to improve and develop cultural approaches. Indeed, culture conditions routinely used reveal only a small fraction of the global microbial community. As an alternative to batch cultures in vials, a gas-lift bioreactor was developed to grow anaerobic and hyperthermophilic microorganisms in continuous culture (Raven et al. 1992). Recently, it has been used to study the metabolism of members of the *Thermococcales*, including *Pyrococcus abyssi* (Godfroy et al. 2000), to develop minimal media and to optimize the growth conditions of *Pyrococcus furiosus* and *Thermococcus hydrothermalis* (Raven and Sharp 1997; Postec et al. 2005a). In addition to the study of pure cultures, the gas-lift bioreactor can also be used to cultivate representative microorganisms from environmental samples in continuous enrichment culture under controlled conditions. The bioreactor allows the long-term cultivation of microbes by enabling a continuous substrate supply, the elimination of volatile metabolic end-products (potentially toxic for microbial growth) by gas sparging and pH and temperature regulation. These features help to grow less dominant microorganisms having poor representation, long latency phase and/or slow growth. In a previous study, the gas-lift bioreactor was used to enrich microorganisms from a black smoker collected at 2275 m depth on the Rainbow hydrothermal field of the Mid-Atlantic Ridge (Postec et al. 2005b). A fifty–days continuous culture at 90°C on a rich medium containing sulphur under anaerobic conditions demonstrated a large diversity inside the cultivated community, including (in addition to archaeal species belonging to the order *Thermococales*) moderately thermophilic members of the orders *Clostridiales* and *Thermotogales*, and members of the *Epsilonproteobacteria* that were not detected in vial cultures. In the present study, the same black smoker chimney was used as inoculum to perform similar experiments on both batch and continuous enrichment cultures under the same conditions, except the temperature lowered to 60°C. The microbial diversity of the communities enriched in vials and bioreactor was analysed using the following

techniques based on the 16S rRNA genes: cloning, sequencing, denaturing gradient gel electrophoresis (DGGE) and whole-cell hybridisation. Molecular results were the guidelines for subsequent isolation of microorganisms from the enrichment cultures.

## Materials and methods

### Samples

During the ATOS cruise (European project VENTOX) on the Rainbow field (36°13'N 33°54W, 2275 m) located on the Mid-Atlantic Ridge (MAR), an active black smoker was collected by the Remotely Operated Vehicle (ROV) Victor, and brought to the surface in an insulated box under aseptic conditions. On board, eight fragments of the chimney were subsampled according to mineral zonations from the inner part to the outer part. The subsamples were crushed in an anaerobic chamber (La Calhene, France) and stored in sterile serum vials filled with sterile seawater containing 0.5 mg $l^{-1}$ of $Na_2S$. All subsamples were pooled to represent the whole chimney and this suspension was used to inoculate the enrichment cultures.

### Continuous enrichment culture in bioreactor: conditions and monitoring

*Medium*

The growth medium was the modified SME medium (Sharp and Raven 1997) in which yeast and peptone were replaced by: 1 g yeast extract (Difco), 0.5 g casaminoacids (Difco), 0.4 g glucose, 0.4 g dextrin (from corn), 0.2 g galactose, 0.2 g dextran, 0.1 g glycogen, 0.2 g pyruvate and 0.1 g acetate (all purchased from Sigma). The medium was supplemented with 3 g $l^{-1}$ colloidal sulphur.

*Culture conditions*

The medium was sterilized by filtration (Sartroban, 0.22 µm) in a 20 litre Nalgene bottle containing the colloidal sulphur, previously sterilized by heating twice at 100°C for 30 min on two successive days. The culture was performed at 60°C and pH 6.5 in a 2 litre glass gas-lift bioreactor as previously described (Raven et al. 1992; Godfroy et al. 2005). The bioreactor was inoculated at 2% (v/v) with the chimney sample suspension. The temperature was controlled by a heated circulating bath filled with water and monitored with a standard PT100

probe covered with Teflon. The pH was monitored using a combination gel pH electrode (Mettler Toledo). Acid (1N HCl) and base (1N NaOH) were added with peristaltic pumps (Masterflex). Temperature and pH were controlled with a 4-20 mA controller and AFS Biocommand system from New Brunswick (Nijmegen, Netherlands). Fresh medium addition and culture withdrawal were performed using peristaltic pumps (Masterflex). The culture was sparged with $N_2$ (0.1 v $v^{-1}$ $min^{-1}$) to maintain anaerobic conditions, and to eliminate volatile metabolic end products that might inhibit the growth of microorganisms (i.e. $H_2S$). The bioreactor was maintained as a batch culture for the first 34 h to prevent wash-out of the cells before they have grown. After 34 h, fresh medium was provided by applying a dilution rate of 0.04 $h^{-1}$ (80 ml $h^{-1}$). To test the effect of the temperature on the composition of the cultivated microbial community, the temperature was increased from 60°C to 70°C at T31 (day 31) and to 80°C at T36 (day 36).

*Culture monitoring and sample preservation*

The culture was maintained for 45 days. Culture samples from the bioreactor were collected every 24 hours, from T0 (day 0) to T45 (day 45). 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). For each sampling, 10 ml of culture were preserved anaerobically at 4°C in a serum vial, and eight cryotubes containing each 1.8 ml of culture were frozen at –20°C with 5% DMSO (v/v). For DNA extraction, cells were recovered from 15 ml of culture by centrifugation (20 min at 8,000 g). Cell pellets were washed with 23 g $l^{-1}$ sterile NaCl, then mixed to 5 ml of lysis buffer TE Na 1X (Tris-HCl pH 8, 100 mM ; NaCl, 100 mM, EDTA pH 8, 50 mM), and stored at -20°C until the DNA extraction procedure was undertaken. For whole-cell hybridisation, 12 ml of culture sample were fixed for two hours with 3% (v/v) formaldehyde. Fixed cells recovered by centrifugation (10 min at 6,000 rpm) were washed with PBS buffer (phosphate-buffered: 8 g NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4$, 0.24 g $KH_2PO_4$, per litre of distilled water, pH 7.4) before storage in 50% (v/v) ethanol in PBS at –20°C. For HPLC analyses, 1.5 ml of culture sample was centrifuged (10 min at 10,000 rpm) and the supernatant was stored at 4°C until analysis.

Batch enrichment cultures in vial: conditions and monitoring

Cultures were performed anaerobically in 100 ml serum vials (Godfroy et al. 1996), using the medium described for the enrichment culture in the bioreactor to which 6.05 g $l^{1}$ PIPES was added and colloidal sulphur was

136 replaced by 10 g $l^{-1}$ sulphur powder. Inoculation was performed with 2% (v/v) of the chimney sample
137 suspension. The same temperature and pH (60°C, pH 6.5) as in the bioreactor were used. Cell pellets dedicated
138 to DNA extractions were recovered from 15 ml of culture after 24 h (sample A1) and 41 h (sample A2) of
139 incubation. The 24 h culture (A1) was subcultured for 17 h in vial in the same conditions (sample B).
140
141 Nucleic acid extraction
142
143 DNA was extracted from frozen cell pellets in lysis buffer (cells recovered from 15 ml of culture, see procedure
144 above). A modified version of the protocol described by Alain et al. (2002) was followed combining chemical
145 and enzymatic lysis. For these culture samples, the applied lysis treatment was 1.5 h and 2.5 h. Afterwards, intact
146 cells could not be observed by microscopy. Supernatants from the 1.5 h and 2.5 h lysis were pooled and
147 extracted twice with equal volumes of buffered (pH 8.0) PCI (phenol/chloroform/isoamylic acid : 25/24/1) and
148 once with an equal volume of chloroform. DNA was finally precipitated by addition of 70 % (v/v) isopropanol.
149 After centrifugation at 11,000×g for 30 min, DNA was air dried before being resuspended in 250 µl TE 1×
150 buffer (10 mM Tris-HCl, 2 mM EDTA, pH 7.5). The extracted DNA quality was routinely checked using 0.8%
151 agarose-TAE-1× gels.
152
153 Amplification of the 16S rRNA gene and DGGE analysis
154
155 The variable v3 region of 16S rRNA genes from extracted DNA were amplified using the primers 341F-GC and
156 907R specific to the bacterial domain (Muyzer et al. 1993; Muyzer and Smalla 1998). The PCR procedure and
157 the analysis of the fragments by DGGE using the Bio-Rad Dcode apparatus are described in Muyzer et al.
158 (1993). Electrophoresis conditions, gel staining, DGGE band extraction, DNA reamplification, and PCR product
159 purification were performed using the conditions described by Postec et al. (2005b).
160
161 Amplification of the 16S rRNA gene and cloning
162
163 Archaeal DNA was amplified using the primer A24F (5'-TTC CGG TTG ATC CTG CCG GA-3') and the
164 reverse primer 1407R (5'-GAC GGG CGG TGW GTR CAA-3') or alternatively A23SR (5'-CTT TCG GTC
165 GCC CCT ACT-3', position 257-234 on *Thermococcus celer* 23S rRNA gene sequence). Bacterial DNA was

amplified using primer E8F (5'-AGA GTT TGA TCA TGG CTC AG-3') and the reverse primer U1492R (5'-GTT ACC TTG TTA CGA CTT-3'). PCR reactions were performed on a Robocycler Gradient 96 (Stratagene) (Wery et al. 2002; Nercessian et al. 2003). PCR products were then checked on a 0.8% (w/v) agarose gel and directly cloned using the TOPO TA Cloning® kit (pCR2.1 vector), according to the manufacturer's instructions (Invitrogen). Clone libraries were constructed by transforming *E. coli* TOP10F' cells. An archaeal and a bacterial library were constructed from two culture samples from the enrichment culture in bioreactor named T7 and T28, collected respectively after 7 and 28 days of culture, and from each sample of enrichment cultures in vials (A1, A2 and B).

16S rRNA gene sequencing and phylogenetic analysis

DNA fragments obtained by DGGE were sequenced by Genome Express S.A. (Grenoble, France). From clone libraries, each clone was cultivated overnight at 37°C with shaking (320 rpm) on deepwell microplates, in 1 ml Luria Bertani broth 2X medium containing ampicillin (50 µg $ml^{-1}$). Plasmids were extracted and purified using Montage Plasmid $Miniprep_{96}$ Kits (Millipore) and partially sequenced using the BigDye Terminator chemistry with an automated capillary sequencer (Applied Biosystem). Sequences were compared to all GenBank, RefSeq Nucleotides, EMBL, DDBJ and PDB sequences using the BLAST (http://www.ncbi.nlm.nih.gov/BLAST) network service (Altschul et al. 1990) in order to determine phylogenetic affiliations and detect chimeric sequences. Alignment of 16S rRNA gene sequences was performed using the CLUSTALW program (Thompson et al. 1994), then refined manually using the SEAVIEW program (Galtier 1996). Sequences displaying more than 97% similarity were considered to be related and grouped in the same phylotype. Complete 16S rRNA gene sequences were obtained for the representative clone of each unique phylotype: the related partial sequences were first assembled using the SEQMAN module of the DNASTAR software (Madison, WI, USA), and the complete sequences were analysed with the BLAST program.

Whole-cell hybridisation

Fixed cells in PBS/ethanol 50% (v/v) (fixation procedure described above) were diluted if necessary and filtered on a 0.2 µm pore size white polycarbonate filter (Isopore Membrane Filters, Millipore) laying on a nitrocellulose membrane. After drying at room temperature, cells were hybridised with the archaeal universal probe ARCH915

196 (5'-GTG CTC CCC CGC CAA TTC CT-3') labelled with indocarbocyanin (Cy3) (Eurogentec) as well as with
197 the universal bacterial probe EUB338 (5'-GCT GCC TCC CGT AGG AGT-3') labelled with fluorescein-
198 isothiocyanate (FITC) (Eurogentec). Whole-cell hybridisations were carried out at 46°C after addition of 1.5 µl
199 ARCH915 and 1.5 µl EUB338 each at 50 ng $\mu l^{-1}$ and 12 µl hybridisation solution [NaCl 5 M: 360 µl, Tris-HCl 1
200 M pH 7.4: 200 µl, formamide: 400 µl, sodium dodecyl sulphate (SDS) 10%: 1 µl, deionised water: 1039 µl] onto
201 each filter. After 2 h hybridisation, the filters were rinsed up 15 min at 48°C in a wash solution (NaCl 5M: 1.8
202 ml, Tris-HCl 1 M pH 7.4: 5 ml, SDS 10%: 25 µl, deionised water: 43.2 ml). After the hybridisation procedure,
203 each sample was stained with 10 µl of 4',6'-diamidino-2-phenylindole (DAPI 1 µg $ml^{-1}$). After addition of
204 Citifluor (Citifluor, UK) to the filters, the hybridised cells were viewed with an Olympus microscope (BX60)
205 equipped with a UV lamp and filters for DAPI (excitation 365 nm, emission 397 nm), FITC (excitation 492 nm,
206 emission 520 nm), or Cy3 (excitation 550 nm, emission 570 nm).
207
208 Analyses of amino acids, organic acids, and glucose
209
210 The HPLC procedure used for analyses of amino acids, organic acids and glucose is in Wery et al. (2001).
211
212 Gas analyses
213
214 The gas outflow from the bioreactor was directly analysed using a MTI M200D micro gas chromatograph
215 equipped with a thermal conductivity detector. A Molecular Sieve column with argon as the carrier gas was used
216 at a temperature of 30°C to detect $H_2$. $CO_2$ and $H_2S$ were detected with a Poraplot U column at 100°C, with
217 helium as the carrier gas.
218
219 Subcultures and isolations
220
221 The media employed for the subcultures were designed to cultivate heterotrophic as well as autotrophic
222 microorganisms that might be expected to grow with different electron acceptors (sulphur, nitrate, sulphate).
223 Isolation of strains whose phylotypes were recovered in the clone libraries was attempted from the enrichment
224 culture samples T7 and T28 from the bioreactor. Four culture media were used. The enrichment medium was
225 used as described above and also according to the three following modifications. Vitamins and minerals were

preserved and organic substrates were replaced by: (1) $NH_4Cl$ 0.03 g $l^{-1}$, acetate 0.016 g $l^{-1}$, yeast extract 0.01 g $l^{-1}$, and sulphur 5 g $l^{-1}$ in the DS medium, or (2) $NaNO_3$ 0.2 g $l^{-1}$ in the DN medium, or (3) $Na_2SO_4$ 0.3 g $l^{-1}$ in the T medium. The DS, DN and T media were adjusted at pH 6.5. The DS medium was sterilized by tyndallisation (twice 30 min at 100°C) while the DN and T media were autoclaved (20 min at 121°C). In the anaerobic chamber, the media were reduced with $Na_2S$ (final concentration: 0.05 g $l^{-1}$), then aliquoted into Hungate tubes or penicillin vials under $N_2/H_2/CO_2$ (90:5:5). The gas phase was then replaced performing 10 cycles of vacuum extraction / addition of $H_2/CO_2$ (80/20, v/v, 2 bar). All the incubations were performed at 60°C and pH6. Strains were isolated by repeated dilutions-to-extinction cultures or streaking on solidified enrichment medium.

Nucleotide sequence accession numbers

The sequence data used in this study have been submitted to the EMBL databases under accession number AJ874300 to AJ874328.

**Results**

Monitoring of the continuous enrichment culture in bioreactor: cell morphologies, whole-cell hybridisation, DGGE, gas chromatography and HPLC

Starting from 2.7 $10^6$ cell $ml^{-1}$ at T0, cell density reached 7.4 $10^8$ cell $ml^{-1}$ at T2 and its maximal value 2.2 $10^9$ cell $ml^{-1}$ at T31 (Fig. 1). Coccoid cells single or in pairs were dominant at T2 (Fig. 2a, 2d). Afterwards rods displaying various morphologies became widely dominant (Fig. 2b, 2e). Short, rod-shaped cells appeared single or in chains within an outer sheath-like structure, similarly to the specific toga of *Thermotogales*. Long rods exhibiting a terminal endospore were observed. From T27, coccoid cells, single or in pairs, increased in density compared to the rod morphologies. At the end of the culture, rod morphologies had nearly disappeared.

The relative proportions of archaeal and bacterial cells were determined by whole-cell hybridisation in eleven culture samples of the bioreactor, from T2 to T41 (Fig. 1). Approximately 2,000 cells were counted on filters for each sample. 99.0% of the cells detected at T2 belonged to the *Archaea* (Fig. 2a, 2d); *Bacteria* were dominant from T4 to T31 (between 94.8 to 99.8% until T24, then 60.0% at T28 and 54.6% at T31). At T36 and T41, *Archaea* became predominant again, representing 61.6% of the cells at T36 and 98.8% of the cells at T41.

Temperature was increased from 60°C to 70°C at T31, and from 70°C to 80°C at T36, which was associated with a significant decrease in the cell density (2.2 $10^9$ cell $ml^{-1}$ at T31 and 1.8 $10^7$ cell $ml^{-1}$ at T41).

The forty-five days enrichment culture was further investigated by DGGE analysis. The v3 hypervariable region of the bacterial 16S rRNA gene was amplified from T1, T4, T9, T13, T16, T24, T28, T31 and T36 culture samples. No amplification was obtained from samples collected after T36, probably due to the low cell density corresponding to the temperature increase at T36. Representative DGGE bands migrating at different distances and originating from various samples were extracted from gels (not shown) and re-amplified directly. The DGGE sequence types (approximately 500 pb) were affiliated to *Thermosipho* MV1063 (99% identity), *Marinitoga camini* (96%), *Caminicella sporogenes* (99-98%) and *Deferribacter abyssi* (87%). *Thermosipho* spp. was detected from T1 to T36, *Marinitoga* spp. at T31, *Caminicella* spp. from T4 to T31 and *Deferribacter* spp. from T1 to T31.

$H_2$, $CO_2$ and $H_2S$ were detected in T8, T21, T30 and T38 samples by gas chromatography. $H_2S$ production was also detected using Zn acetate strips (Lead Acetate, Whatman) from T2 until the end of the culture. From T3 until T41, HPLC analysis of the free amino acids in the culture medium showed that they were all completely consumed, and might be therefore a limiting factor for microbial growth. Analysis of glucose by HPLC indicated an initial concentration of 0.440 g $l^{-1}$, between T4 and T32 glucose was not detected at all showing a complete consumption, and endly detected again and reaching 0.389 g $l^{-1}$ . Also organic acids as pyruvate, succinate, lactate, formate, acetate, propionate, butyrate, isobutyrate and isovalerate were detected and assumed to be metabolic end products.

16S rRNA gene libraries from the continuous enrichment culture in bioreactor

Archaeal and bacterial 16S rRNA genes were amplified from T7 and T28 culture samples from the continuous enrichment culture in bioreactor. All the archaeal sequences from T7 and T28 were related to the genus *Thermococcus* (Table 1). The sequence types A704 and A800 were affiliated to *T. siculi*, and shared more than 97% of identity with a large number of 16S rRNA gene sequences related to members of the group *T. siculi* - *T. celer*, according to the BLAST analysis. Bacterial sequences affiliated with the orders *Clostridiales*, *Thermotogales* and *Deferribacterales* were retrieved in both T7 and T28 libraries (Table 1, Fig. 3) and were closely related to the hydrothermal species *Caminicella sporogenes* (97% 16S rRNA gene identity with clones 775 and 813), *Marinitoga camini* (94% identity with the clone 716, 95% with clone 805) and *Deferribacter*

*abyssi* (98% identity with clones 737 and 820), respectively. The proportion of clones related to *Deferribacter* spp. and *Marinitoga* spp. increased slightly at T28, while the number of clones related to *Caminicella* spp. decreased from 50% to 7% (Fig. 3). Two phylotypes were recovered only in the T28 bacterial library: (i) 12 clones were affiliated to *Thermosipho* spp. and the sequence type 840 shared 99% identity with *Thermosipho* MV1063, the closest species being *T. melanesiensis* (96% identity) and (ii) 3 clones were affiliated to *Thermodesulfatator* spp. and the sequence type 850 shared 96% identity with *T. indicus* (Table 1).

16S rRNA gene libraries from the batch enrichment cultures in vials

Total DNA was extracted from A1, A2 and B culture samples. Archaeal 16S rRNA gene was amplified by PCR only from sample A1, corresponding to the shortest incubated enrichment culture (24 h incubation). No archaeal 16S rRNA gene sequences was amplified after longer incubation (A2: 41 h incubation), and after subculturing from A1 (B). The 55 archaeal clones from the A1 library were all related to the genus *Thermococcus* (Table 1). The sequence type A254 displayed 97 % identity with the closest described strain *T. barophilus* (AY099172), also originating from the Mid-Atlantic Ridge (Snake Pit) (Marteinsson et al. 1999) and growing in the range of 48-95°C under atmospheric pressure. Bacterial 16S rRNA gene was amplified by PCR from all three samples. Sequences related to *Marinitoga* spp. and *Caminicella* spp. were retrieved in each library. Sequences related to the *Thermosipho* spp. were not recovered in the A1 culture, but only after 41 hours of incubation (A2) and after 17 h subculturing (B). Moreover, a shift was observed in the library compositions; sequences related to *Marinitoga* spp. were widely dominant in the A1 library whereas the A2 and B libraries were largely dominated by sequences related to *Caminicella* spp. (Fig. 3). Extending the incubation time or subculturing from the primary A1 culture resulted in similar changes in the composition of the bacterial libraries.

Subcultures and isolations

Several strains were isolated from culture samples (T3, T7 and T28) from the bioreactor: (i) an archaeal strain *Thermococcus* spp. designated as AT1273 (99% 16S rRNA gene similarity with *Thermococcus siculi)* (ii) a strain *Thermosipho* spp. (order *Thermotogales*) designated as AT1272 (98% similarity with *Thermosipho* MV1063, 95% with *T. melanesiensis)* (iii) a new bacterial species of the *Marinitoga* genus (order *Thermotogales*) named *M. hydrogenitolerans* (Postec et al. 2005c); (iv) using the T medium, a new bacterial

316 species among the *Thermodesulfatator* genus capable of sulphate-reduction (96% similarity with the
317 hydrothermal species *T. indicus)*, (v) using the DS and DN media, we isolated a strain closely affiliated to the
318 hydrothermal species *Deferribacter abyssi* (99% identity).
319
320
321 **Discussion**
322
323 In this study, we used an original culture method to enrich thermophilic microorganisms from a hydrothermal
324 black smoker: a continuous culture was performed in a gas-lift bioreactor during 45 days at 60°C and pH 6.5
325 under anaerobic conditions. The microbial diversity in continuous culture and classical batch cultures in vials
326 was compared.
327
328 Methodological considerations
329
330 The molecular inventories gave snapshots of the microbial diversity on a restricted number of samples while
331 DGGE and whole-cell hybridisation revealed a temporal dynamics in the continuous culture. The use of different
332 molecular techniques based on 16S rRNA gene analysis gave complementary data. For example, sequences of
333 *Thermosipho* spp. were detected by DGGE at T7 in the bioreactor but were not evidenced by cloning. Inversely,
334 *Thermodesulfatator* spp. was detected by cloning but not by DGGE. The possible limitations of primer
335 selectivity and cloning biases (Theron and Cloete 2000) or PCR biases (Suzuki and Giovannoni 1996) are well
336 established and can explain the variability in results from different methods.
337 Considering the gas-lift bioreactor as a system for continuous cultivation, the potential adhesion of bacteria on
338 the inner wall and the formation of a biofilm have to be examined, since the bioreactor includes no device to
339 clean surfaces. From a precipitate sampled on the inner wall, no cells were detected by microscopy observation.
340 Culture attempt in vial and DNA extraction failed as well. X-RD analysis indicated that the precipitate was
341 mainly composed of sulphurs (data not shown). The microorganisms detected in this study represent therefore
342 cells in suspension. The dilution rate applied in the bioreactor after 34 h of batch culture was 0.04 $h^{-1}$ and
343 corresponded to a generation time of 17.25 h. Although the continuous culture involves a progressive dilution of
344 the medium inside the bioreactor, four volume changes, corresponding to 100 h (about 4 days) at a dilution rate
345 of 0.04 $h^{-1}$, have been considered sufficient to completely renew the culture medium inside the bioreactor (Raven

et al. 1992). Thus, microorganisms thriving in the continuous culture from T6 were not washed-out and should have grown with a minimal growth rate of 0.04 $h^{-1}$.

*Thermococcales* as early heterotrophic colonizers

*Thermococcales* at deep-sea hydrothermal vents are widespread and members of the genus *Thermococcus* are some of the most numerous hyperthermophiles described from deep-sea vents. Investigation of their natural distribution showed that a viable *Thermococcus* population was present in the surface layers of mature hydrothermal chimneys (Harmsen et al. 1997; Takai et al. 2001; Schrenk et al. 2003). The early growth of *Thermococcales* was observed in enrichment cultures from hydrothermal chimney performed in vials and bioreactor at 90°C (Postec 2005b) and at 60°C (this study). Although all the members of the *Thermococcales* are hyperthermophilic, their early growth at 60°C both in the bioreactor and in vials is possible since (i) some *Thermococcus* sp. are able to grow at 60°C (Godfroy et al. 1997), (ii) an absence of latency phase could explain the early growth (*T. hydrothermalis*; Godfroy, pers. com.), (iii) the number of *Thermococcus*-related cells might be abundant in the chimney sample used as inoculum. This is also suggested by the detection of *Thermococcus* spp. in the molecular inventory performed directly on the studied chimney sample, while the bacteria grown in this study were not detected (Postec 2005d). Their growth at 60°C at the beginning of the enrichment culture and then when temperature was risen from 60 to 80°C after T31 may confer on *Thermococcales* a great ecological advantage to colonize new hydrothermal environments and they may be the first heterotrophs colonizing this ecosystem. This idea is supported by a recent study of nascent vent colonization in which protochimneys were deployed for short time on hydrothermal vents and heterotrophic groups including *Thermococcales* dominated the colonization of mineral surfaces after 72h (McCliment et al. 2006). The temporal sequence of colonization was analysed in the study of *in situ* samplers deployed on hydrothermal vents: the widespread occurrence of *Thermococcales* was demonstrated in short deployments (4-7 days) and decreased with time, suggesting that this group is an early surface colonizer (Nercessian et al. 2003).

Bacterial diversity and insight into microbial interactions

All the bacteria cultivated in this study were related to microorganisms from deep-sea hydrothermal vents. *Caminicella* spp. and *Marinitoga* spp. were detected in both vials and bioreactor, while the autotrophs

376 *Deferribacter* spp. and *Thermodesulfatator* spp. were only detected in the bioreactor. Compared to batch cultures
377 in vials, a larger diversity was described in the enriched community in bioreactor continuous supply of nutrients,
378 gaseous inhibitory by-products removal and and pH regulation over time, what confirms previous results (Postec
379 et al. 2005b).
380 Guided by the results of the molecular analysis, we succeeded in isolating microorganisms (heterotrophs and
381 autotrophs) enriched in continuous in the gas-lift bioreactor. Three new species belonging to the genera
382 *Thermosipho, Marinitoga* and *Thermodesulfatator* were obtained in pure culture in vials. They were related to *T.*
383 *melanesiensis* and *M. camini*, both originating from Atlantic deep sea vents, and *T. indicus*, isolated from the
384 Central Indian Ridge, respectively. *Marinitoga* sp. nov. was recently characterised and named *M.*
385 *hydrogenitolerans* because its growth is not inhibited by high hydrogen concentrations (Postec et al. 2005c). The
386 genus *Thermodesulfatator* is only represented so far by the species *T. indicus*, a thermophilic, anaerobic and
387 strictly chemolithoautotrophic bacterium growing exclusively with $CO_2$ as sole carbon source, $H_2$ as sole
388 electron donor and sulphate as sole electron acceptor (Moussard et al. 2004). A second chemolithoautotrophic to
389 mixotrophic strain isolated from the bioreactor shared 99% 16S rRNA gene similarity with *Deferribacter abyssi*
390 (Miroshnichenko et al. 2003). *D. abyssi* is thermophilic, anaerobic and facultative chemolithoautotrophic using
391 elemental sulphur or nitrate as electron acceptors, similarly to the strain isolated in this study.
392 In the enrichment culture, growth of *Thermococcales* clearly occurred first. Their fermentative metabolism on
393 proteinaceous substrates and in smaller extent carbohydrates through sulphur reduction might have generated a
394 propitious environment for the growth of bacterial heterotrophs (*Caminicella* spp., *Thermosipho* spp. and
395 *Marinitoga* spp) and then autotrophs (*Deferribacter* spp. and *Thermodesulfatator* spp.). The growth of
396 *Thermosipho* sp. and *Marinitoga* sp. is correlated with the diminution of the glucose concentration in the
397 medium and species of these genera are known to be able to use glucose as carbon substrate and to produce
398 acetate as end metabolic product (Antoine et al. 1997; Alain et al. 2002b; Postec et al. 2005c).
399 Indeed 16S rRNA gene sequences related to the autotrophic microorganisms were detected by molecular
400 analysis late at T28 but not at T7. Similarly, a temporal study of *in situ* collectors deployed on deep-sea
401 hydrothermal vents showed that the microbial diversity of the colonizing community increased with time and
402 that chemolithoautotrophs emerged during late stages (Nercessian et al. 2003).
403 The late growth of chemolithotrophs in co-culture with heterotrophs may be explained by interactions between
404 species by means of metabolites exchanges. Organic carbon provided by the medium supported the growth of
405 heterotrophs, afterwards the chemolithotrophs utilized carbon dioxide or acetate as carbon source and hydrogen

and acetate as possible electron donor, both compounds being end-products of fermentation. The study of a natural community showed that acetate and a range of other organic electron donors can be oxidised under sulphate-reducing conditions in hydrothermal vents at high temperature (90°C) (Tor et al. 2003). It has been suggested that acetate and hydrogen are the most prevalent organic fermentation products and important extracellular intermediates in the degradation of organic matter in hot microbial ecosystems, and that cooperative activity between fermentative microorganisms and sulphate reducers is important for the metabolism of fermentable compounds. Syntrophic interactions can also have an impact in hyperthermophilic co-cultures, for example on the metabolism of heterotrophic microorganisms co-cultivated with methanogens (Johnson et al. 2006).

## Conclusions

A gas-lift bioreactor was used to cultivate in continuous a thermophilic microbial community from a deep sea hydrothermal chimney, on an organic-rich medium with sulphur under anaerobic conditions. The enrichment culture was monitored with molecular and chemical analyses. In the long-term running culture, the cultivated populations were evidenced to continuously evolve with time, instead of reaching a stationary state. *Thermococcales* dominated in the first hours of the enrichment cultures suggesting that this group early colonizes hydrothermal edifices, and may represent the first heterotrophic colonizers. A larger diversity was detected in the enrichment culture in bioreactor compared to culture in vials and most of the microorganisms enriched in bioreactor, including three new bacterial species, were successfully isolated by subculturing in vials. Results indicate that the continuous culture in a gas-lift bioreactor, combined with the use of molecular tools, could be of further use to access the "uncultivated" microbial community. The microorganisms isolated in this study displayed a phylogenetic and metabolic diversity. They are involved in the sulphur cycle (suphur- and sulphate-reduction) and in the carbon cycle (autotrophy and heterotrophy). Autotrophic microorganisms were enriched in co-culture with heterotrophs, suggesting that prokaryotic species interact by means of metabolite exchange to support the growth of autotrophs. This emphasizes the importance of microbial interactions with surrounding microorganisms, animals or minerals within ecological niches. Inter-species interactions should be further taken into account to attempt the growth of as-yet uncultivated microorganisms and microbial metabolism need to be examined inside communities rather than extrapolated from pure cultures (Tor et al. 2003). The bioreactor can be considered as a window to investigate *in vitro* interactions between population

interactions that may occur *in situ*. Considering that deep-sea hydrothermal vents are extreme environments encompassing intense thermal and chemical gradient (Karl 1995), the microbial communities inhabiting these disturbed systems must be strongly affected by environmental changes. The gas-lift bioreactor represents a promising tool to investigate *in vitro* the effect of physico-chemical perturbations on the microbial community structure.

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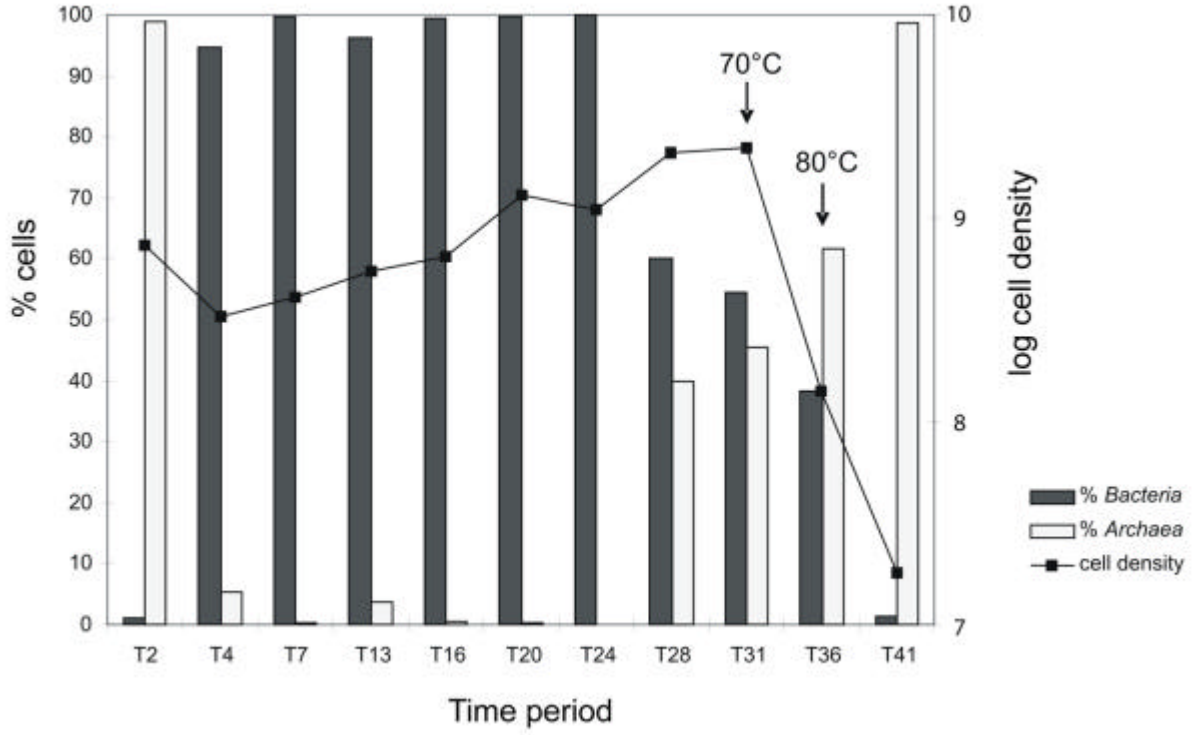


**Fig. 1** Total cell densities of the bioreactor culture from T2 to T41 expressed in cell $ml^{-1}$ and *Archaea* and *Bacteria* ratio expressed in percentages of total cells determined by whole-cell hybridisation using the universal probes ARC915 and EUB338 respectively. The temperature was increased from 60°C to 70°C at T31 and from 70°C to 80°C at T36.

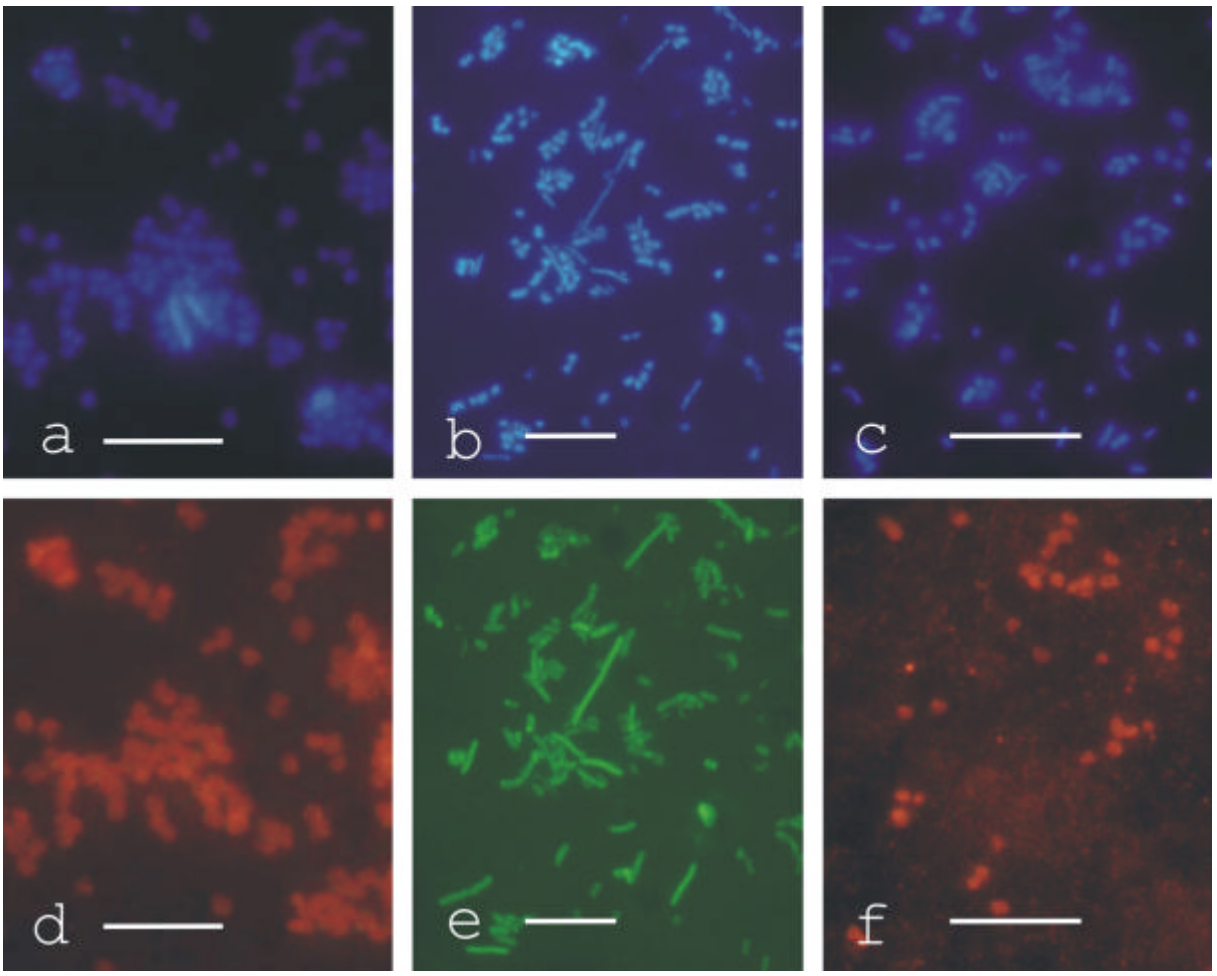


**Fig. 2** Whole-cell hybridisation of fixed cells from 3 samples of the enrichment culture in bioreactor: T2 (a, d), T7 (b, e) and T28 (c, f). Cells were stained with DAPI (a, b and c), and hybridised with the FITC-labelled Eub338 probe (e), or the Cy-3-labeled Arch 915 (d and f). Cells were viewed by epifluorescence microscopy in which DAPI-, FITC- and Cy-3-specific filters were used. The relative proportions of archaeal and bacterial cells were determined by counting approximately 2,000 cells on filter and resulted in: 99.0% of the cells detected at T2 belonged to the *Archaea* (d), 99.0% of the cells detected at T7 belonged to the *Bacteria* (e), 40% of the cells detected at T28 belonged to the *Archaea* (f). Scale bars = 10 µm.

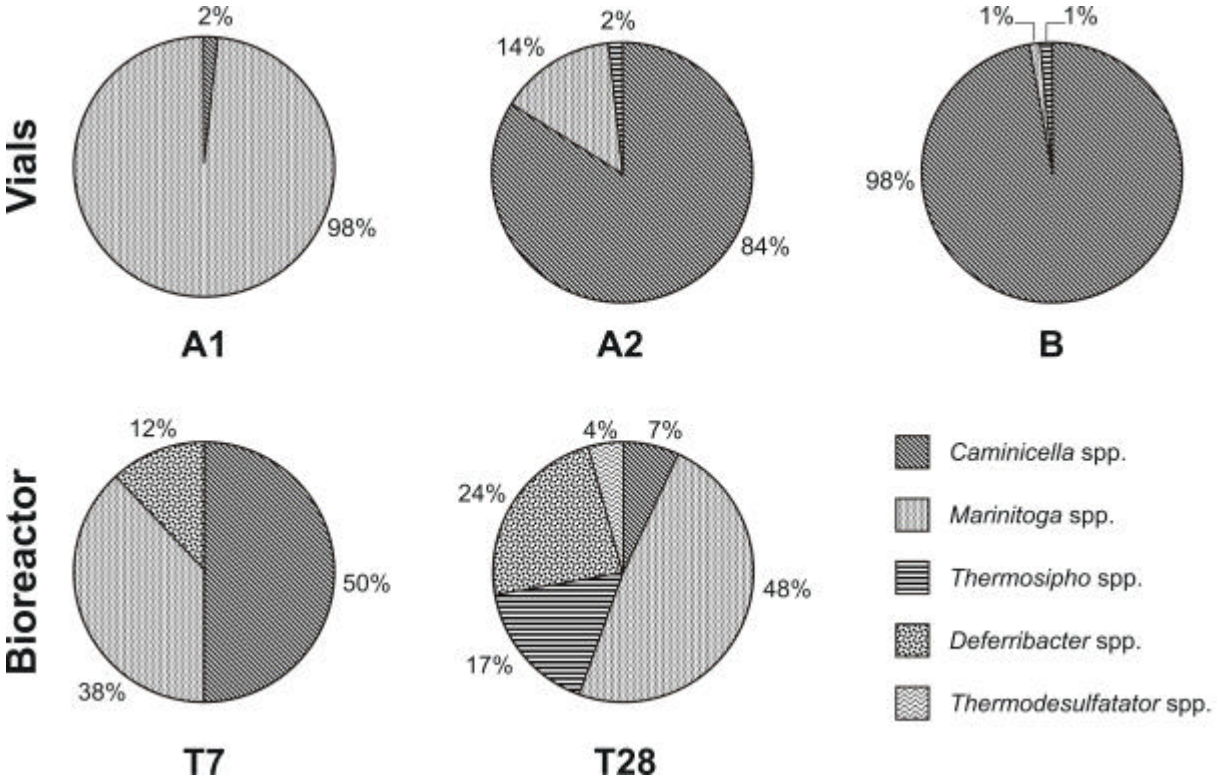


**Fig. 3** Composition of the bacterial clone libraries from enrichment cultures in vials (A1: 24 hours culture, A2: 41 hours culture and B: 17 hours subculture from A1) and from the enrichment culture in bioreactor (T7: 7 days culture, T28: 28 days culture). The percentages of clones of each phylogenetic group are indicated on the pie-charts.

**Table 1.** Distribution and phylogenetic affiliations of archaeal and bacterial 16S rDNA sequences (≥ 97% similarity in each phylotype) from the enrichment culture in bioreactor and from enrichment cultures in flask both performed at 60°C. Representative clones were completely sequenced. The sequence types deposited in GenBank appear in bold.

| | Culture sample | Phylogenetic affiliation | Representative clones | Number of clones | Closest match organism[a] | Identity[a] (%) |
|---|---|---|---|---|---|---|
| **Bioreactor** | T7 | *Thermococcales* | **A704**- A710-A712- A715-A730-A732-A737-A739 | 76 | *Thermococcus siculi* (AY099185) | 98 |
| | T28 | *Thermococcales* | **A800**-A811- A816-A817 | 71 | *Thermococcus siculi* (AY099185) | 98 |
| | T7 | *Clostridiales* | **775**-700-725-728-750-770- | 38 | *Caminicella sporogenes* (AJ320233) | 97 |
| | | *Thermotogales* | **716**-705-706-709-724-768 | 29 | *Marinitoga camini* (AJ250439) | 94 |
| | | *Deferribacterales* | **737**-711-740-754-769 | 9 | *Deferribacter abyssi* (AJ515882) | 98 |
| | T28 | *Clostridiales* | **813**-874 | 5 | *Caminicella sporogenes* (AJ320233) | 97 |
| | | *Thermotogales* | **805**-802-822-823-832 | 35 | *Marinitoga camini* (AJ250439) | 95 |
| | | | **840**-812-821-825-868-884- | 12 | *Thermosipho* MV1063 (AJ419874) | 99 |
| | | *Deferribacterales* | 893 | 17 | *Deferribacter abyssi* (AJ515882) | 98 |
| | | *Thermodesulfo-bacteriales* | **820**-829-856<br>**850**-816-858 | 3 | *Thermodesulfatator indicus* (AF393376) | 96 |
| **Flask** | A1 | *Thermococcales* | **A254** | 55 | *Thermococcus barophilus* (AY099172) | 97 |
| | A1 | *Clostridiales* | **238** | 1 | *Caminicella sporogenes (*AJ320233) | 97 |
| | | *Thermotogales* | **207**-245-219-240-255 | 58 | *Marinitoga camini* (AJ250439) | 94 |
| | A2 | *Clostridiales* | **413**-404-462 | 47 | *Caminicella sporogenes* (AJ320233) | 97 |
| | | *Thermotogales* | **436** | 8 | *Marinitoga camini* (AJ250439) | 95 |
| | | | **440**[b] | 1 | *Thermosipho* MV1063 (AJ419874) | 98 |
| | B | *Clostridiales* | **608**-626-669-635 | 68 | *Caminicella sporogenes* (AJ320233) | 97 |
| | | *Thermotogales* | **660**[b] | 1 | *Marinitoga camini* (AJ250439) | 93 |
| | | | **609** | 1 | *Thermosipho* MV1063 (AJ419874) | 97 |

[a] based on BLAST search. GenBank accession numbers are in brackets.

[b] partial sequence (600pb)