Current Microbiology
March 2005; 50(3) : 138-144
http://dx.doi.org/10.1007/s00284-004-4443-z
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Continuous enrichment culture and molecular monitoring to investigate the microbial diversity of thermophiles inhabiting the deep-sea hydrothermal ecosystems

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Abstract:

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

Introduction

Identifying the populations constitutive of microbial communities is still a major challenge. It was estimated that 90 to 99% of the organisms remained uncultivated [6], due to difficulties in reproducing their physiological niche in the laboratory. The results of molecular approaches dedicated to deep-sea hydrothermal systems were mainly obtained by 16S rDNA analysis. They showed the wealth of microbial diversity [21, 28] and the limitations of traditional cultivation techniques in improving our knowledge of the biodiversity inhabiting deep-sea hydrothermal vents. Nevertheless, *in fine*, cultivation remains necessary to describe new microbial representatives thriving in hydrothermal deep-sea vents [15, 23] and allows the study of *in situ* living microorganisms that express their physiological properties as members of the natural microflora. Since current culture techniques do not always satisfy the need of providing a good balanced picture of the microflora composition, future developments in the study of bacterial diversity should include improvements in the culture methods to approach the physico-chemical conditions of natural habitats [22]. Traditional culture attempts from hydrothermal samples were usually performed by enrichment cultures in flasks. In this study, an enrichment culture was performed in a 2-litre gas-lift bioreactor. This equipment was previously developed to grow anaerobic hyperthermophilic microorganisms [24], to study the metabolism of members of the order *Thermococcales* [14], and to develop a minimal medium for growth of these hyperthermophiles [25]. Enrichment culture in the gas-lift bioreactor was expected to allow the growth of microorganisms (i) poorly represented in the ecosystem, (ii) exhibiting a long latency phase or (iii) having not previously been cultivated so far. This could be induced by the continuous substrate supply, gas elimination by N_2 sparging, pH regulation, and long incubation time (during several weeks). It has already been reported that bacterial and archaeal populations' dynamics can be monitored in anaerobic digester according to 16S rRNA, and 16S rDNA sequences, and changes in the composition of the microflora were then highlighted [9, 17]. In this paper, we describe the use of a gas-lift bioreactor to perform an enrichment culture in controlled conditions. Amongst molecular tools available, denaturing gradient gel electrophoresis (DGGE) and 16S rDNA cloning analysis of two culture samples were chosen to investigate microbial dynamics and diversity. In comparison, enrichment cultures in flasks were performed in the same culture medium and the microflora cultivated was investigated through cloning and sequencing analysis.

Materials and Methods

Samples. During the ATOS cruise (European project VENTOX) located on the Mid-Atlantic Ridge (MAR), on the Rainbow field (36°13'N 33°54W) at 2275 m depth, an active black smoker was collected by the Remote Operated Vehicule (ROV) Victor, and brought to the surface in a decontaminated insulated box. On board, subsamples of the chimney were crushed into an anaerobic chamber and stored in sterile serum vials filled with sterile seawater containing 0.5 mg l^{-1} of Na_2S. The subsamples were pooled to represent the whole chimney, and used as inoculum at 2%.

Enrichment conditions in bioreactor and culture monitoring

Medium. The growth medium was the modified SME medium [29] in which yeast and peptone were replaced by 1 g l^{-1} yeast extract; 0.5 g l^{-1} casaminoacids; 0.4 g l^{-1} glucose; 0.4 g l^{-1} dextrin (from corn); 0.2 g l^{-1} D(+)galactose; 0.2 g l^{-1} dextran; 0.1 g l^{-1} glycogen; 0.2 g l^{-1} pyruvate, 0.1 g l^{-1} acetate and 3 g l^{-1} colloidal sulphur.

Culture conditions. The medium was sterilized by filtration (Sartroban, 0.22 µm) in 20 l 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 90°C and pH 6.5. The temperature was regulated and the pH was controlled by addition of either 1 N NaOH or 1 N HCl solutions. Moreover, the culture was sparged with N_2 (0.1 $v.v^{-1}.min^{-1}$) to maintain anaerobic conditions, and to eliminate possible volatile fermentation end products that might inhibit the growth of fermentative microorganisms.

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

Sample preservation. For each sampling, eight 1.8 ml-cryotubes of culture were frozen at –20°C in the presence of 5% DMSO. Cells were also recovered from 15 ml of culture by centrifugation (30 min at 8000×g), washed with 23 g l^{-1} sterile NaCl, and the cell pellets were stored at -20°C.

Enrichment conditions in flask and culture monitoring. Enrichment cultures were performed in 100 ml serum vials as previously described [13], with the same medium, temperature and pH as in the bioreactor. Cell pellets for molecular analysis were recovered from 15 ml of culture in flask, after 45h and 65h of incubation, and also after a 20h subculture from the first 45h culture.

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

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

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

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

more than 97% sequence similarity, only one of the sequences was retained for phylogenetic analysis. One representative of each unique phylotype was completely sequenced.

Results

Monitoring of the enrichment culture in bioreactor. The bioreactor was inoculated at 2% (vol/vol) with a chimney sample suspension. Very low cell densities were measured during the first 34 hours of batch. Then a dilution rate of 0.04 h^{-1} (80 ml h^{-1}) was applied. The period of batch culture was fixed arbitrarily in order to avoid washing out of the cells before they had become adapted to the growth conditions. Cell density stabilized at around 5 x 10^5 cell ml^{-1} until 105 h of culture. By 124 h, cell number had increased to 1.8 x 10^8 cell ml^{-1}. This was associated with H_2S production (detected using Zn acetate strips). Amongst observed morphologies, coccoid cells, single or in pairs, were dominant throughout the enrichment culture. Short rod-shaped cells appeared singly or in chains within an outer sheath-like structure, comparable to the 'toga' specific to *Thermotogales* [1]. From 350 h until the end of the culture, only single or paired coccoid cells were observed.

DGGE monitoring of the culture in bioreactor. Total DNA was successfully extracted on samples between the 4th to the 50th day of culture. From the earlier samples, difficulties in DNA extraction were encountered, probably due to the very low cell density. The DGGE procedure was tested with 16S rDNA fragments from reference strains. Conditions were optimised to analyse separately archaeal and bacterial fragments. Fragments from the two *Pyrococcus* species, *P. abyssi* and *P. glycovorans,* could not be separated (data not shown). Archaeal 16S rDNA gene were amplified with the DGGE primers from 29 distinct culture samples covering all the culture duration. One band was obtained for each sample, at the same height in all cases. 12 bands were excised, amplified by PCR, checked on a DGGE gel, and directly sequenced. A similarity of more than 97% was shared between those sequences on about 320 bp of the variable v3 region of the 16S rRNA gene. The type sequence Apa10 (Table 1) presented 100% identity on 417 bp with seven matching organisms from the BLAST analysis, all belonging to the *Pyrococcus* genera. As a result only sequences related to *Pyrococcus* could be detected throughout the culture. Using the same procedure, the v3 region of bacterial 16S rDNAs was amplified with specific DGGE primers (Table 1). Bacterial 16S rDNA genes were amplified successfully from nine distinct culture samples, from day 5 to day 35. Several dense bands were obtained at different heights. Amongst the 14 sequences obtained from excised bands, five of them represented by the sequence Apb7 (Table 1), matched by a BLAST analysis with sequences of the *Bacillus* genus. These sequences were retrieved from samples between day 5 and 9. Nine sequences obtained from excised bands were closely related to the genus *Marinitoga* and

were retrieved between day 7 and 35. DGGE analysis revealed the phylogenetic affiliation of the dominant cultivated strains and showed a larger diversity among *Bacteria* than *Archaea*.

Cloning-sequencing analysis of the culture in bioreactor. In the two archaeal libraries corresponding to the two culture samples T7 and T28, all the sequences belonged to the order *Thermococcales* (Fig. 2.). Within 59 clones partially sequenced from the earlier culture sample T7, two phylotypes were identified and were closely related to the genus *Pyrococcus*. The type sequences T7a-44 and T7a-60 shared 99% of similarity with *Pyrococcus horikoshii* as well as with *Pyrococcus abyssi* and *Pyrococcus furiosus* based on about 1500 pb. However, the complete sequences of these two representative clones shared only 96% of similarity, so that two distinct *Pyrococcus* species could have been cultivated. Within 49 clones partially sequenced from the second culture sample T28, 4% represented by the sequence type T28a-17 were related to the genus *Pyrococcus,* while 96% were closely related to the genus *Thermococcus*, represented by the sequence T28a-18 (Fig. 2.). The nearest strain according to the BLAST analysis was "*Thermococcus sulfurophilus"* (AF394925). Among the T7 bacterial library, six phylotypes were defined: one within the γ-*Proteobacteria* (3% of the sequences), two within the ε-*Proteobacteria* (38% of the sequences), two within the *Bacillus/Clostridiales* group (36% of the sequences), and one within the *Thermotogales* order (23% of the sequences) (Fig. 3.). Interestingly, all the ε-*Proteobacteria* sequences were affiliated to uncultured bacteria, all originated from deep-sea hydrothermal environments. Within the *Bacillus/Clostridiales* group and the *Thermotogales* order, the retrieved sequences were related (with 99% of identity) to *Caminicella sporogenes* and *Marinitoga piezophila* respectively. Within the T28 bacterial library, 17% of the sequences were affiliated to uncultured ε-*Proteobacteria*. Nine per cent of the sequences were closely related to the genus *Caminicella*, and 61% to the genus *Marinitoga*. No sequences related to γ-*Proteobacteria* were retrieved in the T28 bacterial library and 13% of the sequences were assigned to deep-sea hydrothermal sequences of the *Aquificales* order, which were not retrieved in the earlier culture sample.

Cloning-sequencing analysis of the cultures in flask. The medium was inoculated at 2% (vol/vol) with the same suspension of chimney sample. Total DNA was successfully extracted from the enrichment cultures in flask, after 45h and 65h of incubation at 90°C, and from a 20h subculture from the 45h culture. Only archaeal 16S rRNA gene could be amplified. Three archaeal clone libraries were constructed and 64 clones of each were sequenced. As a result, the sequences obtained were all affiliated to the *Thermococcus* genus. The nearest described species according to the BLAST analysis was *Thermococcus barophilus* (AY099172). No *Pyrococcus* neither bacteria could be detected among the strains cultivated in batch.

Discussion

Compared to conventional enrichment culture in batch, a novel approach was used to investigate the microbial diversity of a hydrothermal vent chimney sample. The use of the gas-lift bioreactor technology to perform a continuous culture provide a controlled laboratory environment for the enrichment and cultivation of different microbial groups in hyperthermophilic conditions (90°C). pH and temperature were continuously monitored and adjusted. In addition, the culture was continuously sparged with N_2, thus homogenising the medium and also eliminating possible toxic products from fermentation processes. Since the medium was continually renewed, the enrichment culture could be maintained a long time during which the analysis of the microbial population was performed. A dynamics in the cultivated microflora could be highlighted all through the enrichment culture in bioreactor, as it was already observed in anaerobic digestors [8, 17]. Actually, an evolution of the cell morphologies including rods and cocci could be observed, and modifications of the microbial diversity was ascertained through 16S rDNA sequences analysis.

The diversity of microbial population was first assessed using the DGGE technique. Here we demonstrate the efficiency of the DGGE technique for studying thermophilic *Archaea* and *Bacteria* diversity and monitoring the population dynamics. Using this technique, we demonstrated that 16S rDNA sequences, retrieved from the continuous culture, were closely related to members of the genera *Bacillus*, *Marinitoga* and *Pyrococcus*. Cloning analysis of the microbial population was performed on two culture samples at day 7 (T7) and at day 28 (T28). Similarly to the DGGE technique, *Pyrococcus*, *Caminicella*, and *Marinitoga* related sequences were detected at day 7, whereas *Pyrococcus* and *Marinitoga* were detected at day 28. Cloning analysis revealed a larger biodiversity than DGGE, since members of *Thermococcus*, γ- and ε-*Proteobacteria* and *Aquificales* were only detected by cloning. Therefore our results confirm the need for combining several molecular methods to avoid the bias inherent in each one and to have a better knowledge of the microbial diversity.

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

Experiments conducted to look for the occurrence of microorganisms of the domain *Bacteria* indicated that no bacterial sequences could be obtained from the batch cultures in flask. In contrast, studies within the continuous bioreactor revealed a large bacterial diversity through the cloning-sequencing analysis of 16S rDNA (Fig. 2.). This again confirms the interest of using a different methodological approach to investigate the biodiversity of microorganisms inhabiting deep-sea hydrothermal vents. Amongst the 16S rDNA sequences detected in this study, close relatives of *Caminicella sporogenes* [3] and *Marinitoga piezophila* [1] were found. These species are known to grow at temperatures much lower than 90°C (maximal growth temperature of 65°C and 75°C respectively). The presence of these bacteria at 90°C may indicate (i) the existence of unknown species belonging to both genera and thriving in these conditions, (ii) a possible growth of close representatives of previously isolated *Caminicella* and *Marinitoga* spp. under hyperthermophilic conditions due to thermoprotective molecules released by the other members of the enrichment culture, or (iii) unknown interactions between bacteria and archaeons. Many sequences retrieved during this study were affiliated to uncultivated microorganisms belonging to the ε-*Proteobacteria*. They were in particular closely related to the hydrothermal sequences VC2.1 Bac31 and VC2.1 Bac1, from the Snake Pit deep-sea vent site, on the Mid-Atlantic Ridge [26]. In all the deep-sea vent microbial communities studied to date, ε-*Proteobacteria* phylotypes were shown to be dominant, accounting for 40-98% of the bacterial clone libraries. The ε-*Proteobacteria* detected in our enrichment culture at 90°C were adapted at higher temperatures than those described for all ε-*Proteobacteria* characterised so far, which do not grow over 70°C in pure culture [4]. These results confirm the importance of pursuing efforts for isolating new populations of hyperthermophiles from their natural environments. With regards to numerous reports, it is now more and more obvious that the combination of both molecular approach and cultivation is the best way so far to assess the microbial diversity of hydrothermal vent chimney. Molecular studies suffer from the drawback that metabolic properties can only rarely be inferred from the 16S rRNA sequences data alone. Likewise, limitations of molecular tools must be kept in mind [2]. In this respect, the use of both cultivation and molecular methods may help to answer these questions.

The gas-lift bioreactor technology appears therefore to be an improved method to perform enrichment cultures. During the enrichment culture in the continuous bioreactor, different microorganisms were cultivated and a much larger diversity was exhibited as compared to cultures performed in flasks (batch conditions). Even new microorganisms inhabiting deep-sea hydrothermal vent could be evidenced, and isolation of two strains by subcultures in flask has already been successful. The use of the bioreactor for further enrichment experiments should contribute to better simulate hydrothermal physiological niches in the laboratory, and to reproduce environmental perturbations *in vitro* (temperature, pH, O_2, nature and concentration of the substrates ...). This should improve our understanding of the key ecological roles played by microorganisms in the hydrothermal ecosystems.

Nucleotide sequence accession numbers

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

ACKNOWLEDGEMENTS

The authors want to thank P.M. Sarradin, chief scientist of the ATOS cruise, as well as the captain and crew of the Atalante and the Victor team. We thank Dr P. Roger and L. Casalot (IRD) for revising the manuscript. This work was supported by Ifremer, European VENTOX Program and Région Bretagne.

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Figures and tables

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

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

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

Table 1.

Band	Number of bands associated[d]	Closest relative	% similarity
Apa10[a]	12	*Pyrococcus abyssi* L19921	100
Apb7[b]	5	*Bacillus* sp. USC14 AF346495	99
Apb9[c]	9	*Marinitoga piezophila* AF326121	99

[a] 417 bp used for BLAST analysis

[b] 553 bp used for BLAST analysis

[c] 555 bp used for BLAST analysis

[d] more than 97% of similarity between the sequences of a same group

Fig. 1.

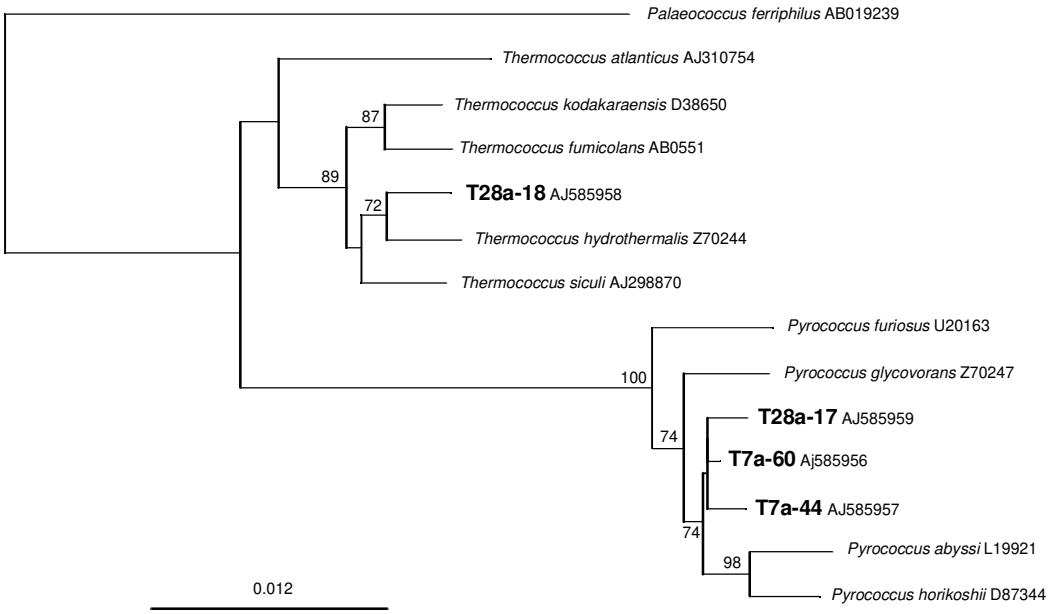
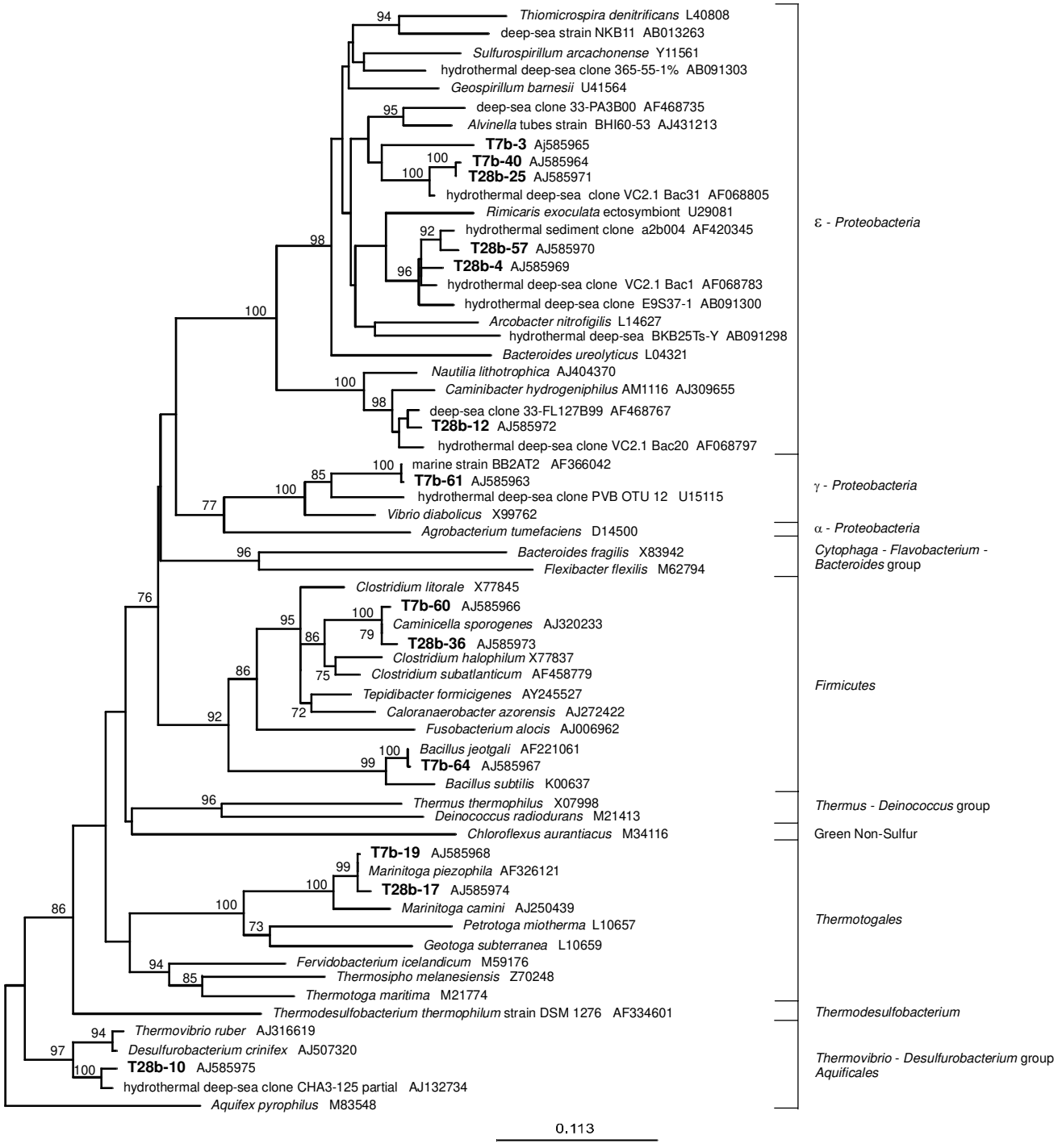

Palaeococcus ferriphilus AB019239
Thermococcus atlanticus AJ310754
Thermococcus kodakaraensis D38650
87
Thermococcus fumicolans AB0551
89
T28a-18 AJ585958
72
Thermococcus hydrothermalis Z70244
Thermococcus siculi AJ298870
Pyrococcus furiosus U20163
100
Pyrococcus glycovorans Z70247
T28a-17 AJ585959
74
T7a-60 Aj585956
T7a-44 AJ585957
74
Pyrococcus abyssi L19921
98
Pyrococcus horikoshii D87344
0.012

Fig. 2.


Thiomicrospira denitrificans L40808
deep-sea strain NKB11 AB013263
Sulfurospirillum arcachonense Y11561
hydrothermal deep-sea clone 365-55-1% AB091303
Geospirillum barnesii U41564
deep-sea clone 33-PA3B00 AF468735
Alvinella tubes strain BHI60-53 AJ431213
T7b-3 Aj585965
T7b-40 AJ585964
T28b-25 AJ585971
hydrothermal deep-sea clone VC2.1 Bac31 AF068805
Rimicaris exoculata ectosymbiont U29081
hydrothermal sediment clone a2b004 AF420345
T28b-57 AJ585970
T28b-4 AJ585969
hydrothermal deep-sea clone VC2.1 Bac1 AF068783
hydrothermal deep-sea clone E9S37-1 AB091300
Arcobacter nitrofigilis L14627
hydrothermal deep-sea BKB25Ts-Y AB091298
Bacteroides ureolyticus L04321
Nautilia lithotrophica AJ404370
Caminibacter hydrogeniphilus AM1116 AJ309655
deep-sea clone 33-FL127B99 AF468767
T28b-12 AJ585972
hydrothermal deep-sea clone VC2.1 Bac20 AF068797
marine strain BB2AT2 AF366042
T7b-61 AJ585963
hydrothermal deep-sea clone PVB OTU 12 U15115
Vibrio diabolicus X99762
Agrobacterium tumefaciens D14500
Bacteroides fragilis X83942
Flexibacter flexilis M62794
Clostridium litorale X77845
T7b-60 AJ585966
Caminicella sporogenes AJ320233
T28b-36 AJ585973
Clostridium halophilum X77837
Clostridium subatlanticum AF458779
Tepidibacter formicigenes AY245527
Caloranaerobacter azorensis AJ272422
Fusobacterium alocis AJ006962
Bacillus jeotgali AF221061
T7b-64 AJ585967
Bacillus subtilis K00637
Thermus thermophilus X07998
Deinococcus radiodurans M21413
Chloroflexus aurantiacus M34116
T7b-19 AJ585968
Marinitoga piezophila AF326121
T28b-17 AJ585974
Marinitoga camini AJ250439
Petrotoga miotherma L10657
Geotoga subterranea L10659
Fervidobacterium icelandicum M59176
Thermosipho melanesiensis Z70248
Thermotoga maritima M21774
Thermodesulfobacterium thermophilum strain DSM 1276 AF334601
Thermovibrio ruber AJ316619
Desulfurobacterium crinifex AJ507320
T28b-10 AJ585975
hydrothermal deep-sea clone CHA3-125 partial AJ132734
Aquifex pyrophilus M83548
0.113
ε - Proteobacteria
γ - Proteobacteria
α - Proteobacteria
Cytophaga - Flavobacterium - Bacteroides group
Firmicutes
Thermus - Deinococcus group
Green Non-Sulfur
Thermotogales
Thermodesulfobacterium
Thermovibrio - Desulfurobacterium group
Aquificales