ELSEVIER

FEMS Microbiology Letters 244 (2005) 61–68

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Diversity of thermophilic and non-thermophilic crenarchaeota at 80 °C

Thomas Kvist, Anett Mengewein, Stefanie Manzei, Birgitte K. Ahring, Peter Westermann *

Environmental Microbiology & Biotechnology Group (EMB), BioCentrum – Danmarks Tekniske Universitet Søltofts Plads, Building 227, DK-2800 Lyngby, Denmark

Received 15 November 2004; received in revised form 10 January 2005; accepted 12 January 2005

First published online 21 January 2005

Edited by C. Edwards

Abstract

A hot spring in the solfataric field of Pisciarelli (Naples – Italy) was analysed for Archaeal diversity. Total DNA was extracted from the environment, archaeal 16S rRNA genes were amplified with Archaea specific primers, and a clone library consisting of 201 clones was established. The clones were grouped in 10 different groups each representing a specific band pattern using restriction fragment length polymorphism (RFLP). Members of all 10 groups were sequenced and phylogenetically analyzed. Surprisingly, a high abundance of clones belonging to non-thermophilic Crenarchaeal clusters were detected together with the thermophilic archaeon *Acidianus infernus* in this thermophilic environment. Neither *Sulfolobus* species nor other hyperthermophilic Crenarchaeota were detected in the clone library. The relative abundance of the sequenced clones was confirmed by terminal restriction fragment analyses. Amplification of 16S rRNA genes from Archaea transferred from the surrounding environment was considered negligible because DNA from non-thermophilic Crenarchaeota incubated under conditions similar to the solfatara could not be PCR amplified after 5 min.

Keywords: Thermophilic; *Sulfolobus*; Non-thermophilic archaea; Solfatara; Clone library; 16S rRNA gene; DNA stability

1. Introduction

Microbial biodiversity in extreme environments is generally considered to be scarce compared to most other environments. Although Archaea have been estimated to represent up to 20% of the biomass found on Earth [1], and thus are believed to play a major role the global ecosystem, only a few different species have so far been found and isolated from solfataric environments. Up to now, most biodiversity information about solfataric environments has been derived from isolation and cultivation studies [2]. It is, however, a general assumption that enrichment cultures underestimate the actual diversity because different organisms require different conditions to support growth [3–5]. Other studies have suggested that only as little as 0.1–1% of the prokaryotic organisms can be successfully cultivated by traditional techniques [6,7].

The kingdom of Crenarchaeota is at present divided into seven major groups: One group constituted by the cultivated thermophilic and hyperthermophilic isolates and six groups represented by sequences retrieved from low-temperature environments [8]. So far only one member of the groups from non-thermophilic environments has been cultivated (''Crenarchaeum symbiosum'') [9] but still not in axenic culture. Knowledge

* Corresponding author. Tel.: +45 45 25 61 88; fax: +45 45 88 32 76.
E-mail address: pw@biocentrum.dtu.dk (P. Westermann).

0378-1097/$22.00
doi:10.1016/j.femsle.2005.01.021

about non-thermophilic Crenarchaeota is, therefore, solely based on sequence data collected from various low-temperature environments such as soil, freshwater, deep drillings, and seawater [4,10–13].

Although there are numerous examples of different monophyletic prokaryotic groups having related ecological requirements (e.g., methanogenic Archaea and anaerobic Gram positive bacteria), there are also examples of highly diverse ecological requirements within related monophyletic groups (e.g., methanogenic and halophilic Archaea). In this study we demonstrate that members previously supposed to be limited to the low temperature Crenarchaeal cluster can be found in hyperthermophilic acidic environments in similar amounts as members of the hyperthermophilic/thermophilic Crenarchaeal cluster.

2. Materials and methods

2.1. Sediment sampling

A sample containing an estimated ratio of 1:1 hot spring water and surface sediment (0–2 cm) was collected by means of a sterile spoon from a hot spring in the Pisciarelli solfataric field of Naples (Italy). The sample was collected in 20 ml sterile bottles and distributed in aliquots for parallel analyses. All samples were kept on ice until the return to the laboratory (approximately 9 h), and samples for DNA analysis were afterwards frozen at −80 °C in 20% glycerol. The temperature of the hot spring was 80 °C and the pH was 3.0 (on-site measurements).

2.2. Nucleic acid extraction

Extraction was carried out as described by Yu and Mohn [14] with a number of minor modifications listed below. About 1 g of sample was transferred to a bead beat screw-top tube containing 1 g of 0.2 mm silica beads (Bio Spec. Products – USA). One ml of extraction buffer [14] containing 30 μl of DEPC was added and the sample was bead beated twice for 2 min with intermediate ice cooling. The extract was centrifuged at 14,000*g* for 3 min, and the DNA-containing supernatant was collected. The beating procedure was repeated with fresh buffer followed by centrifugation and pooling of the two supernatants. SDS was removed by first adding ammonium acetate to a final concentration of 2 M. The mixture was then incubated 5 min on ice followed by centrifugation for 10 min at 14,000*g* (4 °C). The supernatant was transferred to a double volume of 7 M guanidine–HCl and mixed gently, and transferred to a spin column obtained from Genomic Mini Kit – (Aabiot – Poland). Liquid was removed from the column by applying a vacuum to the column using a spin column compatible manifold, and washing was carried out as described by the manufacturer. DNA was eluted from the columns using 100 μl of Tris buffer (75 °C) after 5 min of incubation at room temperature. Four parallel samples were extracted from the same source, followed by subsequent pooling of the eluted DNA.

2.3. Clone library

Partial 16S rRNA gene sequences were amplified using Archaea specific primers Arch21F (5′-TTC CGG TTG ATC CYG CCG GA-3′) [15] and Ar9R (5′-CCC GCC AAT TCC TTT AAG TTT C-3′) (906–927 *Escherichia coli* numbering) [10] using Ready-To-Go PCR-Beads (Amersham Biosciences) in 25 μl reactions. The PCR program was initiated with 5 min denaturation at 94 °C, followed by 30 cycles of: 90 s denaturation at 94 °C – 90 s annealing at 55 °C – 90 s elongation at 72 °C. The run was terminated after a final 7 min elongation. Negative controls were prepared with both H_2O and with non-target template from *E. coli* extracted by the method described above. The PCR products were purified by cutting out the products from a 1% agarose gel using Gel-Out Kit supplied by Aabiot (Poland) as described by the manufacturer.

Cloning of the PCR product was carried out using TOPO TA cloning (Invitrogen), and successfully transformed cells were picked from LB agar plates containing 50 μg/ml kanamycin after 24 h of incubation at 37 °C.

Each clone was transferred to Euro-Taq PCR mixture (Eurogentec – Belgium) and PCR with primers Arch21F and Ar9R were carried out for all clones. The presence of a PCR-product of correct size was verified by electrophoresis of the products on a 1% agarose gel. Each product from the colony PCR was cut in parallel reactions using restriction enzymes *Alu*I and *Bsu*RI, respectively, and the pattern from each restriction reaction was visualized by electrophoresis of the product on a 3% agarose gel. The size/migration of each fragment was measured using Gel-Pro Analyzer 3.1 (Media Cybernetics), and a representative of each pattern was chosen for sequencing.

2.4. Sequencing and phylogenetic analysis

Forward and reverse sequencing were done by MWG-Biotech (Germany) using either T3/T7 or M13 uni/M13 rev sequencing primers (Invitrogen) matching sequences on the TOPO cloning vector. Contigs of forward and reverse sequences were constructed using "Cap Contig Assembly"(BioEdit) [16]. All retrieved sequences were tested for chimeric properties using the "Chimera Check" [17] function and the closest relatives were detected using the "Sequence Match" function [17] and "Blastn" from NCBI [18]. Alignment of all sequences, their closest relatives and the out-group was