A nested PCR approach for improved recovery of archael 16S rRNA gene fragments from freshwater samples

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Abstract

In a survey on the presence of archaela in a number of European lakes, it was found that known archaela primer sets for PCR were not suited for use in freshwater environment, as some lack selectivity, while others were too selective. A nested PCR was developed for denaturing gradient gel electrophoresis (DGGE) with primer sets 21F–958R and Parch519f–Arch915r, respectively. After sequencing of the DGGE bands obtained by this nested method, 93% of the sequences were of archaela origin. More diverse archaela DGGE patterns were found as compared with other PCR methods. The nested PCR-DGGE method presented here is therefore a reliable tool to analyze the archaela diversity in freshwater habitats, revealing even more widespread diversity of the archaela.

Introduction

The abundance, the high diversity and the low number of cultured archaela representatives has led to intense research efforts into this domain of life. Besides the frequent occurrence in extreme environments, archaela have been studied for their paleotemperature proxy quality (e.g. Schouten et al., 2002; Wuchter et al., 2005), their metabolism (e.g. Boetius et al., 2000; Brasen et al., 2008) and their phylogeny (Simon et al., 2000; Ochsenreiter et al., 2003). For the latter purpose, several PCR-based methods have been used.

While analyzing the archaela species composition in several European lakes, we found that the described archaela primers gave no result or were not specific enough. Using a direct PCR approach with the primer pair Parch519f–Arch915r (Coolen et al., 2004) on lake samples, we amplified 16S rRNA gene fragments of bacteria and, in particular, of cyanobacteria rather than of archaela.

To circumvent the molecular detection problems related to low numbers of free-living planktonic archaela in a stratified sulfurous lake in Spain, Lliros et al. (2008) applied different combinations of archaela primers in a nested approach. The nested approach for detecting general archaela resulted mostly in smeared and fuzzy bands.

In this article, we describe a new nested PCR approach meant to improve the selective amplification of general archaela 16S rRNA genes from freshwater environments using denaturing gradient gel electrophoresis (DGGE) analysis. We amplified the 16S rRNA genes of archaela using the primer pair 21F–958R (Delong, 1992) and used the amplicons obtained as a template in a subsequent PCR with the primer pair Parch519f–Arch915r (Coolen et al., 2004). The final PCR products were analyzed using DGGE to compare the archaela community composition of the samples taken from different lakes throughout Europe and to identify the archaela present.

Materials and methods

Sample description

For this study, we used lake sediment and water from above (t, top) and below (b, bottom) the thermocline of lakes Lago Albano (Alb), Lago Maggiore (Mgr), Lago di Motticiano...
(Monti), Lago di Vico (Vic) and Lago Trasimeno (Tsm) in Italy, Lac du Joux (Jox), Pfäffikersee (Pfa), Sempachersee (Smp) and several arms of Vierwaldstättersee (Vws) in Switzerland, Lac du Bourget (Bgt) in France, Mondsee (Mond) in Austria and Titisee (Tts) in Germany.

Depending on cell density, water samples of 1L or 2L were filtered over 0.7-μm pore glass fiber filter with a diameter of 47 mm. Sediment samples were taken from the top 2–5 cm of sediment cores. Filters were kept frozen at −20 °C, while sediment samples were kept at 4 °C during transportation to the laboratory, after which they were freeze dried.

DNA extraction

DNA was extracted following a modified protocol of Zwart et al. (2003). In brief, 0.5 g of zirconium/silica beads (0.1 mm in diameter), 0.5 mL of TE buffer [10 mM Tris (pH 8), 1 mM EDTA] and 0.5 mL of buffer-saturated phenol (pH 7–8) were added to the tubes containing a quarter of the filter with the collected cells. The tubes were vigorously shaken (5000 r.p.m.) on a Mini-Beadbeater (Biospec Products, Bartlesville, OK) four times for 30 s with intermittent cooling on ice and then centrifuged for 5 min at 9168 g. The upper, aqueous phase was collected and extracted twice with phenol–chloroform–isoamyl alcohol (25:24:1 v/v/v). The DNA was precipitated by adding 0.1 volume of 3 M sodium acetate (pH 5), 2 volumes of 96% (v/v) ethanol and 2 μL of 20 g·mL−1 glycogen, followed by incubation overnight at −20 °C and centrifugation for 30 min at 15 493 g. Subsequently, the DNA was dissolved in 100 μL of molecular biology grade water (Sigma-Aldrich, St. Louis, MO).

DNA from the sediment samples was extracted as described by Coci (2007). Briefly, 0.5 g of freeze-dried sediment was homogenized by vortexing with 1 mL CTAB buffer (Zhou et al., 1996) plus 0.5 g sterilized zirconium–silica beads (0.1 mm diameter) and subsequently subjected to disruption by bead-beating at 5000 r.p.m. two times for 30 s, with intermittent cooling on ice. After addition of 5 μL proteinase K (20 mg·mL−1), samples were homogenized and incubated for 30 min at 37 °C, with quick vortexing after 15 min. After addition of 150 μL of a 20% (w/v) sodium dodecyl sulfate (SDS) solution, samples were incubated for 1 h at 65 °C in a heat block apparatus, with quick vortexing every 15–20 min. After centrifugation at 10 000 g for 8 min, 600 μL of supernatant was collected into 2-mL clean screw-cap tubes. The rest of the sample was re-extracted with 450 μL CTAB buffer and 50 μL of 20% (w/v) SDS solution, vortexed for 10 s, incubated for 10 min at 65 °C and centrifuged at 6000 g for 10 min. Again, 600 μL was collected, added to the previous extracted supernatant, mixed with 1 mL phenol : chloroform : isooamyl-alcohol solution (25:24:1 v/v) and centrifuged at 6000 g for 10 min. One milliliter of the supernatant was collected into new screw-cap tubes containing 700 μL isopropanol and incubated for 1 h at 24 °C. After 20 min of centrifugation at 16 000 g, isopropanol was decanted and the pellet was resuspended and washed with 1 mL of 70% (v/v) ethanol of −20 °C. This was followed by 5 min of centrifugation at 15 000 g, decantation of the ethanol and drying of the pellet under vacuum centrifugation and final suspension in 100 μL molecular biology reagent water (Sigma-Aldrich).

After extraction, the DNA was purified on a Wizard column (Promega, Madison, WI) and the quantity of DNA was determined spectrophotometrically using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technology, Wilmington, DE).

PCR amplification and DGGE analysis

16S rRNA genes were amplified by PCR using different primer pairs. Table 1 gives details of the primers and the PCR conditions. PCR amplification was performed in a total volume of 50 μL containing 1× PCR Mg-free buffer, 200 μM of each deoxynucleotide, 1.5 mM MgCl2, 1.25 U of Taq DNA Polymerase (Invitrogen, Tech-Line), 400 ng μL−1 bovine serum albumin (New England BioLabs, Beverly, MA), 0.5 μM of each primer, and 5 μL of purified DNA solution as template. The reactions were performed in an MBS 0.5S

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Temperature cycling</th>
<th>Primer sequences</th>
<th>References</th>
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<tr>
<td>21F–958R</td>
<td>30 cycles of 94 °C for 45 s; 55 °C for 60 s; 72 °C for 90 s</td>
<td>21F: 5′-TTC CGG TTG ATC CYG CCG GA-3′</td>
<td>Delong (1992)</td>
</tr>
<tr>
<td>21–univ-1389-arch</td>
<td>35 cycles of 94 °C for 25 s; 50 °C for 45 s; 72 °C for 120 s</td>
<td>21F: 5′-TTC CGG TTG ATC CYG CCG GA-3′</td>
<td>Delong (1992)</td>
</tr>
<tr>
<td>344–958</td>
<td>30 cycles of 94 °C for 60 s; 56 °C for 60 s; 72 °C for 60 s</td>
<td>344: 5′-ACG GGG IGC AGC AGG CGC GA-3′</td>
<td>Raskin et al. (1994); Delong (1992)</td>
</tr>
<tr>
<td>Parch519–Arch915</td>
<td>30 cycles of 94 °C for 40 s; 57 °C for 40 s; 72 °C for 40 s</td>
<td>Parch519: 5′-CAG CCG CCG CGG TAA-3′</td>
<td>Coolen et al. (2004)</td>
</tr>
</tbody>
</table>

*A 40-bp-long GC-clamp (5′-CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG C-3′) (Muyzer et al., 1993) was attached to the 5′-end of primers ARC915r to prevent complete melting of the PCR products during DGGE.
ThermoHybaid thermocycler (Ashford, UK). Five microliters of each PCR product was visualized on 1.5% (w/v) agarose gels, stained for 1 h with 0.5 g L\(^{-1}\) ethidium bromide solution.

DGGE was carried out with a D-Code system (Bio-Rad Laboratories, CA) using an 8% polyacrylamide gel with a 20–80% gradient of a DNA denaturant agent (Schäfer & Muyzer, 2001). The gel was run at 100 V for 19.5 h at 60 °C in 0.5 × Tris-acetate-EDTA buffer. The gel was stained with 1 g L\(^{-1}\) ethidium bromide solution for 30 min. Bands were visualized under UV light and photographed using an IMAGO system (B&L Systems, the Netherlands).

**Comparative sequence analysis**

The middle of the DGGE bands was excised and eluted from the gel in molecular biology reagent water (Sigma-Aldrich) overnight at 4 °C. The DNA was reamplified with Parch519f–Arch915r primers without a GC clamp. The DNA was purified using a DNA Clean & Concentrator-5 kit (Zymo Research, CA) and sequenced by a commercial company (Macrogen Inc., South Korea).

The sequences were first compared with sequences stored in GenBank using the NBLAST tool (Altschul et al., 1997) and subsequently imported into the SILVA database (Pruesse et al., 2007) using the software program ARB (Ludwig et al., 2004). The sequences were aligned using the automatic align tool in ARB and the alignments were corrected manually. Phylogenetic trees were calculated using the neighbor-joining distance matrix method, with the Jukes–Cantor method for correction. Bootstrap analysis was performed in MEGA (Tamura et al., 2007). The sequences determined in this study were deposited in GenBank under the following accession numbers: FJ475241–FJ475349.

**Results**

The first analysis of the archaeal community in a number of European lakes, which was based on PCR-DGGE of the 16S rRNA gene, was set up with the Parch519f–Arch915r primer set of Coolen et al. (2004). Figure 1a shows a DGGE gel with bands of DNA obtained from seven different lakes amplified with this archaeal primer set. Sequencing of the bands obtained with this method from several gels and a subsequent BLAST analysis showed that only 6.7% of the bands belonged to the archaea.

In a further attempt to recover archaeal sequences, freshwater samples were subjected to PCR amplification with different primer sets. On agarose gels, PCR amplifications with different combinations of the forward primers 21, 344 and Parch519f and the reverse primers Arch915r, 958 and univ-1389-arch gave generally no band, bands at unexpected places or smears (not shown). Only the Parch519f–Arch915r primer set yielded good results on the agarose gels.

In order to obtain a more specific recovery of archaeal DNA for lake samples, the amount of magnesium chloride in the PCR mix and the annealing temperature in the PCR program were altered. As this did not yield a better result, a nested approach was tested. In the first round, a PCR protocol with primer set 21F–958R was used, as this primer set showed no occurrence of false positive bands on the agarose gel. In the second round, the Parch519f–Arch915r PCR primer set was used. The product of this nested PCR was placed on a DGGE gel (Fig. 1b) and the bands from that gel were sequenced once more. The specificity of this approach turned out to be much higher than that of the direct method. The DGGE gel after nested PCR showed a less dense banding pattern. Moreover, the number of archaeal species identified after sequencing DGGE bands was much higher. The number of nonarchaeal species found with the nested approach was very small; i.e. four out of 58 analyzed bands were of bacterial origin. In contrast, when using the Parch519f–Arch915r primer set directly on the environmental DNA, 54 out of 59 DGGE bands were recognized by a BLAST search to be of bacterial, mainly cyanobacterial, origin.
Discussion

The observation of a large number of nonarchaeal sequences when applying an archaea-specific primer set on DNA samples from a number of European lakes was in great contrast to other studies analyzing 16S rRNA genes of archaea in various environments, such as marsh water (Koch et al., 2006), paleosol (Chandler et al., 1998), gastrointestinal samples (Yu et al., 2008) and grassland soil (Nicol et al., 2003), where only archaeal 16S rRNA gene sequences were found.

When applying the primer set Parch519f–Arch915r for the first time in Antarctic Ace Lake, a meromictic saline basin, Coolen et al. (2004) obtained good, specific results. In this study, 15 archaeal sequences were recovered by DGGE, representing all visible bands on the respective DGGE. In a comparative analysis, it was shown that the melting quality of the retrieved 16S rRNA gene fragments of 396 bp was the best for DGGE analysis among the primer pairs tested. These results show that the Parch519f–Arch915r primer set is very well suited for saline water and marine environments. This is in contrast to our lake samples.

The initial choice for the Parch519f and Arch915r primers made it possible to retrieve a large number of the known archaeal sequences with a coverage of 56% and 79%, respectively. Because the Parch519f primer binds to 178 known cyanobacterial sequences and because freshwater systems generally have a large number of cyanobacteria, this primer set may have a significant bias in analysis of freshwater systems than in other environments.

The limited selectivity of the Parch519f–Arch915r primer set against bacteria was investigated more thoroughly by construction of a phylogenetic tree consigning the species found by the different methods (Fig. 2). A large part of the nonarchaeal sequences found with the direct approach are related to cyanobacteria.

The Parch519f–Arch915r primer set seemed to bind more easily to the relatively large number of cyanobacteria present in many lakes studied (e.g. Plankthorix and Synechococcus).
species). Consequently, the archaeal sequences are under-represented in PCR analyses based on the Parch519f–Arch915r primer set. After amplifying the archaeal 16S rRNA genes with the primer set 21F–958R in a first round of PCR, the competition with the cyanobacterial species present in the sample no longer biases the amplification of archaeal 16S rRNA genes.

The archaeal sequences that did emerge from the direct approach were also found with the nested approach, as they cluster together closely in the phylogenetic tree. This indicated that the nested method does not neglect the archaeal sequences found by the direct method.

The choice of a nested approach in which the second round provides the possibility to perform a DGGE using a GC clamp-enhanced primer was also used by Nicol et al. (2006). Their study, however, focused on soil crenarchaeal groups 1.1b and 1.1c. Therefore, the primer sets used by Nicol et al. (2006) in the first round of nested PCR were not applicable for a study in which a broader spectrum of archaeal lineages was to be studied. Other studies have used nested PCR methods on freshwater samples before (Jurgens et al., 2000; Abreu et al., 2001): however, with these methods, the PCR fragment obtained was larger than can be used in DGGE. Ochsenreiter et al. (2003) reported that they could not obtain pelagic freshwater amplicons with the archaeal PCR primers used in their study.

From the analysis presented here, we conclude that the proposed nested approach performs better in freshwater environments than the direct approaches described previously. This holds true for both water and sediment samples. When comparing the nested and direct methods on samples from sediment and water, it was observed that the high yield of archaea found with the nested approach is equal for water and sediment samples (results not shown).

The use of this nested PCR method will facilitate more accurate analysis of the phylogeny of archaea present in the freshwater environment. Future analysis with this method will give a good overview of archaeal species composition in lacustrine systems, revealing a more widespread nature of the archaeal community.

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