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SHORT COMMUNICATIONS

Designing and Testing Oligonucleotide Primers for Amplification and Sequencing of Archaeal 16S rRNA Genes

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The analysis of 16S rRNA genes is the main tool for investigations in molecular ecology that allows obtaining primary data on biodiversity of prokaryotes in various natural ecosystems. As distinct from the common microbiological techniques, this approach does not require isolation of pure cultures of the members of the natural microbial community, but is based on evaluation of the diversity of 16S rRNA genes (phylotypes) involved in the community. This technique is based on the amplification of a total set of 16S rRNA genes followed with cloning, sequencing, and phylogenetic analysis. It provides for the identification of microorganisms for which pure cultures are still unavailable (noncultivated microorganisms). Moreover, the high sensitivity of this technique allows the detection of very low contents of specific microorganisms, even of single cells [1].

This approach was used for the study of various marine, soil, thermal, and hypersaline habitats and led to the discovery of new phylogenetic groups of both bacteria and archaea. Multiple new noncultivated forms (phylotypes) of archaea belonging to two main phylogenetic subdivisions (*Euryarchaeota* and *Crenarchaeota*) were found in various ecosystems [2–9]. In addition to this, molecular studies of hot springs in Yellowstone National Park led to the suggestion that some newly discovered forms of noncultivated archaea may be the representatives of a new phylogenetic subdivision called *Korarchaeota* [10]. Thus, the search for new forms of noncultivated archaea in a vast range of natural habitats is a very promising line of research in the molecular ecology of prokaryotes.

The main procedure in the amplification and sequencing of 16S rRNA genes is the polymerase chain reaction performed with oligonucleotide primers specific for certain regions of the gene. The use of the so-called universal primers, corresponding to highly conserved regions of the 16S rRNA gene [11, 12], allows genes from both bacteria and archaea to be amplified and read, but the extent of universality of these primers is far from ideal. Various sets of primers specific for certain domains of prokaryotes were also designed [13, 14], but their effectiveness with respect to archaea is, according to computer analysis, lower than that of universal primers [15]. Thus, studies on the modification of previously designed primers and the design of new domain-specific primers remain pertinent.

The aim of our study was to construct a primer set that would be able to amplify and sequence the regions of archaeal 16S rRNA genes with a length suitable for phylogenetic analysis. This set was to be tested during the investigation of two prokaryotic communities of different origins: (1) An enrichment culture of thermophilic sulfate-reducing microorganisms obtained from an oil sample containing seawater [16]. The sample was taken from the White Tiger high-temperature oil field (Vietnam) from a depth of 2895 m. The outlet temperature was 106°C. The enrichment culture was grown on marine Widdel medium II containing Na-butyrate and selenium as microelements [17]. The cultivation temperature was 70°C. According to the data of preliminary morphological, cultural, and physiological analyses, these sulfate reducers belong to archaea. (2) Samples of bacterial mats from aragonite-like structures found at methane seeps in the Black Sea. The samples of bacterial mats were obtained during the 45th cruise of the research vessel *Professor Vodyanitskii* by trawling in the region of methane seeps (44°46'680 N; 31°58'970 E; depth, 180–190 m). According to the results of a preliminary microscopic and cytological study, the microbial community of these samples contains several types of cells. It was supposed that the minor components belong to (eu)bacteria and the major component belongs to archaea [18].

DNA extraction, amplification, and cloning of PCR products were performed as described previously [19]. The search for conserved regions suitable for primer design was performed using the alignments of complete nucleotide sequences of 16S rRNA genes available from the Ribosomal Database Project (RDP) [http://rdp.cme.msu.edu]. Phylogenetic screening for sequences similar to the sequenced 16S rRNA genes

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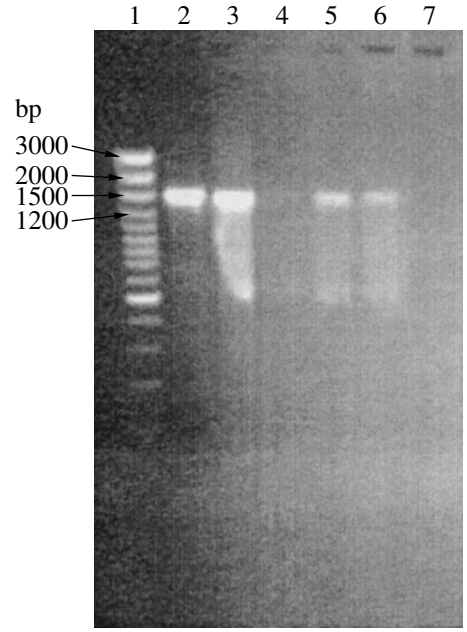

Fig. 1. Selective amplification of a long PCR fragment of archaeal 16S rRNA genes. 1, Ladder GeneRuler 100 bp DNA Ladder, MBI Fermentas, SM0321; 2, PCR on DNA from the enriched culture of sulfate-reducing archaea from a high-temperature oil field, 20 ng/reaction; 3, PCR on total DNA from the natural community of bacterial mats on aragonite coral-like structures found at Black Sea methane seeps, 20 ng/reaction; 4, PCR on *E. coli* DH5α DNA, 20 ng/reaction; 5, PCR on archaeal DNA from *Methanosarcina lacustris* ZS^T, 20 ng/reaction; 6, PCR on the mixture of *E. coli* DH5α DNA, 15 ng/reaction, and *Methanosarcina lacustris* ZS^T DNA, 5 ng/reaction; 7, control PCR without template DNA.

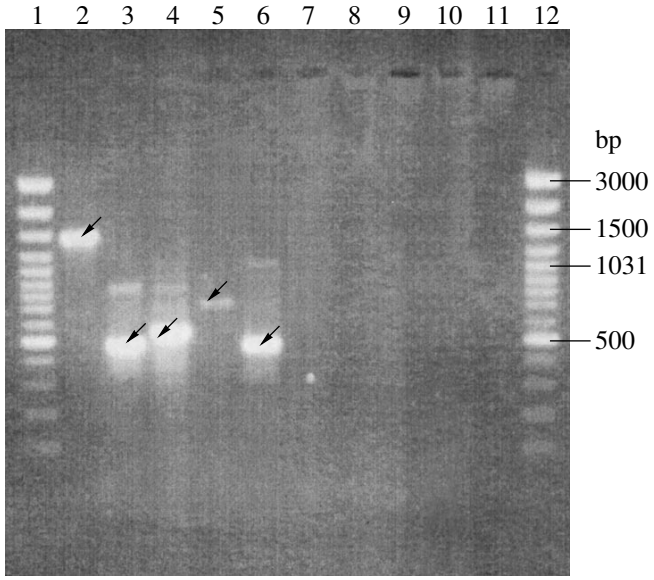

Fig. 2. Amplification of various regions of a cloned archaeal 16S rRNA gene (BSeua2 clone). 1 and 12, Ladder GeneRuler 100 bp DNA Ladder, MBI Fermentas, SM0321; 2, PCR with the A8F–1492R primer pair; 3, PCR with the A680F–A1041R primer pair; 4, PCR with the A8F–A517R primer pair; 5, PCR with the A800F–1492R primer pair; 6, PCR with the A1041F–1492R primer pair; 7, control reaction with the A8F–A517R primer pair and *E. coli* DH5α DNA; 8, control reaction with the A8F–1492R primer pair and *E. coli* DH5α DNA; 9, control reaction with the A680F–A1041R primer pair and *E. coli* DH5α DNA; 10, control reaction with the A800F–1492R primer pair and *E. coli* DH5α DNA; 11, control reaction with the A1041F–1492R primer pair and *E. coli* DH5α DNA. Arrows point on the expected PCR products.

was carried out through the GenBank database using BLASTA software [http://ncbi.nlm.nih.gov].

At the first stage, a computer analysis of complete (longer than 1500 nucleotides) 16S rRNA gene sequences available from RDP was made. The criteria of choice of the regions suitable for primer design were maximum conservativeness of the region for all archaeal genes examined and, simultaneously, its difference from the corresponding regions of (eu)bacterial genes. After a detailed analysis of aligned genes, the most conservative region at the 5'-end was chosen, which corresponded to the *E. coli* positions 8–25. Using the consensus sequence of this region, we constructed the forward primer A8F. This primer, together with the universal primer 1492R [11], was used for the amplification of a contiguous fragment of the 16S rRNA gene corresponding to an almost complete gene sequence. In addition to A8F, a set of forward/reverse

Oligonucleotide primers for selective amplification and sequencing of archaeal 16S rRNA genes

Primer	Sequence	Position, *E. coli* nomenclature
A8F	5'-TCCGGTTGATCCTGCCGG-3'	8–25
A517R	5'- GGTRTTACCGCGGCGGCTGAC-3'	537–517
A680F	5'-CSRGGGTAGGGGYGAAATCC-3'	680–699
A680R	5'-GGATTTCRCCCCTACYCCSG-3'	699–680
A800F	5'-GTAGTCCYGGCYGTAAAC-3'	800–817
A800R	5'-GTTTACRGCCRGGACTAC-3'	817–800
A1041F	5'-GAGAGGWGGTGCATGGCC-3'	1041–1058
A1041R	5'-GGCCATGCACCWCCTCTC-3'	1058–1041

primers was designed, which allows us to amplify and sequence short fragments involving different regions of the archaeal gene. The sequences of these primers are given in the table.

The specificity of the primer pair A8F–1492R was checked using the following DNA samples: enrichment culture, natural community, *Escherichia coli* DH5α (in all cases, 20 ng DNA per the reaction mixture, 25 μl), the archaea on *Methanosarcina lacustris* ZS^T (20 ng), and a mixture of the DNAs of *E. coli* DH5α (15 ng) and *M. lacustris* ZS^T (5 ng). The results of PCR are shown in Fig. 1. These data prove that even a 4-fold excess over the standard content of the DNA template had no influence on the PCR result. Simultaneously, the presence of *E. coli* DH5α (15 ng) [20] in the PCR mixture did not affect the quality of the PCR products.

A short fragment of a full-size PCR copy of the 16S rRNA gene from the enrichment culture of sulfate-reducing microorganisms was amplified using the primer pair A800F–A1041R for the estimation of the phylogenetic position of the gene. Since this fragment corresponds to a conserved region of the gene, it was possible to sequence its PCR product directly. The amplified fragment was determined to be closely related to the 16S rRNA gene of archaea of the kingdom *Euryarchaeota*; however, it remains unknown whether it belongs to a single species or represents a mixture of closely related species.

To test the designed primer set more accurately, PCR on the DNA template extracted from the natural community was performed. Long PCR gene fragments (primers A8F–1492R) were cloned in the pGEM3fz+ vector. To determine their phylogenetic position, 12 clones from the resulting library were sequenced using the A8F primer (corresponding to the 5'-end of the gene, where several variable regions are located). Upon comparison with RDP database sequences, all sequenced clones showed relatedness with archaea. This fact confirms the specificity of the designed PCR primers. Among the sequenced clones, 6 clones belonged to the *Crenarchaeota* kingdom and 6 clones to the *Euryarchaeota* kingdom. One of the clones belonging to *Euryarchaeota*, BSeua2, was chosen for further testing of inner primers, and complete sequencing of this clone was carried out. Using various combinations of forward and reverse primers from the designed set, it was possible to amplify various fragments of the 16S rRNA gene (Fig. 2). As a result, a large portion (1300 bp) of the BSeua2 sequence was read, and it corresponded to the positions 30–1450 according to the *E. coli* nomenclature. Phylogenetic analysis of this sequence showed that the BSeua2 phylotype belongs to methane-producing archaea from the kingdom *Euryarchaeota*, but it was not close to any cultivated microorganism of this kingdom.

Thus, the designed primer set is able to selectively detect and sequence 16S rRNA gene fragments whose length is enough for correct phylogenetic analysis (up to the complete copy of the gene). This fact allows these primers to be used for the investigation of microbial communities from various habitats without cultivation. This study also showed that bacterial mats from aragonite-like structures at the methane seeps in the Black Sea require additional investigations, which should involve both novel molecular ecological and common microbiological and biogeochemical methods.

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