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PCR-Based Identification of Hyperthermophilic Archaea of the Family *Thermococcaceae*

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A method for rapid detection and identification of hyperthermophilic archaea of the family *Thermococcaceae* based on PCR amplification of 16S rRNA gene fragments with primers TcPc 173F (5′-TCCCCCATAGGYCT GRGGTACTGGAAGGTC-3′) and TcPc 589R (5′-GCCGTGRGATTTCGCCAGGGACTTACGGGC-3′) was developed and used for identification of new isolates.

The family *Thermococcaceae* (order *Thermococcales*, kingdom *Euryarchaeota*, domain *Archaea* [14]) includes the phenotypically close genera *Thermococcus* (17), comprising 20 valid species, and *Pyrococcus* (6), comprising 4 valid species. *Thermococcaceae* have attracted consistent attention from researchers because of their evolutionary significance (1, 16), as well as their biotechnological potential, connected with production of thermostable enzymes. The present work focused on elaboration of a method for rapid identification of *Thermococcaceae* based on PCR amplification of 16S rRNA gene fragments with specific oligonucleotide primers.

BlastN (2) of the National Center for Biotechnology Information site (http://www.ncbi.nih.gov/BLAST/) showed that GenBank contained 142 sequences of 16S rRNA genes with 95 to 100% similarity with the 16S rRNA gene of *Thermococcus celer* (including sequences pertaining to valid species of *Thermococcus* and *Pyrococcus)*. Other GenBank sequences had less than 91% similarity with the 16S rRNA gene of *T. celer* as a query. The 142 sequences of 16S rRNA genes related to *Thermococcaceae* were retrieved from GenBank and aligned with the MultAlin program (5; http://prodes.toulouse.inra.fr/multalin/). Consensus sequences of *Archaea*, *Bacteria*, and *Eucarya* were deduced, by using a specially written program with a straightforward algorithm, from aligned small-subunit (SSU) rRNA sequences available from RDP, release 8.0 (9), at the website http://rdp.cme.msu.edu/html/ in files SSU_Prok.gb and SSU_Euk.gb (1,107 archaeal sequences apart from *Thermococcaceae*, 15,104 bacterial sequences, and 2,054 eukaryotic sequences). The MultAlin-generated consensus sequences of *Thermococcaceae* were aligned, again with the use of MultAlin, with consensus sequences of *Archaea*, *Bacteria*, and *Eucarya* and examined for sites that are conserved among *Thermococcaceae* and that at the same time exhibit strong signatures at and/or close to the extending (3′) ends of prospective primers. The sites corresponding to primers TcPc 173F and TcPc 589R were finally chosen for this work. As seen from Fig. 1, most of the nontarget sequences belonging to the groups whose rRNA genes could be aligned with *Thermococcaceae* at the site of interest exhibit mismatches with primer TcPc 173F in positions 1, 2, and 4, counting from the 3′ end of the primer, and mismatches in positions 1 and 4 with primer TcPc 589R; this should be sufficient for reliable discrimination. However, in higher-level consensus sequences of *Archaea* and *Bacteria*, the above-mentioned alignment positions were strongly degenerated (occupied by Ns), making final conclusions on the primer specificity impossible. Therefore, assessment of the affinity of candidate primers for individual sequences of SSU rRNA genes was performed with individual 16S rRNA gene sequences of *Thermococcaceae* and individual SSU rRNA sequences available from RDP, release 8.0, in the aforementioned files SSU_Prok.gb and SSU_Euk.gb. The candidate primers were applied to all sites (taken with a 1-nucleotide step) of all sequences (with the help of a specially written program with a straightforward algorithm). The affinity of a primer for a particular sequence was expressed in terms of the number of mismatches and of the total weight of mismatches recorded for the sequence site exhibiting the highest affinity. Weighting of mismatches was undertaken because the discriminating ability of a mismatch in a 3′-to-5′ exonuclease-free reaction mixture is higher the closer the mismatch is to the extending (3′) end of the primer (see, e.g., references 7 and 8). It was performed with the geometric progression formula $w_i = w_1 \times q^{(i-1)}$, where w_i is the weight of a given mismatch, w_1 is the weight of the 3′-terminal mismatch, i is the position number of the mismatched nucleotide (counting from the 3′ end of the primer), and q is a coefficient specifying the decrease in the mismatch weight with each step from the 3′ primer end to the 5′ primer end. The value of w_1 was chosen in such a way as to make the average value of the weights of all possible mismatches equal to 1. This condition is fulfilled if the weight of the 3′-terminal mismatch is specified as $w_1 = N \times (q-1)/(q^N-1)$, where N is the total number of nucleotides in the primer. Thus, the formula used was as follows:

$$w_i = N \times (q-1)/(q^N-1) \times q^{(i-1)} \quad (1)$$

Three variants of formula 1 were used, differing in the q value (0.95, 0.9, and 0.85). In silico testing of a number of candidate primers led us to choose the primer pair TcPc 173F-TcPc 589R, since these primers exhibited low affinity for nontarget sequences in terms of both unweighted mismatches and mismatches weighted by any of the three variants of the geometric

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Primer TcPc 173F,  5'->3'            TCCCCCATAGGYCTGRGGTACTGGAAGGTC
                                     ||||||||||||||||||||||||||||
Valid TcPc species, 90%, +, 3'->5'   AGGGGGTATCCRGACYCCATGACCTTCCAG
96 relatives,       90%, +, 3'->5'   AGGGGGTATCCGGACYCCATGACCTTCCAG
Archaea,            85%, +, 3'->5'   ADGRSSTATYBNVWNHHNHvGACCTTrCba


Primer TcPc 589R,  3'->5'            CGGGCATTCAGGGACCGCTTTAGRGTGCCG
                                     ||||||||||||||||||||||||||||||
Valid TcPc species, 95%, -, 5'->3'   GCCCGTAAGTCCCTGGCGAAATCYCACGGC
96 relatives,       95%, -, 5'->3'   GCCCGTAAGTCCCTGGCGAAATCYCACGGC
Archaea,            90%, -, 5'->3'   yYBdDYMAGTYYBYBSkKAAAKYYNNNNGC
Bacteria,           90%, -, 5'->3'   hHNdDYRMGYYdkNBGtKAAADBYYNBRGC
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FIG. 1. Primers TcPc 173F and TcPc 589R and the corresponding sites of consensus sequences of 16S rRNA genes of organisms related to *Thermococcaceae* and of consensus sequences deduced for *Archaea* (without *Thermococcaceae*) and *Bacteria.* 173 and 589 refer to the locations of the sites in the 16S rRNA molecules (*Escherichia coli* numbering). The percentages show the consensus levels. + and −, plus and minus DNA strands, respectively. Mismatches are in lowercase. The site 173 to 193 is highly variable among *Bacteria* and *Eucarya*, including variations in size; therefore, the consensus for *Thermococcaceae* in this region could be compared only with the archaeal consensus. The site 589 to 618 is highly variable among *Eucarya*, including variations in length, making comparison with *Eucarya* at this site impossible.

progression formula. Apart from a few environmental clones of 16S rRNA genes, the nontarget organisms exhibiting the highest affinity for the forward primer were *Archaeoglobus fulgidus* and *Acidianus infernus*; *Acidilobus aceticus* was the organism showing the highest affinity for the reverse primer, and *Archaeoglobus fulgidus* exhibited the highest affinity for the primer pair (Fig. 2). These cultures were used along with other negative controls during in vitro verification of primer specificity.

Tests of the primer specificity used DNAs of the following archaeal and bacterial strains: positive controls *Thermococcus celer* DSM 2476^T, *Thermococcus pacificus* DSM 10394^T, *Thermococcus litoralis* DSM 5473^T, *Thermococcus sibiricus* DSM 12597^T, *Thermococcus gorgonarius* DSM 10395^T, *Thermococcus peptonophilus* DSM 10343^T, *Thermococcus alcaliphilus* DSM 10322^T, *Thermococcus profundus* DSM 9503^T, *Thermococcus chitonophagus* DSM 10152^T, and *Pyrococcus woesei* DSM 3773^T and negative controls *Acidianus infernus* DSM3191^T, *Haloferax mediterranei* DSM 1411^T, *Methanopyrus kandleri* DSM6324^T, *Methanococcus jannaschii* DSM2661^T, *Sulfolobus solfataricus* DSM1616^T, *Archaeoglobus fulgidus* DSM 4304^T, *Methanosarcina barkeri* DSM 800^T, *Desulfurococcus amylolyticus* DSM 3822^T, *Acidilobus aceticus* DSM 11585^T, *Thermoanaerobacter wiegelii* DSM 10319^T, *Thermotoga maritima* DSM 3109^T, *Thermoterrabacterium ferrireducens* DSM 11255^T, and *Moorella glycerini* DSM 11254^T. The strains were grown on media and under conditions recommended by the Deutsche Sammlung von Mikroorganismen und Zellkulturen. DNA was isolated by a standard procedure (10).

PCR was run in a reaction mixture (20 μl) containing 1 μl of template DNA, primers (0.1 μM each), 2 μl of 10× PCR buffer, 1.0 mM $MgCl_2$, deoxyribonucleotide triphosphates (200 μM each), and 1.5 U of *Taq* DNA polymerase (set #K0163; Fermentas, Vilnius, Lithuania) on a Tertsik multichannel thermal cycler (DNK-Tekhnologiya, Moscow, Russia). The temperature profile was as follows: initial DNA denaturation for 3 min at 94°C, 25 to 30 cycles of denaturation at 92°C for 20 s and primer annealing and extension at 72°C for 1 min 40 s, and final extension for 5 min at 72°C. Amplification products (10 μl) were visualized in agarose gel according to standard protocols (3).

In specificity tests, the annealing temperature was varied from 65 to 75°C. A single PCR product of the expected size (about 400 bp) was produced on the DNA of all *Thermococcaceae* strains at all annealing temperatures tested except 75°C, at which no amplification occurred. No products were formed on the DNAs of negative controls in the entire range of the annealing temperatures tested. For further work, the annealing temperature of 72°C was chosen as providing a safety margin for primer specificity.

The proposed primer pair TcPc 173F-TcPc 589R was used for identification of hyperthermophilic isolates maintained at

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Primer TcPc 173F, 5'->3'          TCCCCCATAGGYCTGRGGTACTGGAAGGTC
Corresponding sites, +, 3'->5':
Archaeoglobus fulgidus            AGGGGGTATCCcctaCCCATGACCTTaCAG  5 ( 4.5)
Acidianus infernus                AGGccccgTCCGcttCCCATGACCTTgCAG  9 ( 5.3)
Acidilobus aceticus               AGGGGcTATCCGctCCCCcgGACCTTgCca  8 (11.7)

Primer TcPc 589R, 3'->5'          CGGGCATTCAGGGACCGCTTTAGRGTGCCG
Corresponding sites, -, 5'->3':
Archaeoglobus fulgidus            GCtgGTAAGTCCtccGgGAAATCTggCGGC  8 ( 8.3)
Acidianus infernus                cCCtGcAAGTCaCTGcttAAAgaCCcgGGC 11 (11.1)
Acidilobus aceticus               cCCCGTAAGTCCCTcctGAAAgCCCtgGGC  7 ( 5.8)
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FIG. 2. Negative controls that were deduced to be the most appropriate for the primers TcPc 173F and TcPc 589R. + and −, plus and minus DNA strands, respectively. Mismatches are in lowercase. The numbers of mismatches and, in parentheses, the total weights of mismatches according to formula 1 at $q = 0.9$ are specified on the right.

TABLE 1. Identification of new isolates by PCR with the primer pair TcPc 173F-TcPc 589R, specific for *Thermococcaceae*

Strain	Isolation source	Cultivation conditions[a]				Amplification result[b]
		Mineralization of the medium	Electron donor	Electron acceptor	Reference[c]	
SN531	Hydrothermal vent chimney, East Pacific Rise	Marine	Peptone	Fe(III)	12	+
515	Hydrothermal vent chimney, East Pacific Rise	Marine	H_2	S^0	11	+
AM4	Hydrothermal vent chimney, East Pacific Rise	Marine	CO	None	13	+
AM8	Hydrothermal vent chimney, East Pacific Rise	Marine	Peptone	S^0	11	+
SB611	Oil well, North Sea	Marine	Peptone	None	12	+
SM402	Shallow-water marine hydrothermal sediment, Kuril Islands	Marine	Peptone	Fe(III)	12	+
SM4022	Shallow-water marine hydrothermal sediment, Kuril Islands	Marine	Peptone	Fe(III)	12	+
204	Continental hot spring, Kuril Islands	Freshwater	Peptone	S^0	4	−
313	Continental hot spring, Kamchatka	Freshwater	Peptone	S^0	4	−

[a] In all cases, the media contained 200 mg of yeast extract (Sigma)/liter, the medium pH was 6.5, and the incubation temperature was 85°C.
[b] +, single band of the expected size; −, absence of any amplification products.
[c] References are sources for medium compositions.

the laboratory culture collection and morphologically resembling *Thermococcaceae* (Table 1). A specific amplification product was obtained for seven strains, four of which had been isolated and cultivated under conditions unusual for *Thermococcaceae*: strains SN531 and SM402 used Fe(III) as the electron acceptor, and strains 515 and AM4 used inorganic energy sources, H_2 and CO, respectively (M. L. Miroshnichenko, unpublished data; T. G. Sokolova, J. M. Gonzalez, N. A. Kostrikina, N. A. Chernyh, T. P. Tourova, E. A. Bonch-Osmolovskaya, and F. T. Robb, Abstr. 4th Int. Congr. Extremophiles, abstr. P275, p. 417, 2002). Identification of strain SB611, isolated from a North Sea oil well, as a representative of *Thermococcaceae* provides additional evidence of the presence of these microorganisms in high-temperature oil reservoirs (15). The sequence of the strain SB611 amplification product (GenBank accession no. AY591754) showed 99% similarity (BlastN) with that of *T. litoralis*.

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