Phylogenetic analysis of several *Thermus* strains from Rehai of Tengchong, Yunnan, China

Lianbing Lin, Jie Zhang, Yunlin Wei, Chaoyin Chen, and Qian Peng

Abstract: Several *Thermus* strains were isolated from 10 hot springs of the Rehai geothermal area in Tengchong, Yunnan province. The diversity of *Thermus* strains was examined by sequencing the 16S rRNA genes and comparing their sequences. Phylogenetic analysis showed that the 16S rDNA sequences from the Rehai geothermal isolates form four branches in the phylogenetic tree and had greater than 95.9% similarity in the phylogroup. Secondary structure comparison also indicated that the 16S rRNA from the Rehai geothermal isolates have unique secondary structure characteristics in helix 6, helix 9, and helix 10 (reference to *Escherichia coli*). This research is the first attempt to reveal the diversity of *Thermus* strains that are distributed in the Rehai geothermal area.

Key words: *Thermus*, diversity, phylogenetic analysis, RNA secondary structure.

Materials and methods

Media

Based on the medium described by Wiegel, the modified medium (MD) was prepared as follows (Wiegel 1986). Nitritotriacetate acid, 100 mg; NaCl, 8 mg; Na$_3$HPO$_4$, 111 mg; MgSO$_4$·7H$_2$O, 100 mg; KNO$_3$, 103 mg; NaNO$_3$, 689 mg; Na$_2$MoO$_4$·2H$_2$O, 0.025 mg; FeCl$_3$, 0.28 mg; CuSO$_4$, 0.016 mg; MnSO$_4$·H$_2$O, 2.2 mg; H$_3$BO$_3$, 0.5 mg; ZnSO$_4$·7H$_2$O, 0.5 mg; CoCl$_2$·6H$_2$O, 0.046 mg; CaSO$_4$·H$_2$O, 60 mg; tryptone, 1 g; and yeast extract, 1 g, were weighed. The ddH$_2$O was added up to 1000 mL, and the pH value was adjusted to 7.8–8.0 with 3 mol NaOH/L. The medium was autoclave at 121 °C for 40 min.


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Isolation of bacterial strains

As described by Chung et al. (2000), 100 mL of water was filtered through membrane filters (Gelman type GN-6; pore size 0.45 μm, diameter 47 μm), which were placed on the surface of MD agar plates containing 4% agar. These preparations were wrapped in plastic bags and incubated at 70 °C for 4 days. Cultures were purified by subculturing the laboratory and were preserved at −70 °C in MD containing 15% glycerol.

16S rRNA gene sequence determination and phylogenetic analyses

The following primers of 16S rRNA gene were designed according to Robb and Place (1995): forward primer c1 (39 bases including a polylinker) 5’-GGGATCCGCGGTGCGTGGGAC-3’ and reverse primer c2 (37 bases including a polylinker) 5’-GGCTGAGGGTGCTGCTTAC-3’, complementing positions 8–27 and 1510–1492, respectively, of Escherichia coli 16S rRNA. PCR amplification of the 16S rRNA gene was carried out in a thermocycler (iCYCLE) using the La Taq PCR kit (Takara Bio Inc., Japan). The temperature profile was set as follows: 1 cycle at 95 °C for 5 min, 30 cycles each (94 °C for 30 s, 55 °C for 30 s, 68 °C for 30 s), 1 cycle at 68 °C for 15 min, and a final soak at 4 °C for 15 min. The PCR product was ligated with pCR4-TOPO vector using the TA cloning kit (Invitrogen, USA). A reaction solution (2 μL) was transformed into the competent cell TOP10. The recombinant plasmid was isolated using the Qiagen spin Miniprep kit. Sequences were finished by Shimadzu corporate and the full-length sequences were assembled by program Editseq 4.01 and Seqman 4.03 (DNASTAR Inc., Madison, Wisconsin, USA). The 16S rDNA sequences obtained in this study were aligned against previously determined sequences in the public databases by the BLASTn program in NCBI. The 16S rDNA sequences of other species of Thermus and (or) Meiothermus (Nobre et al. 1996; Chung et al. 2000) available from the public databases were chosen for phylogenetic analysis. A phylogenetic dendrogram was generated by using the treeing algorithm contained in the ClustalX v1.8 (Thompson et al. 1997) and TreeView v1.5 package (Page 1996).

Nucleotide sequence accession numbers

The strain designations and accession numbers of the 16S rDNA and 16S rRNA reference sequences used in the phylogenetic and secondary structure analyses are Meiothermus ruber DSM 1279T, L09672; M. cerbereus GY-1T or DSM 11376, Y13594; M. chlorophilus ALT-8T or DSM 9957T, X84212; M. silvanus VI-R2T or DSM 9946T, X84211; M. taiwanensis WR-220, M. cerbereus M. chlorophilus and M. silvanus.

Fig. 1. Neighbor-joining tree of Thermus spp. 16S rDNA. The Thermus isolates from Rehai are underlined. Four branches formed by strains from Tengchong were labeled A, B, C, and D. A total of 1000 bootstrap replicates were performed. The sequences of Meiothermus were used as the outgroups in the phylogenetic analysis.

16S rRNA secondary structure prediction

The 16S rRNA secondary structures were predicted by using free-energy minimization algorithm with the program RNA Structure 3.71 (Mathews et al. 1999) at positions from 70 to 100 and from 178 to 220 (refer to the positions in the E. coli 16S rRNA). Program RnaViz (De Rijk and De Wachter 1997) was used to display the model structures. The structures of helix 6, helix 9, and helix 10 focused on the appearance of their internal loop, terminal loop, and the number of base pairs in the helix.

Results

Isolation of strains and morphological characteristics

Twenty isolates were obtained from water samples from 10 hot springs. These isolates formed yellow or orange, round, smooth colonies with a diameter of 2–4 mm. Short rods of 0.5–0.8 μm in width and 3.0–8.0 μm in length were seen by phase-contrast microscopy. These strains were not motile and no spores were produced. In this study, six strains (named RH-0401 to RH-0406) isolated from different hot springs were selected for 16S rRNA sequence determination and analysis.

16S rRNA gene sequence determination and analysis

The almost complete 16S rRNA gene sequences approximate to 1475 nucleotides were determined for the six Thermus strains. Then the sequences were deposited into NCBI GenBank under accession Nos. AY731822 for RH-0401, AY731823 for RH-0402, AY731824 for RH-0403, AY731825 for RH-0404, AY731826 for RH-0405, and AY731827 for RH-0406. The sequences were aligned against other sequences in the public databases by the BLASTn pro-
The 16S rRNA gene sequence similarity values among the nine strains isolated from the Rehai geothermal area and the other validly described Thermus species were in a range of 92%–99%. Phylogenetic analysis showed that the partial Thermus 16S rDNA sequences from the Rehai geothermal area formed four branches in the phylogenetic tree (Fig. 1). Branch A included strains RH-0406 and RH-0405, which was close to T. oshimai (similarity 98%). Branch B included strains RH-0403 and RH-0404, which clustered with "T. rehai" (similarity 99%), and their similarity to T. thermophilus was 97%. Branch C included strains RH-1214 and RH-1514 and appeared to form the monophyletic branch among other Thermus species (similarity 96%). Branch D included RH-0401 and RH-0402 and was very close to T. brockianus (similarity 99%). These four monophyletic branches were supported with bootstrap values of 95.9%, 98.9%, 98.0%, and 98.5%, respectively.

### Secondary structure comparison

Comparison of the 16S rRNA secondary structures showed that the strains RH-1214, RH-1514, RH99-02, RH-0401, and RH-0402 had unique structural features at positions 178–220 (Fig. 2). At positions 178–198 (helix 9) characterized by the appearance of the internal loop (four bases), these were more similar to T. filiformis than to other taxa of genus Thermus. But there were differences in the number of base pairs of the helix region between the internal loop and the terminal loop. Another helix (helix 10) at positions 199–220
Table 1. Comparison of special fragments of 16S rDNAs and their secondary structure at helices 6, 9, and 10 in *Thermus* spp.

<table>
<thead>
<tr>
<th>Branch</th>
<th>Species</th>
<th>Nucleic acid sequence</th>
<th>Secondary structure characteristics$^a$</th>
<th>Internal loop</th>
<th>Terminal loop</th>
<th>Nucleic acid sequence</th>
<th>Secondary structure characteristics$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>—</td>
<td><em>T. antranikianii</em></td>
<td>GG—GTAGGTTTATGCCT—ACCC</td>
<td>8(4)</td>
<td>UGUG</td>
<td></td>
<td>GGGGTGGATAGCCCCG</td>
<td>5(5)</td>
</tr>
<tr>
<td>—</td>
<td><em>T. scotoductus</em></td>
<td>GG—GCAGGTTTATACCT—GTTC</td>
<td>8(4)</td>
<td>UGUG</td>
<td></td>
<td>GGG-TGGATAGCCCC-G</td>
<td>4(5)</td>
</tr>
<tr>
<td>—</td>
<td><em>T. filiformis</em></td>
<td>GGCTGCGGGGTTTTACTCCGGTC</td>
<td>8(5)[4]</td>
<td>UGUG</td>
<td>UUGG</td>
<td>GGG-TGAAGAG-CCC-G</td>
<td>4(5)</td>
</tr>
<tr>
<td>—</td>
<td><em>T. thermophilus</em></td>
<td>GGCCGCGGGGTTTTACTCCGGTC</td>
<td>10(5)</td>
<td></td>
<td></td>
<td>GGG-CTTTG—CCC-G</td>
<td>4(3)</td>
</tr>
<tr>
<td>A</td>
<td><em>T. oshima</em></td>
<td>GG—GTGGTTCCGCCAC—CC</td>
<td>6(4)</td>
<td>CUUG</td>
<td></td>
<td>GGGCC—AAACGCC-G</td>
<td>3(5)</td>
</tr>
<tr>
<td>A</td>
<td>RH-0405</td>
<td>GG—GTGGTTCCGCCAC—CC</td>
<td>6(4)</td>
<td>CCUG</td>
<td></td>
<td>GGGCC—AAACGCC-G</td>
<td>3(5)</td>
</tr>
<tr>
<td>A</td>
<td>RH-0406</td>
<td>GG—GTGGTTCCGCCAC—CC</td>
<td>6(4)</td>
<td></td>
<td>UUGG</td>
<td>GGGCC—AAACGCC-G</td>
<td>3(5)</td>
</tr>
<tr>
<td>B</td>
<td>“<em>T. rehai</em>”</td>
<td>GGCGGTGGGGGAAATCACTGAC—GGTC</td>
<td>9(4)</td>
<td>GUGU</td>
<td>UUGG</td>
<td>GGGGTTTATGCCC-G</td>
<td>4(6)</td>
</tr>
<tr>
<td>B</td>
<td>RH-0403</td>
<td>GGCGGTGGGGGAAATCACTGAC—GGTC</td>
<td>9(4)</td>
<td>CCUG</td>
<td></td>
<td>GGGGTTTATGCCC-G</td>
<td>4(6)</td>
</tr>
<tr>
<td>B</td>
<td>RH-0404</td>
<td>GGCGGTGGGGGAAATCACTGAC—GGTC</td>
<td>9(4)</td>
<td></td>
<td>UUGG</td>
<td>GGGGTTTATGCCC-G</td>
<td>4(6)</td>
</tr>
<tr>
<td>C</td>
<td>RH-1214</td>
<td>GGCGGTGGGGGAAATCACTGAC—GGTC</td>
<td>9(4)</td>
<td>GUGU</td>
<td>UUGUG</td>
<td>GGGGC—GA-CCC-G</td>
<td>3(4)</td>
</tr>
<tr>
<td>C</td>
<td>RH-1514</td>
<td>GGCGGTGGGGGAAATCACTGAC—GGTC</td>
<td>9(4)</td>
<td></td>
<td>UUGUG</td>
<td>GGGGC—GA-CCC-G</td>
<td>3(4)</td>
</tr>
<tr>
<td>—</td>
<td><em>T. aquaticus</em></td>
<td>GGCGGTGGGGGAAATCACTGAC—GGTC</td>
<td>9(4)</td>
<td>UGUG</td>
<td></td>
<td>GGGGT—TTGCC-G</td>
<td>3(5)</td>
</tr>
<tr>
<td>—</td>
<td><em>T. igniterrae</em></td>
<td>GGCGGTGGGGGAAATCACTGAC—GGTC</td>
<td>9(4)</td>
<td></td>
<td>UGUG</td>
<td>GGGGT-GACAG-CCC-G</td>
<td>4(5)</td>
</tr>
<tr>
<td>D</td>
<td><em>T. brockianus</em></td>
<td>GGCCATGGGGTTTTACTCCGTTGTC</td>
<td>10(5)</td>
<td>UGUG</td>
<td></td>
<td>GGGC—GAGAG-CCC-G</td>
<td>4(4)</td>
</tr>
<tr>
<td>D</td>
<td>RH-0401</td>
<td>GGCCATGGGGTTTTACTCCGTTGTC</td>
<td>10(5)</td>
<td>GUGU</td>
<td></td>
<td>GGGC—GAGAG-CCC-G</td>
<td>4(4)</td>
</tr>
<tr>
<td>D</td>
<td>RH-0402</td>
<td>GGCCATGGGGTTTTACTCCGTTGTC</td>
<td>10(5)</td>
<td>GUGU</td>
<td></td>
<td>GGGC—GAGAG-CCC-G</td>
<td>4(4)</td>
</tr>
</tbody>
</table>

$^a$The number outside the parentheses and square brackets indicates the number of base pairs in the helix. The number in the parentheses indicates the number of bases in the terminal loop. The number in the square brackets indicates the number of bases in the internal loop.
consisted of a different shorter helix region, and the sequences of the terminal loop varied from species to species. Analysis of the structure of helix 6 was conducted but not shown in this paper. In helices 6, 9 and 10 the differences in the secondary structures and nucleic acid sequences were prominent (Table 1). Based on the secondary structures of helices 6, 9, and 10, a key to the species was proposed. According to the key, the nine strains from Tengchong fall into branches A, B, C, and D. This result is consistent with the comparison of the similarity of the 16S rDNA molecules. The key was shown as follows:

1. Helix 6, 6 (4) Branch A
   a. Helix 9, STL (CCUG) *T. oshimai*
   b. Helix 9, SIL (UGUG) RH-0405, RH-0406
2. Helix 6, 9 (4)
   a. Helix 10, 4 (6) Branch B
      a) Helix 9, Internal loop (+) “T. rehai”
      b) Helix 9, Internal loop (−) RH-0403, RH-0404
   b. Helix 10, 3 (4) Branch C (RH-1214, RH-1514)
   c. Helix 10, 3 (5) *T. aquaticus*
   d. Helix 10, 4 (5) *T. ignitae*
3. Helix 6, 10 (5)
   a. Helix 10, 4 (3) *T. thermophilus*
   b. Helix 10, 4 (4) or 5 (4) Branch D
      a) Helix 9, Internal loop (+) RH-0401, RH-0402
      b) Helix 9, Internal loop (−) *T. brockianus*
4. Helix 6, 8 (4)
   a. Helix 10, 5(5) *T. antranikianii*
   b. Helix 10, 4(5) *T. scotoductus*
5. Helix 6, 8 [4(5)] *T. filiformis*
   or Helix 10, 4(5), Helix 9, Internal loop (+) *T. filiformis*

In the key, the number outside of the parentheses and square brackets indicates the number of base pairs in the helix. The number in parentheses indicates the number of base sequences in the terminal loop. The number in the square brackets indicates the number of base sequences in the internal loop. The “STL” indicates the sequence of bases of the terminal loop. The “SIL” indicates the sequence of bases of the internal loop. The opening quote indicates the appearance of the internal loop in the helix. The closing quote indicates the disappearance of the internal loop in the helix.

**Discussion**

The Rehai geothermal area is one of the most famous volcanic geothermal regions in China. Previous research had shown that some of the *Thermus* spp. were identified in those hot springs with water temperatures ranging from 50 to 80 °C and with pH ranging from neutral to alkaline. The *Meiothermus* strains (Chen et al. 2002) and thermoacidophilic archaeon *Sulfolobus tengchongensis* strains have also been isolated from hot springs (Xiang et al. 2003) in this area. da Costa et al. (2001) proposed that chemical and biological parameters of the hydrothermal area are two important factors for the distribution of *Thermus* species over the globe. The number of *Thermus* species is widely distributed, but others have a circumscribed geographical distribution. Strains of *T. filiformis* and *T. aquaticus* were only isolated from New Zealand and Yellowstone National Park, respectively. *Thermus brockianus* strains were firstly discovered in Yellowstone National Park, and similar strains have also been found in New Mexico, Portugal, and the Azores. These results indicated that factors other than mere geographical isolation might play important roles in the colonization of hot springs by different *Thermus* species (Williams et al. 1996).

In the Rehai geothermal area, the water chemistry is mainly HCO₃⁻-Cl-Na type and Cl-HCO₃⁻-Na type and the pH value ranges from 1.5 to 10.5. This varied chemical characteristics may be the main reason for the distribution of *Thermus* strains. The phylogenetic tree that was obtained showed that all nine 16S rDNA sequences of *Thermus* strains from the Rehai geothermal area fell into four distinct groupings. The phylogenetic diversity was also demonstrated by the comparison of the 16S rRNA sequence of hypervariable regions combined with the secondary structure analysis should be a very useful tool to identify the *Thermus* species. These nine Tengchong Rehai strains were isolated from hot springs with different pH values and water temperature. For the further investigation of the diversity of *Thermus* spp. in Rehai hot springs, the chemical composition of the hot spring may be a useful clue to determine what influences the distribution of *Thermus* strains.

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**References**


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