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The Phylotype of *Thermus* from the Rehai Geothermal Area, Tengchong, China

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Through enrichment on two nutrient agars, 57 *Thermus* isolates were recovered from 15 hot spring samples taken from the Rehai geothermal area, Tengchong, China. Unique growth characteristics were observed when the strains were transferred from YIM14 medium to *Thermus* medium. Phylogenetic analysis showed that the 16S rDNA sequences of the isolates and clones from the Rehai geothermal area formed a monophyletic group on the phylogenetic tree. A secondary structure comparison showed that their 16S rRNAs have unique secondary structure characteristics.

Key words: *Thermus*, phylogenetic analysis, monophyletic group

It has been well established that the genus *Thermus* is widely distributed in the neutral to alkaline hot springs of geothermal areas with water temperatures higher than 55°C (Kristjansson *et al.*, 1983; Williams *et al.*, 1992; da Costa *et al.*, 2001). Strains of *Thermus* are not restricted to natural environments. Many strains have been recovered from artificial environments (Brock and Freeze, 1969; Ramaley and Bitzinger, 1975; Beffa *et al.*, 1996) and many others have even been isolated from abyssal geothermal sites (Marteinsson *et al.*, 1995; Marteinsson *et al.*, 1999). Phylogenetic research showed that *Thermus* spp. is abundant in many more extreme environments (Hugenholtz *et al.*, 1998; Skirnisdottir *et al.*, 2000; Hjorleifsdottir *et al.*, 2001).

In this study, we recovered *Thermus* strains from samples taken from the Rehai geothermal area on two nutrient agars. Our results show that *Thermus* spp. is widely distributed in the hot springs of this geothermal. Strains from YIM14 medium were physiologically more diverse than strains from *Thermus* medium. Phylogenetic analysis showed that all the *Thermus* 16S rDNA sequences of the isolates or clones from the Rehai geothermal area formed a monophyletic group as compared with the reference sequences of *Thermus* genus. The secondary structure comparison performed also showed that the 16S rRNAs of the monophyletic group had unique secondary structure characteristics.

Materials and Methods

Sample collection and strain isolation

Twenty sites with water temperatures ranging from 20 to 96°C and pH values ranging from 2.5 to 8.5 were sampled. Water or mat samples were collected in sterile flasks and stored at 4°C after being transported to laboratory without temperature control.

Two media were used for strain isolation, i.e., *Thermus* medium (Williams *et al.*, 1992) and YIM14 medium. The later of which had basal salt composition designed from the chemical analysis of DaGunGuo hot spring water, one of the most famous hot springs in the Rehai geothermal area (Tong and Zhang, 1989; Liao and Zhao, 1999). The YIM14 medium contained (per liter) 1000 mg of NaCl, 300 mg of KCl, 40 mg of NaF, 80 mg of H_3BO_3, 15 mg of LiCl, 3 mg of KBr, 3 mg of $(NH_4)_2SO_4$, 80 mg of $MgSO_4 \cdot 7H_2O$, 1000 mg of $Na_2S_2O_3 \cdot 5H_2O$, 100 mg of KH_2PO_4, 1600 mg of $NaHCO_3$, 1 ml of a trace mineral solution, 1 g of yeast extract (Oxoid) and 1 g of tryptone (Oxoid). The pH of the medium was adjusted to 7.5 with H_2SO_4 at 25°C before autoclaving. Trace mineral solution contained (per liter) 12.8 g nitrilotriacetic acid, 0.1 g $FeSO_4 \cdot 7H_2O$, 0.1 g $MnCl_2 \cdot 4H_2O$, 0.16 g $CoCl_2 \cdot 6H_2O$, 0.1 g $CaCl \cdot 2H_2O$, 0.1 g $ZnCl_2$, 0.02 g $CuCl_2$, 0.01 g H_3BO_3, 0.01 g $NaMoO_4 \cdot 2H_2O$, 1 g NaCl, 0.026 g $NiSO_4 \cdot 6H_2O$, and 0.02 g $Na_2SiO_3 \cdot 9H_2O$. The modified *Thermus* medium was *Thermus* medium (Williams *et al.*, 1992) supplemented with 0.1% (w/v) $Na_2S_2O_3 \cdot 5H_2O$. The methods used for bacterial isolation and enrichment were as described previously (Williams *et al.*, 1992; da Costa *et al.*, 2001), and the optimal growth temperature was determined as described by Chung *et al.* (2000).

16S rRNA gene sequence determination and phylogenetic analysis

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The bacterial genomic DNA was extracted as described previously (Ausubel *et al.*, 1998). The 16S rRNA gene was amplified by PCR (Lin *et al.*, 2002) employing primers 5'AGAGTT TGATCCTGGCTCAG 3' and 1492R 5'GGTTACCTTGTTACGACTT 3', which correspond to positions 8-27 and 1510-1492, respectively, in the *Escherichia coli* 16S rDNA (Robb *et al.*, 1995). After being purified using a gel extraction mini kit (Watson Biotechnologies, Inc), the PCR products were cloned into the pGEM-T easy vector (Promega, USA) and sequenced by the deoxynucleotide chain-termination method, using an ABI PRISM™ 377XL DNA Sequencer (Applied Biosystems, USA). The 16S rDNA sequences were aligned with a subset of 16S rDNA sequences obtained from the GenBank database by using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990) at the National Center for Biotechnology Information (NCBI). The obtained sequences and the reference sequences were aligned and compared using ClustalX (Thompson *et al.*, 1997). The phylogenetic tree was constructed from the evolutionary distance matrix calculated by the neighbour-joining method (Saitou and Nei, 1987) with Kimuras two parameter (Kimura, 1980) and illustrated using the TreeView drawing program (Page, 1996).

16S rRNA secondary structure prediction

The 16S rRNA secondary structure was predicted by using the free-energy minimization algorithm with the program RNA Structure (Mathews *et al.*, 1999). The model structure was displayed using the RnaViz program (De Rijk and De Wachter, 1997).

Nucleotide sequence accession numbers

The 16S rDNA sequences obtained in this study were deposited in the GenBank database under accession numbers AF521186 for strain RH-914, AF521187 for strain RH-1214, and AF521188 for strain RH-1514. The GenBank accession numbers of the 16S rDNA sequences of the organisms or clones used in the phylogenetic and secondary structure analysis are as follows: *T. rehai* RH99-GF7504^T (AF331969) (Lin *et al.*, 2002), DFQ6 (AY082363), DFQ28 (AY082364), DFQ34 (AY082365), DFQ37 (AY082366) (Wang *et al.*, 2002), *T. igniterrae* RF-4^T (Y18406), *T. antranikianus* HN3-7^T (Y18411), *T. brockianus* YS038^T (Y-18409), *T. scotoductus* ITI-252^T (Y18410), *T. thermophilus* HB8^T (X07998), *T. filiformis* Wai33.A1^T (L09667), *T. aquaticus* YT-1^T (L09663), *T. oshimai* SPS-17^T (Y18416) and *Aquifex pyrophilus* (M83548).

Results

Strain isolation and physiology

From 15 samples taken from water temperatures ranging from 50°C to 86°C and pH values ranging from 5.0 to 8.4, colonies with the morphology and pigmentation of *Thermus* were selected. These colonies were re-streaked onto fresh plates to ensure purity and 57 isolates were obtained. When the strains firstly isolated from YIM14 agar were transferred to *Thermus* agar and incubated at 70°C for 2-3 days, three growth types were observed. Type I represented by strain RH-1514 grew well on *Thermus* agar, type II represented by strain RH-914 grew poorly and type III represented by strain RH-1214 grew only on modified *Thermus* agar. However, strains from *Thermus* agar grew well on YIM14 agar. Interestingly, strains of type I which grew well on *Thermus* agar were all isolated from source temperatures no higher than 80°C. Strains of type II and type III were from higher temperature samples. When the strains of type III were inoculated into YIM14 medium and incubated, the cultures were turbid, while the cultures of type II and type III were clear or transparent, and formed yellow films on the broth surface. However, no differences in terms of their optimum growth temperatures were found (Table 1).

Phylogenetic analysis

Phylogenetic analysis showed that all the *Thermus* 16S rDNA sequences of the isolates or clones from the Rehai geothermal area that could be searched in the GenBank database formed a monophyletic group on the phylogenetic tree (Fig. 1). This monophyletic group was supported by a bootstrap value of 874 (total of 1000 replicates). Within this group, three major branches A, B and C, were supported by bootstrap values of 964, 1000 and 874, respectively. Branch A included the three representative strains of this study and a clone DFQ6. Branch B included the three other clones DFQ28, DFQ34, DFQ37. Branch C had only one strain RH99-GF7504^T.

The mean sequence dissimilarity (π) within branch A (π=0.0034±0.0014) and within branch B (π=0.0018±0.0004) were less than the mean sequence dissimilarity (π=0.0116±0.0013) between the two branches. Branch A and branch B diverged from branch C, with a mean sequence dissimilarity of 0.0369±0.0032. Sequence dissimilarities between the monophyletic group and the other taxa of *Thermus* phylum were all more than 4%.

Table 1. Growth characteristics of the tree physiological types

Type	Strain	Growth on *Thermus* agar	Growth on *Thermus*$^+$ agar	Culture on YIM14 medium	Temperature of sampled sites (°C)	T_{opt}(°C)
I	RH-1514	Good	Good	Turbid	55	65-70
II	RH-914	Poor	Good	Clear	84	65-70
III	RH-1214	No growth	Good	Clear	88	65-70

Thermus agar$^+$, *Thermus* agar supplemented with thiosulfate; T_{opt}, optimum growth temperature.

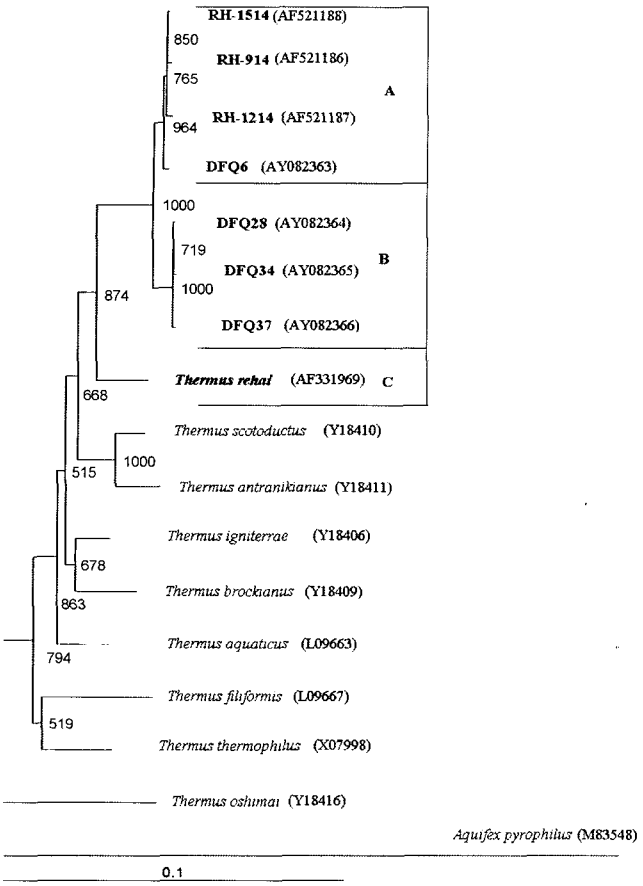

Fig. 1. Neighbour-joining tree of Rehai *Thermus* 16S rDNA. The eight validly described *Thermus* species and the one unvalidly described *Thermus* isolate from Rehai are indicated in italics. The GenBank accession numbers of analyzed sequences are shown in parentheses. The three branches are labeled A, B and C. The scale bar indicates 10 nucleotide substitution per 100 nucleotides. A total of 1000 bootstrap replicates were performed, and the bootstrap values are indicated at the branching points.

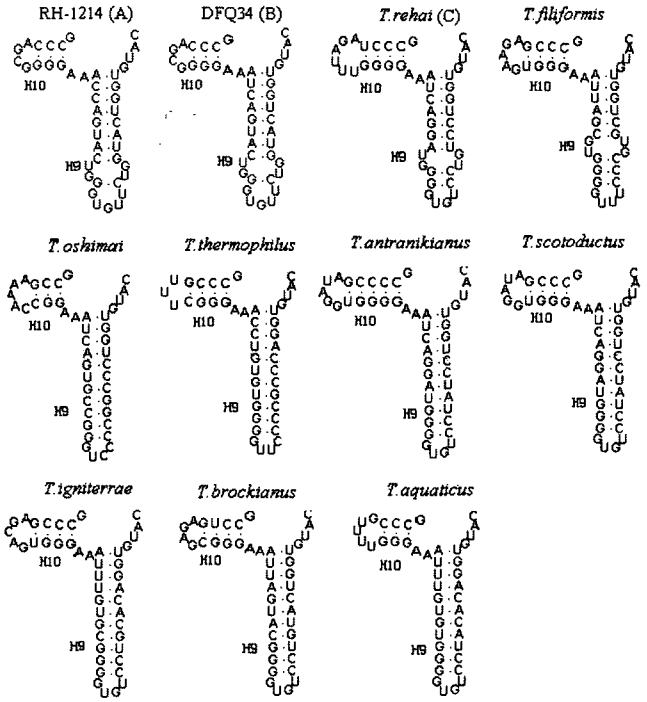

Fig. 2. Comparative secondary structures of the 16S rRNAs of Rehai *Thermus* phylotypes and some other *Thermus* species at positions 178-220 (positional and helix numbers refer to *E. coli* numbering) (Broslus *et al.*,1978) estimated using the free-energy minimization algorithm (Mathews *et al.*, 1999).

Secondary structure comparison

A comparison of the 16S rRNA secondary structures showed that the three branches A, B and C had unique structural features at positions 178-220 (Fig. 2). In helix 9, characterized by an internal loop, the three branches were more similar to *T. filiformis* than to other taxa of the *Thermus* phylum. However, for the three branches and *T. filiformis*, differences were evident in the number of base pairs of the helix region between the internal loop and the capping loop. The sequences of the capping loop were also different. Another helix at positions 199 to 220 (H10) of all the compared sequences consisted of a different short helix region, and the sequences of the capping loops also varied.

Discussion

Rehai geothermal area is a famous volcanic region in China. Hundreds of hot springs, including 20 boiling hot springs, are located in this region (Tong and Zhang, 1989; Liao and Zhao, 1999). In this study, we undertook to isolate *Thermus* strains from the Rehai geothermal area. Our results show that *Thermus* spp. is widely distributed in hot springs with water temperatures ranging from 50 to 86°C and pH values ranging from neutral to alkaline, which is consistent with previous reports (Williams *et al.*, 1992; da Costa *et al.*, 2001). The higher physiological diversity of strains from YIM14 medium than strains from *Thermus* medium demonstrates that YIM14 medium was more suitable for strain isolation from samples of the Rehai geothermal area than standard medium.

The phylogenetic similarities of the three physiological types in branch A were higher than 99%, and were in agreement with a previous report which claimed that minor differences in 16S rDNA sequences are indicative of physiological variance between *Thermus* strains (Saul *et al.*, 1997). Growth enhancement by thiosulfate is a rare characteristic of *Thermus* strains, and has been reported only once (Skirnisdottir *et al.*, 2001). The growths of type II and type III in branch A of this study were also enhanced by thiosulfate. This finding demonstrates once again that the metabolism of *Thermus* spp. is more diverse than previously believed (Skirnisdottir *et al.*, 2001). Furthermore, isolates from hot springs with water temperatures higher than 80°C and of strains from moderate temperature samples had different appearances in *Ther-*

mus agar. Chemical analysis showed that the water compositions of the high temperature and low temperature hot springs in the Rehai geothermal area are different (Tong and Zhang, 1989; Liao and Zhao, 1999). Then, there is probably a chemical reason for the different appearances of the Rehai *Thermus* strains.

Thermus species seem to be distributed differently over the globe. Some are widely distributed, whereas some others seem to have a circumscribed geographical distribution (da Costa *et al.*, 2001; Williams *et al.*, 1996). Strains of *T. filiformis* have only been isolated from New Zealand, and strains of *T. aquaticus* have only been isolated from Yellowstone National Park (da Costa *et al.*, 2001). The phylogenetic tree we obtained showed that all the *Thermus* 16S rRNAs of the isolates and clones from the Rehai geothermal area formed a monophyletic group. This was also supported by 16S rRNA secondary structure comparisons. Costa proposed two equally possible reasons for the different distributions of *Thermus* species (da Costa *et al.*, 2001). One concerned the lack of intensive sampling and characterization of isolates or the lack of culture-independent researches. The other concerned the physical, chemical and biological parameters of the hydrothermal areas. However, the real reason remains unclear. The unique growth characteristics of branch A provides a useful clue as to how the chemical compositions of hot springs influence the metabolism of *Thermus* strains, and may also shed light on the reasons for the different distributions of *Thermus* species.

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References

Altschul, S.F., W. Gish, W. Miller, E.W. Myers, and D.J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215, 403-410.

Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl. 1998. Short protocols in molecular biology, 3rd ed. p. 39-40. Science Press, Beijing.

Beffa, T., M. Blanc, P.F. Lyon, G. Vogt, M. Marchiani, J.L. Fischer, and M. Aragno. 1996. Isolation of *Thermus* strains from hot composts (60 to 80). *Appl. Environ. Microbiol.* 62, 1723-1727.

Brock, T.D. and K.L. Freeze. 1969. *Thermus aquaticus* gen. n. and sp. n., a nonsporulating extreme thermophile. *J. Bacteriol.* 98, 289-297.

Broslus, J., J.L. Palmer, J.P. Kennedy, and H.F. Noller. 1978. Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli. Proc. Natl. Acad. Sci. USA.* 75, 4801-4807.

Chung, A.P., F.A. Rainey, M. Valente, M.F. Nobre, and M.S. da Costa. 2000. *Thermus igniterrae* sp. nov. and *Thermus antranikianii* sp. nov., two new species from Iceland. *Int. J. Syst. Evol. Microbiol.* 50, 209-217.

Da Costa, M.S., M.F. Nobre, and F. Rainey. 2001. Genus 1. *Thermus*, p. 404-414. *In* D.R. Boone, and R. Castenholz (eds.), Bergeys mannual of systematic bacteriology, 2nd ed. Volume one: The archaea and the deeply branching and phototrophic bacteria. Springer, New York.

De Rijk, P. and R. De Wachter. 1997. RnaViz, a program for the visualization of RNA secondary structure. *Nucleic Acids Res.* 25, 4679-4684.

Hjorleifsdottir, S., S. Skirnisdottir, G.O. Hreggvidsson, O. Holst, and J.K. Kristjansson. 2001. Species composition of cultivated and noncultivated bacteria from short filaments in an Icelandic hot spring at 88. *Microbiol. Ecology.* 42,117-125.

Hugenholtz, P., C. Pitulle, K.L. Hershbergerand, and N.R. Pace. 1998. Novel division level bacterial diversity in a Yellowstone hot spring. *J. Bacteriol.* 180, 366-376.

Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* 16, 111-120.

Kristjansson, J.K. and G.A. Alfredsson. 1983. Distribution of *Thermus* spp. in Icelandic hot springs and a thermal gradient. *Appl. Environ. Microbiol.* 45, 1785-1789.

Liao, Z.J. and P. Zhao. 1999. Yunnan-Tibet geothermal belt-geothermal resources and case histories, p, 103-127. Science Press, Beijing.

Lin, L.B., C.Y. Chen, Q. Peng, K.L. Ben, and Z.M. Zhou. 2002. *Thermus rehai* sp. nov., isolated from Rehai of Tengchong,Yunnan Province, China. *J. Basic Microbiol.* 42, 337-344.

Marteinsson, V.T., J.L. Birrien, J.K. Kristjansson, and D. Prieur. 1995. First isolation of thermophilic aerobic non-sporulating heterotrophic bacteria from deep-sea hydrothermal vents. *FEMS Microbiol. Ecol.* 18, 163-174.

Marteinsson, V.T., J.L. Birrien, A.L. Reysenbach, M. Vernet, D. Marie, A. Gambacorta, U.B. Sleytr, and D. Prieur. 1999. *Thermococcus barophilus* sp. nov., a new barophilic and hyperthermophilic archaeon isolated under high hydrostatic pressure from a deep-sea hydrothermal vent. *Int. J. Syst. Bacteriol.* 49, 351-359.

Mathews, D.H., J. Sabina, M. Zuker, and D.H. Turner. 1999. Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. *J. Mol. Biol.* 288, 911-940.

Page, R.D.M. 1996. Treeview: an application to display phylogenetic trees on personal computers. *Comp. Appl. Biosci.* 12, 357-358.

Ramaley, R.F. and K. Bitzinger. 1975. Types and distribution of obligate thermophilic bacteria in man-made and natural thermal gradients. *Appl. Microbiol.* 30, 152-155.

Robb, F.T. and A.R. Place. 1995. Archaea, a laboratory mannual, Thermophiles. p. 101-105. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Saitou, N. and M. Nei. 1987. The Neighbor-joining Method:a new method for reconstructing phylogenetic tree. *Mol. Biol. Evol.* 4, 406-425.

Saul, D.J., R.A. Reeves, H.W. Morgan, and P.L. Bergquist. 1999. *Thermus* diversity and strain loss during enrichment. *FEMS Mircobiol. Ecol.* 30, 157-162.

Skirnisdottir, S., G.O. Hreggvidsson, S. Hjorleifsdottir, V.T. Marteinsson, S.K. Petursdottir, O. Holst, and J.K. Kristjansson. 2000. Influence of sulfide and temperature on species composition and community structure of hot spring microbial mats.

Appl. Environ. Microbiol. 66, 2835-2841.

Skirnisdottir, S., G.O. Hreggvidsson, O. Holst, and J.K. Kristjansson. 2001. Isolation and characterization of a mixtrophic sulfur-oxidizing *Thermus scotoductus*. *Extremophilus*. 5, 45-51.

Tao, W., L.H. Chai, C.L. Guo, X.L. Cui, and L.H. Xu. 2003. Study on nine 16S rDNA clones came from two hot springs in the west of Yunnan, *Wei Sheng Wu Xue Tong Bao.* in press.

Thompson, J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin, and D.G. Hinggis. 1997. The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 25, 4876-4882.

Tong, W. and M.T. Zhang. 1989. Geothermics in Tengchong, p. 81-100. Science Press, Beijing.

Williams, R.A.D. and M.S. da Costa. 1992.The genus *Thermus* and related microorganisms, p. 3745-3753. *In* A. Balows, H.G. Truper, M. Dworkin, W. Harder, and K.H. Schleifer (eds.), The Prokaryotes, 2nd ed. Springer, New York.

Williams, R.A.D., K.E. Smith, S.G. Welch, and J. Micallef. 1996. *Thermus oshimai* sp. nov., isolated from hot springs in Portugal, Iceland and the Azores, and comment on the concept of a limited geographical distribution of *Thermus* species. *Int. J. Syst. Bacteriol.* 46, 403-408.