Phylogeny of Twenty *Thermus* Isolates Constructed from 16S rRNA Gene Sequence Data

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The sequences of the 16S rRNA genes of 20 *Thermus* isolates were determined to a high fidelity by using automated DNA sequencing and fluorescent-dye-labelled primers. The strains tested included members of the three validly named *Thermus* species and representatives of major taxonomic clusters defined previously for this genus. The parsimony method was used to reconstruct the phylogeny of the strains from the aligned sequences, and a bootstrap analysis revealed a number of well-supported clades. Our results are not consistent with groupings inferred from numerical taxonomy data but support the conjecture that the genus *Thermus* contains more species than the three currently recognized species.

Since the original description of the genus *Thermus* by Brock and Freeze (4), the ubiquitous nature of members of this taxon has been demonstrated by the ready isolation of strains from neutral-pH thermal areas around the world (reviewed in reference 48). The genus *Thermus* represents a deep eubacterial branch (16) and contains three validly named species: *Thermus aquaticus* (4), *Thermus ruber* (26), and *Thermus filiformis* (20). Many other *Thermus* isolates have been described in some detail but have not yet been validly named; the taxonomy of the genus is still incomplete. While there is general agreement that *T. ruber* and *T. aquaticus* are taxonomically and phylogenetically distinct (17), it is still unclear whether the yellow-pigmented isolates that grow at 70°C constitute a single species or more than one species. In a numerical taxonomic study Hudson et al. (21) discerned eight species groups which were separated at a simple matching coefficient value of 65%, while Williams (47) suggested that there are at least four genospecies on the basis of DNA-DNA hybridization data and other properties. In both cases there were strong indications that strains isolated from the same thermal region shared common properties and grouped together. A comparison of results is difficult because different strains have been used in different studies.

In this study the sequences of the 16S rRNA genes of 20 *Thermus* strains were determined by using a procedure developed specifically to provide high-fidelity data with an automated DNA sequencer, and the aligned sequences were subjected to phylogenetic analysis. The complete 16S rRNA gene sequence of "*Thermus thermophilus*" HB8 (29) and a partial sequence of *T. aquaticus* (45) were available from other sources, but the results described below were derived from our own sequence versions. The 20 strains included representatives of the major clusters defined in the study of Hudson et al. (21) and the three validly named species, as well as two "*T. thermophilus*" strains (HB8 and HB27) and "*Thermus flavus*."

Phylogenetic systematics is a two-stage process. The first stage is the estimation of a phylogenetic tree, and for this we used aligned sequence data and reconstructed the phylogeny by using the criterion of maximum parsimony (44). The

second stage is the translation of the phylogenetic tree into a classification; at the present time there are two methods available for doing this, and they have been described in full by Wiley (46). One approach is the method of "subordination," in which every nested clade is given a new taxonomic rank. The problem with this procedure is that depending on the number of taxa and the topology of the phylogenetic tree, the number of ranks can exceed practical limits. The method used in our analysis is the "phyletic sequencing" method, in which all clades that arise from the same major branch or "trunk" of a phylogenetic tree are recognized as having the same taxonomic rank. This method circumvents the problem of having too many ranks, and as a result, the method has been adopted as the de facto standard.

Our results indicate that there is significant cladistic structure in the data with statistically supported clades. Although these clades largely disagree with the numerical classification data (21), their presence supports the suggestion that the genus *Thermus* may contain more species than the three currently recognized species.

MATERIALS AND METHODS

Design of oligonucleotide primers. The sequences of the 16S rRNA genes of eubacteria belonging to a variety of genera were aligned by using the multiple-sequence alignment program PILEUP (13). The genes chosen for alignment were from *Escherichia coli* (10), "*T. thermophilus*" (29), *Bacillus subtilis* (15), *Clostridium perfringens* (14), and *Anaerocystis nidulans* (25). The primer sequences used were based on regions with high levels of homology which were optimally spaced for DNA sequencing (Table 1).

Bacterial strains, media, and culture conditions. The *Thermus* strains used in this study are listed in Table 2. M13 mp10 recombinant phages were grown in *E. coli* JM101 (49) which was grown in YT broth (0.8% Bacto Tryptone, 0.5% yeast extract, 0.5% NaCl). The *Thermus* species were grown at temperatures appropriate for each isolate (Table 2) in 162 salts (6) supplemented with 0.3% Bacto Tryptone and 0.3% yeast extract. Solid media contained 2.7% Davis agar. For M13 recombinants, 5-bromo-4-chloro-3-indoxyl- β -D-galactoside and isopropyl-1-thio- β -D-galactopyranoside were added to solid YT media to final concentrations of 20 and 4 μ g/ml, respectively.

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TABLE 1. Positions and sequences of primers used for PCR isolation and sequencing of *Thermus* 16S rRNA genes

Primer	Strand	Position ^a	Sequence
PCR1	F	(1→21)	TTGTTGGAGAGTTTGATCCTG
PCR2	R	(1519→1494)	AAAGGAGGTGATCCAGCCGCACCTTC
RNA1	F	337→356	ACTCCTACGGGAGGCGAGCAG
RNA2	F	516→535	GCCAGCAGCGCGGTAAATAC
RNA3	F	784→805	GGATTAGATACCC(G/T)(G/T)GTAGTCC
RNA4	F	1086→1105	GGGTTAAGTCCCGCAACGAG
RNA5	R	356→337	CTGCTGCCCTCCCGTAGGAGT
RNA6	R	535→516	GTATTACCGCGGCTGCTGGC
RNA7	R	805→784	GGACTAC(C/A)(C/A)GGGTATCTAATCC
RNA8	R	1105→1086	CTCGTTGCGGGACTTAACCC

^a The positions of primers RNA1 to RNA8 are the coordinates of the primers on the 16S rRNA sequence of *E. coli* (10). The sequences of PCR1 and PCR2 were derived from the 16S rRNA sequence of "*T. thermophilus*" (29), and the positions of these primers are the positions on the gene of this organism.

Genomic DNA preparation, PCR isolation, and cloning of 16S rRNA genes. Total cellular DNA was isolated from 30- to 50-ml *Thermus* cultures by using a minor modification of the method of Saito and Miura (36). Polymerase chain reactions (PCR) for 16S rRNA genes were carried out by using 10 pmol of primers PCR1 and PCR2 (Table 1) and 0.2 U of *Taq* polymerase (AmpliTa; Cetus Corp.) in 50-μl portions of a buffer containing deoxynucleoside triphosphates at concentrations of 250 μM, 2.5 mM MgCl₂, 10 mM Tris (pH 8.8), 50 mM KCl, and 0.001% gelatin. The following thermal cycle was used for 30 cycles: 94°C for 1 min, 57°C for 1 min, and 72°C for 4 min. Each amplified fragment was "polished" and phosphorylated by using standard techniques described by Maniatis et al. (27). The fragments were gel purified on 1% agarose (acetate) gels, removed from the agarose with Gene-Clean (Bio 101, San Diego, Calif.), extracted once with phenol, and inserted into *Sma*I-cut M13 mp10 (28) by using T4 DNA ligase.

Synthesis and dye labelling of oligonucleotides. All oligonucleotides were synthesized with an Applied Biosystems model 392 synthesizer by using the cyanoethyl phosphoramidite method with Aminolink 2 attached at the 5' terminus (1a). Four 10-nmol portions of each oligonucleotide were labelled with 0.2-mg portions of the four fluorescent dyes used by Applied Biosystems automated sequencers under the conditions specified by the manufacturers. Excess label was removed from the labelled oligonucleotides by precipitation in ethanol. Pellets were extensively washed, dried, and finally resuspended in 200 μl of 10 mM Tris-1 mM EDTA (pH 7.6); 1 μl of a 50-fold dilution of this stock solution was found to be close to the correct concentration for sequencing reactions, although some optimization was occasionally required.

DNA sequencing. Sequencing reactions were carried out by using an ABI Catalyst 800 robotic workstation and an ABI model 373A automated sequencer. In general, the

TABLE 2. Bacterial isolates used in this study

Organism	Abbreviation ^a	Source	Culture collection no. ^b	Growth temp (°C)	Accession no. ^c	Reference
" <i>T. thermophilus</i> " HB8	HB8	Hot spring, Japan	ATCC 27634	75	X07998	30
" <i>T. thermophilus</i> " HB27	HB27	Hot spring, Japan		70	L09659	24
" <i>T. flavus</i> " AT-62	" <i>T. flavus</i> "	Japan	ATCC 33923	75	L09660	35
<i>T. ruber</i>	<i>T. ruber</i>	Kamchatka, USSR	DSM 1279	50	L09672	26
<i>T. aquaticus</i> YT-1	<i>T. aquaticus</i>	Yellowstone Park, Wyo.	ATCC 25104	70	L09663	4
<i>Thermus</i> sp. strain NMX2 A.1	NMX2	Jemez, N. Mex.	TG 263	70	L09661	18
<i>Thermus</i> sp. strain YSPID A.1	YSPID	Octopus Spring, Yellowstone Park, Wyo.	TG 287	70	L10070	18
<i>Thermus</i> sp. strain ZHGI A.1	ZHGI	Haegindi, Iceland	TG 201	70	L09664	18
<i>Thermus</i> sp. strain ZFI A.2	ZFI	Fludir, Iceland	TG 166	70	L09662	18
<i>Thermus</i> sp. strain ZHGI A.4	ZHGI	Haegindi, Iceland	TG 218	70	L10071	18
<i>Thermus</i> sp. strain Fiji3 A.1	Fiji3	Savu Savu Beach, Fiji	TG 113	70	L10067	18
<i>Thermus</i> sp. strain Rt41A	Rt41A	Kuirau Park, Rotorua, New Zealand	TG 52	72	L09669	32
<i>T. filiformis</i>	<i>T. filiformis</i>	Waimangu, New Zealand	TG 80	70	L09667	20
<i>Thermus</i> sp. strain Tok3 A.1	Tok3	Tokaanu, New Zealand	TG 49	75	L10069	40
<i>Thermus</i> sp. strain Tok8 A.1	Tok8	Tokaanu, New Zealand	TG 12	72	L09666	19
<i>Thermus</i> sp. strain Tok20 A.1	Tok20	Tokaanu, New Zealand	TG 119	70	L09665	19
<i>Thermus</i> sp. strain W28	W28	Waimangu, New Zealand	TG 29	70	L10068	18
<i>Thermus</i> sp. strain OK6 A.1	OK6	Orakei Korako, New Zealand	TG 50	70	L09668	23
<i>Thermus</i> sp. strain T351	T351	Whakarewarewa, New Zealand	TG 8	72	L09671	18
<i>Thermus</i> sp. strain HS A.1	HS1	Hamner Springs, New Zealand	TG 271	70	L09670	18
<i>Thermotoga maritima</i> MSB-8	<i>Th. maritima</i>	Naples, Italy	DSM 3109	85	M21774	1

^a Abbreviations used in Fig. 1.

^b ATCC, American Type Culture Collection, Rockville, Md.; DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany; TG, Thermophile Group, University of Waikato, Hamilton, New Zealand.

^c GenBank accession number of the 16S rRNA gene sequence.

mobility shift file provided by Applied Biosystems for sequencing primer SP6 worked well enough for the home-made dye primers, but in some cases, existing files proved to be inadequate and we required Applied Biosystems to generate new mobility shift files specific for individual primers. The length of sequence obtained varied for each primer, but the methods which we used gave sufficient data to provide accurate, overlapping sequences of both strands of the genes. The sequences of the forward and reverse strands were generated from separate PCR isolates because the use of two or more isolates, rather than a single recombinant, highlighted errors caused by misincorporation of bases during the PCR. Any discrepancies in the forward and reverse sequences were resolved by sequencing additional recombinants. However, on a few occasions, a base could not be resolved, and we assumed that this was due to base differences between the two genes present on the *Thermus* genome (see below). In these instances, a sequence uncertainty code was used. The 5'- and 3'-terminal sequences corresponding to the PCR primers cannot be considered a reliable representation of the original genome because of the possibility of slight differences between the primers and their binding sites. These bases were not included in the computer analysis or the sequences submitted to GenBank.

Phylogenetic methods. The full-length sequences were constructed by using the GCG (Wisconsin) package of Devereux et al. (8) run on a Silicon Graphics 4D-30 workstation. Sequences were aligned by the algorithm PILEUP (13) with a low gap weight, and the 16S rRNA sequence of *Thermotoga maritima* (1) was included as an outgroup sequence so that the phylogenetic tree could be rooted. Because of the high levels of similarity among the *Thermus* sequences, the choice of alignment was clear-cut, and no corrections for secondary structure were required. Minor modifications were made for alignment of the sequence of the outgroup organism, *Thermotoga maritima*. Aligned sequences were analyzed by using the computer program PAUP 3.0 (43), which estimates a phylogenetic tree by using the criterion of maximum parsimony. All 1,515 sites were used, and gaps were treated as missing data. The tree bisection-reconnection branch-swapping option was used to search for the most parsimonious tree. A total of 100 bootstrapped trees were sampled (11) to determine a measure of the support for each clade on the tree. The neighbor-joining method of phylogenetic reconstruction (37) was also applied to the data by using the programs DNADIST (to generate evolutionary distances) and NEIGHBOR (to construct the neighbor-joining tree), which form part of the PHYLIP, version 3.5, computer package (12). Neighbor-joining bootstrapping was also performed.

Nucleotide sequence accession numbers. The nucleotide sequence accession numbers for the isolates which we studied are shown in Table 2.

RESULTS AND DISCUSSION

DNA sequencing. A number of methods are available for sequencing 16S rRNA. However, with closely related organisms, sequencing errors can lead to quite major changes in the assessed phylogeny (5). Direct sequencing of rRNA by using reverse transcriptase has been used in a number of studies, including one examining *Thermus* strains (2). The approach taken in this study was to sequence one of the two 16S rRNA genes isolated by the PCR. Several methods for automated sequencing of 16S rRNA genes were tested for reliability. An obvious technique was to sequence PCR

products directly by using internal primers and dye-labelled terminators. However, this method has inherent problems because of the nature of the 16S rRNA gene sequence. rRNA has a strong secondary structure, and after denaturation single-stranded templates derived from rRNA genes mimic portions of the secondary structure to form hairpins which inhibit polymerase extension. One solution is to use elevated reaction temperatures and a heat-stable enzyme such as *Taq* polymerase, but unfortunately, dye terminator chemistry is presently limited to reaction temperatures of less than 65°C (below the temperature optimum of *Taq* polymerase) because at higher temperatures the enzyme has difficulty incorporating dideoxynucleoside triphosphate terminators linked to a dye ligand. Although a temperature of 65°C is considerably higher than the temperatures required by mesophilic enzymes, secondary structure within templates can still be a problem. Denaturing agents such as dimethyl sulfoxide can alleviate the secondary structure problem to some extent, but in our experiments the results were still of insufficient quality for comparisons between such similar genes. The sequences reported in this paper were obtained by using rRNA-specific dye-labelled primers and standard dideoxynucleoside triphosphates, which allowed reactions to be performed at 70°C; at this temperature, the problem of secondary structure is greatly reduced. A further improvement in sequence quality was gained by cloning all PCR products in M13 vectors. This provided a more consistent sequencing template than purified PCR fragments.

Comparison of two 16S rRNA genes in "*T. thermophilus*" HB8. "*T. thermophilus*" has two 16S rRNA genes which have been mapped to separate *Nde*I restriction fragments of the genome (3). Consequently, it was necessary to confirm that the two genes have identical or very similar DNA sequences. The two genes of "*T. thermophilus*" HB8 were individually isolated by cutting genomic DNA with *Nde*I, and fragments 1 (706 kb) and 8b (30 kb) (3) were separated by pulsed-field gel electrophoresis. A PCR was performed with DNA present in cores removed from the agarose gel, and 500-bp sequences at the 5' and 3' ends of the two genes were obtained and compared. There were no detectable differences between the two genes in these regions (data not shown), and as these sequences cover much of the gene, including five variable regions, we inferred that it was acceptable to isolate either of the two genes at random by performing a PCR with total genomic DNA. The complete sequence of the 16S rRNA gene of HB8 has been published previously by Murzina et al. (29). However, Hartmann et al. (16) observed two discrepancies in their data, one in the V6 region and the other, a missing AC, at positions 1245 and 1246 (*E. coli* numbering). The sequence obtained by us is in accord with the results of Hartmann et al., and this version was used in our analyses.

Phylogenetic analysis. Table 3 shows the pairwise differences between the sequences, and from these data two most parsimonious trees were identified, each with a length of 682 and a consistency index of 0.603. The strict consensus tree with the percentage of bootstrapped trees supporting each clade is shown in Fig. 1. There are no substantial differences between the results obtained by the neighbour-joining method and the results obtained by using parsimony analysis, and so the neighbor-joining data are not given in this paper.

Although there appears to be superficial agreement between the clades of the phylogenetic tree and the clusters of Hudson et al. (21), inconsistencies exist (namely, the poly-

TABLE 3. Differences between sequences

Organism	No. of differences ^a	
Tok20 A.1	2	0.001
Tok3 A.1	5	0.003
Tok8 A.1	5	0.003
W28	5	0.003
OK6 A.1	5	0.003
T351	5	0.003
Rt41A	5	0.003
<i>T. filiformis</i>	5	0.003
" <i>T. flavus</i> " AT-62	5	0.003
HB27	5	0.003
HB8	5	0.003
HS A.1	5	0.003
Fiji3 A.1	5	0.003
ZHGI A.1	5	0.003
ZHGIB A.4	5	0.003
<i>T. aquaticus</i> YT-1	5	0.003
YSPID A.1	5	0.003
ZFI A.2	5	0.003
NMX2 A.1	5	0.003
<i>T. ruber</i>	5	0.003
<i>Thermotoga maritima</i> MSB-8	5	0.003

^a The values on the lower left are the absolute numbers of nucleotide differences. The values on the upper right are the numbers of differences expressed as proportions of the sequence length.

FIG. 1. Strict consensus phylogenetic tree constructed from two most parsimonious trees. The scale bar represents an expected nucleotide substitution rate of 0.01 change per site. The values on the branches indicate the levels of support derived from 100 bootstrapped trees. The shaded boxes delineate clades supported by more than 80% of the bootstrapped trees. Also included are the geographic origins of the isolates and the cluster assignments made by Hudson et al. (21) (letters in brackets). For abbreviations see Table 2.

phyly of Tok20 A.1 and Fiji3 A.1 [clustered in group G by Hudson and coworkers], the polyphyly of "*T. flavus*" and *T. ruber* [clustered in group N], the polyphyly of HS A.1 and NMX2 A.1 [clustered in group A], and the paraphyly of Tok8 A.1 and W28 [group E]. Such a result is not unexpected; phenotypic markers do not necessarily provide phylogenetic conclusions, and in some cases the groups are represented by only one or two sequences which may not be representative. The degree of correlation between the available sequence data and the clusters of Hudson et al. was tested by constraining taxa grouped in the same clusters to monophyly and then calculating the lengths of the trees generated. Six constrained trees were found, each 104 steps longer than the most parsimonious trees, implying that if we were to accept the hypothesis that the clusters defined by Hudson et al. represent monophyletic assemblages, we would have to postulate 104 more homoplastic base substitution events (parallelisms, convergences, or reversals) than is necessary in the evolution of the 16S rRNA sequences. As a consequence, there is good evidence that these clusters do not represent phylogenetic groups.

There are clear and well-supported differences between the clades on the tree, which is evident from the bootstrap values obtained. Felsenstein (11) suggested that the monophyly of a clade should be accepted only if the clade occurs in more than 95% of the bootstrapped trees. Other workers

(for example, Sanderson [38]) have argued that the 95% value is too conservative; by calibrating the bootstrap test of monophyly for four taxa, Rodrigo (33) noted that a value of 80% may be more appropriate. If a critical value of 80% is used, the clades shown in Fig. 1 emerge as being significantly supported. Three major clades (clades A, C, and H) each contain one of the currently recognized species, thus allowing for the possibility that these clades and the strains that they contain delimit the nominal species. However, it is possible to split clades A and C further. For example, according to one method of translating a phylogenetic tree into a classification (the method of phyletic sequencing [46]), the following clades may be recognized as species: clades A, D, F, and G. Because the clades are significantly supported by the bootstrap, we believe that there is initial evidence to indicate that the *Thermus* isolates used in this study represent more than three species.

The phylogenetic tree is consistent with the results of several other reports (2, 17, 41) in separating *T. ruber* from *T. aquaticus* (and by implication from other yellow-pigmented high-temperature *Thermus* strains). The validity of the *T. ruber* genospecies was established and supported by good phenotypic markers (41), although recently the membrane lipid composition of *T. ruber* was shown to be very similar to that of *T. aquaticus* (9). Williams (47), using DNA-DNA homology data, and Donato et al. (9), using lipid

analysis data, concluded that *T. aquaticus* and "*T. thermophilus*" are distinct genospecies. These organisms are clearly separated in the numerical taxonomies of Hudson et al. (21) and Santos et al. (39) and in our phylogenetic tree.

Clade A and particularly subclade B in this study conform fairly closely to clusters H and E of Hudson et al. (21). However, the morphology of *T. filiformis*, a validly named species and a member of clade A, is not typical of the morphology of other organisms in the clade. The most obvious morphological characteristic of *T. filiformis* is its ability to form filaments on basal CMD medium containing 3 g of Trypticase per liter and yeast extract. Other *Thermus* species can be induced to form filaments, but only in the presence of high concentrations of glycine and other amino acids. The levels used in the induction experiments are unlikely to be found in the native habitat, and Janssen et al. (22) suggested that *T. filiformis* forms filaments by a different mechanism than other *Thermus* species. In addition, *T. filiformis* has a unique membrane lipid composition (9) and remained unclustered in the numerical taxonomy of Hudson et al. Therefore, this organism may not be the organism of choice to typify this genospecies.

There is an apparent geographical correlation within the clades which could be tested by identifying strains in their native environments. A strain present in a pool may remain undetected because it is selectively removed during enrichment, and indeed, it could be argued that the geographical grouping seen in the data may be an artifact of different approaches to enrichment. The sequence data should be useful for designing clade-specific probes as each major clade has distinctive sequence motifs that are located in definite regions within the 16S rRNA genes (data not shown). By using fluorescent-dye-labelled oligonucleotide probes capable of hybridizing with these variable regions, it should be possible to categorize individual *Thermus* cells and to examine the diversity and distribution of these organisms in nonenriched samples (7, 42).

The phylogeny inferred from the 16S rRNA data and the degree of bootstrap support for some of the major clades provide some evidence for the conjecture that there are more than three *Thermus* species. However, as other authors have argued (31, 34), evolutionary trees estimated from a single gene should be treated with caution. The degree to which lateral transfer of genetic material has occurred and the effects of ancestral polymorphism and selection cannot be readily estimated. It is only with the application of different data that we can corroborate or disprove the results of the phylogenetic analysis described above. With this aim in mind, we are sequencing the genes for *Thermus* DNA polymerases and DNA ligases, and in addition, a collaborative project is under way which will combine phenotypic, molecular, and DNA sequence data to provide a definitive taxonomy of the genus *Thermus*.

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