DNA Relatedness of *Thermus* Strains, Description of *Thermus brockianus* sp. nov., and Proposal To Reestablish *Thermus thermophilus* (Oshima and Imahori)

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Aerobic, thermophilic, gram-negative bacteria obtained from Yellowstone National Park that were placed in the genus *Thermus* on the basis of phenotypic data were examined by chemotaxonomic techniques to determine their peptidoglycan compositions, their respiratory quinones, their mean DNA base compositions, and their levels of DNA-DNA homology as determined by both the filter hybridization and reassociation rate methods. These isolates from hot springs included *Thermus aquaticus* strains and strains of a new genospecies. We propose the name *Thermus brockianus* for this new genospecies; strain YS38 is the type strain of this taxon. A collection of 10 strains, including the type strain of "*Thermus thermophilus*," which were isolated from widely separated geothermal sites, exhibited high levels of DNA-DNA homology with each other and had similar physiological properties. Therefore, we propose that the species *Thermus thermophilus* (Oshima and Imahori) should be reestablished, with strain HB8 as the type strain.

The thermophilic genus *Thermus* and its type species, *Thermus aquaticus*, were described by Brock and Freeze (3) as nonsporulating, heterotrophic, rod-shaped organisms whose optimum pH was between 7.0 and 8.0 and whose optimum temperature for aerobic growth was 70°C. The Gram reaction of these organisms is negative, and the cell walls of *Thermus* strains have an outer layer that balloons out from the peptidoglycan layer at regular intervals to give a corrugated appearance to the cell surface (47). *Thermus* strains are usually yellow pigmented because of carotenoids, but colorless and brown strains (20, 35) have been isolated from unilluminated sources (2, 31, 44). *Thermus ruber*, which contains red carotenoids and grows optimally at 60°C, has also been validly described (22, 23), but this species exhibits low levels of DNA-DNA homology with the yellow-pigmented strains (42) and is easily distinguished from them phenotypically. *Thermus filiformis* is remarkable for its unusual filamentous habit and is a validly described species (15), but a number of isolates from New Zealand exhibit high levels of DNA-DNA homology with the type strain of *T. filiformis* despite their nonfilamentous morphology (9). *Thermus* sp. strain HB8^T (T = type strain) was isolated in Japan and was originally named "*Flavobacterium thermophilum*" (28); this organism was validly redescribed as "*Thermus thermophilus*" (29), but subsequently this name was not included on the Approved Lists of Bacterial Names (43) because of the phenotypic similarity of this organism to *T. aquaticus* (6). Two names for Japanese isolates, "*Thermus flavus*" (37) and "*Thermus caldophilus*" (45), have not been validly published. Many *Thermus* strains have been isolated from thermal areas in the United States (24), Japan (46), Iceland (1, 5, 16, 19–21, 32), Belgium (6), Britain (31), New Zealand (14, 15), Portugal (39), the Czech Republic (34), Kenya (48a), and Thailand (17). Thermostable enzymes, including some of biotechnological importance, have been isolated from *Thermus* strains, but the majority of *Thermus* isolates have been described only as strains of the genus (48, 49). The only validly described species besides *T. aquaticus* are those with striking

visual properties, including the red-pigmented organism *T. ruber* (22, 23), the filamentous organism *T. filiformis* (15), and the recently described, unusual, brownish organism *Thermus scotoductus* (20). A valid taxonomy can be constructed only on the basis of detailed knowledge of large collections of isolates. In this paper we describe DNA-DNA homology data and other properties which support reestablishment of *T. thermophilus* (Oshima and Imahori) as a valid species, with strain HB8 as the type strain, and a proposal to establish a new species, *Thermus brockianus*, with YS38 as the type strain.

MATERIALS AND METHODS

Strains and their culture. The *Thermus* strains which we used and their sources are listed in Table 1. Cells were grown at 65°C in shake flask cultures in medium containing 0.3% tryptone, 0.1% yeast extract (Difco), and Castenholz mineral salts (35). Cells were harvested by centrifugation at 6,000 × g and were stored at –20°C.

Respiratory quinones. Cell pastes were freeze-dried and extracted with a 2:1 mixture of chloroform and methanol in the dark overnight (4). Each extract was filtered and concentrated under reduced pressure, applied as a band to a preparative Keisegel 60F thin-layer chromatography plate, and then developed with 15% diethyl ether in light petroleum (bp 60 to 80°C). The band at *R_f* 0.8 that absorbed UV (254-nm) light, which presumptively corresponded to menaquinones, was dissolved in chloroform, concentrated under a stream of nitrogen, and then applied to a reverse-phase thin-layer plate (type RP18F254; Merck) and developed with acetone. The *R_f* values of extracts of *Thermus* strains were observed under UV (254-nm) light and compared with the *R_f* values of the menaquinones of *Bacillus stearothermophilus* and *T. aquaticus* YT1^T. UV absorption spectra of extracts of the preparative plates in iso-octane were recorded at wavelengths between 230 and 350 nm.

Peptidoglycan preparation and analysis. Cells were extracted by the rapid peptidoglycan preparation method (41) and were hydrolyzed with 4 M HCl; the preparations were then separated by thin-layer chromatography on cellulose plates and tested for the presence of ornithine (11) and the absence of diaminopimelic acid (36). The amino acids and amino sugars in some samples were quantitatively analyzed with an LKB model 4000 amino acid analyzer.

DNA purification and mean base compositions. Cells were lysed and DNA was purified as described previously for *T. ruber* (42), except that cesium chloride density gradient centrifugation was performed for 16 h at 18°C in a Sorvall type TV-865 vertical rotor at 45,000 rpm (relative centrifugal force, 177,000 × g). The mean base composition was determined by thermal denaturation in dilute saline citrate with a Gilford Response spectrophotometer or a Pye-Unicam model 8800 spectrophotometer equipped with a model SPX876 programmed heating accessory. Mean base compositions were calculated with reference to the DNA of *Escherichia coli* DSM 2840 (G+C content, 52 mol%) by using the following

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TABLE 1. Sources of the *Thermus* isolates used

Strain(s)	Source
<i>T. aquaticus</i>	
YT1 ^T (= ATCC 25104 ^T = DSM 625 ^T = NCIB 11243 ^T).....	ATCC ^a
YS4, YS8, YS9, YS10, YS16, YS28, YS31, YS32, YS41, and YS48	R. J. Sharp
<i>T. filiformis</i> Wai33A1 ^T	ATCC
<i>T. Brockianus</i> YS7, YS11, YS19, YS36, YS37, YS38 ^T , YS44, and YS45	R. J. Sharp
" <i>T. flavus</i> " AT62 (= DSM 674 = ATCC 33932)....	T. Saiki
" <i>T. caldophilus</i> " GK24	T. Ohta
<i>T. thermophilus</i>	
HB8 ^T (= ATCC 27624 ^T = DSM 579 ^T = NCIB 11244 ^T).....	T. Oshima
RQ1 and RQ3.....	M. S. da Costa
B (= NCIB 11247)	R. A. D. Williams
ZK1, ZK2, and ZK3.....	K. Horikoshi
IB21	G. Alfredsson

^a ATCC, American Type Culture Collection.

formula: (30): $G+C \text{ content} = 52 + 2.08(T_m - T_m \text{ of DSM 2840 DNA})$, where T_m was the thermal denaturation temperature.

DNA-DNA homology. Hybridization of sheared fragments of ³H-labeled DNA with single-stranded DNA bound to 5-mm nitrocellulose discs was carried out as described previously (42). Levels of DNA-DNA homology were also determined by measuring the rates of reassociation of pairs of single-stranded sheared DNA samples (7) under optimum conditions with a Gilford Response spectrophotometer. DNA samples in dilute saline citrate were adjusted accurately to an optical density at 260 nm of 0.70 and sheared by six passages through a 0.625-in. (ca. 1.59-cm) 25-gauge needle under pressure. The fragment size range of each sheared DNA preparation was checked by electrophoresis in 0.3% agarose gels with TAE buffer (38). DNA samples of each pair of strains, together with a 1:1 mixture of each pair (300-μl volumes), were denatured by heating the preparations at 90°C in microcuvettes for 10 min. The final sodium concentration used for renaturation (0.515 M) gave an estimated T_m of 101°C according to the following equation (10): $T_m = 0.41 \times G+C \text{ content} + 78.7 + 13.3 \log[Na^+]$. Renaturation temperatures that were 23 and 27°C below the T_m gave similar homology results. The temperature of the cuvettes was reduced to the renaturation temperature, and 33 μl of 5 M sodium chloride at the same temperature was rapidly mixed into each cuvette. The decrease in A_{260} at the renaturation temperature was monitored for 30 min. The rates of reassociation were calculated over the time interval that gave the lowest level of homology with DNAs from *T. aquaticus* YT1^T and *T. thermophilus* HB8^T (from 8 to 18 min after the renaturation temperature was reached).

RESULTS AND DISCUSSION

The two instrument systems which we used yielded different T_m values for *E. coli* DSM 2840 DNA, but gave the same base compositions for *Thermus* strains when they were standardized by using the *E. coli* DNA. The mean base compositions of the DNAs of 11 strains of *T. aquaticus*, 8 strains of *T. Brockianus*, and 10 strains of *T. thermophilus* were 61 to 64, 61 to 63, and 63 to 65 mol%, respectively, values which are consistent with the values obtained for other strains of the genus *Thermus*.

The respiratory quinones were menaquinones, as judged by their R_f values on the preparative thin-layer plates and by their UV absorption spectra. The R_f values of the menaquinones of the following strains on the reverse-phase plate were identical to the R_f value of the menaquinone of strain YT1^T (menaquinone 8): *T. aquaticus* YS31, *T. Brockianus* YS38^T, YS44, and YS45, *T. thermophilus* HB8^T, B, ZK3, and IB21, and *T. filiformis* Wai33A1^T.

Our qualitative analysis confirmed that ornithine was the major dibasic amino acid in the peptidoglycan of all strains and that lysine occurred as a minor substituent (33). This characteristic distinguishes *Thermus* strains, which have peptidoglycan cross-linked by ornithine, from the thermophilic bacilli, which are sometimes yellowish when they are first isolated and

do not always sporulate freely, but for the most part have peptidoglycans containing diaminopimelic acid. The molar ratios of amino compounds to ornithine were as follows: glutamate, 1.52 to 2.35; glycine, 2.85 to 3.40; alanine, 2.02 to 3.50; and glucosamine, 0.58 to 1.20. These values agree with values obtained in previous analyses of *Thermus* strains. The rather high glutamate content and the presence of other amino acids indicate that the peptidoglycan was not free of protein.

The strains identified as members of the genospecies *T. thermophilus* exhibited high levels of intragroup DNA-DNA homology (Table 2), but low levels of homology with strains of both *T. aquaticus* and *T. filiformis*, as well as with strains belonging to the new genospecies described below as *T. Brockianus* (Table 3). The *T. thermophilus* homology cluster includes the Japanese isolates of *T. thermophilus*, "*T. flavus*," and "*T. caldophilus*," as well as the more recently isolated organisms strains ZK1, ZK2, and ZK3 from Japan (46), strains RQ1 and RQ3 from Portugal (39), strain B from a terrestrial source in Iceland (47), and strain IB21 from a submarine hot spring (21) off the coast of Iceland. This species is distributed widely in hot springs.

The results of molecular biology studies, including the catalog of oligonucleotides derived from hydrolysis of 16S rRNA (27), indicated that there is a distinct, if remote, relationship between the genus *Thermus* (represented by *T. aquaticus* and *T. ruber*) and the genus *Deinococcus* (13). Alignment of the 16S RNA gene of *T. thermophilus* HB8^T (12, 25) with other 16S RNA sequences led to the conclusion that *T. thermophilus* is a green nonsulfur bacterium, along with *Thermomicrobium roseum*, *Chloroflexus aurantiacus*, and *Herpetosiphon aurantiacus*. This is the second deepest bacterial branchpoint after *Thermotoga maritima*. Many more 16S rRNA and 16S ribosomal DNA (rDNA) sequences are now available for *Thermus* strains (25, 26, 40). The sequences of the genes for the 16S rRNAs of *T. ruber*, *T. Brockianus* YS38^T, and strains Vi7 and SPS14 (8) confirmed that the yellow-pigmented *Thermus* strains are closely related to each other, but not to *T. ruber*. The lower "thermophilic bias" evident in the sequence of the *T. ruber* gene, which is associated with the lower growth temperature of this organism compared with the yellow strains, was important in confirming the relationship between the thermophilic genus *Thermus* and the mesophilic genus *Deinococcus* (8).

The sequences of the 16S rDNAs of *T. aquaticus* YT1^T, "*T. flavus*" AT62, *Thermus* sp. strains HSA1, OK6A1, Rt41A, T351, Tok8A1, Tok20A1, ZHG1A1, and *T. filiformis* Wai33A1^T, and *T. ruber* were determined by Saul et al. (40) and deposited in the GenBank database. When these sequences were aligned with those of *T. aquaticus* YS25 and YS52 (sequences determined at the Deutsche Sammlung von Mikroorganismen und Zellkulturen for R. J. Sharp [unpublished data]), *T. thermophilus* HB8^T (12, 25), *T. thermophilus* HB27 (40), and *T. Brockianus* YS38^T (8), a region including helix 10 (Table 4) was found to be species specific. The sequence of strain HSA1 (isolated on the South Island of New Zealand [15]) is very similar to that of *T. thermophilus* HB8^T; there are only 13 differences between the two sequences. The status of "*T. flavus*," which was identified as a member of the genospecies *T. thermophilus* by DNA-DNA homology (Table 2), was confirmed by the 16S rDNA sequence of this organism, which differed from the sequence of *T. thermophilus* HB8^T (12) at only six positions. Strain HB27, a strain that is frequently used as a host for cloning in the genus *Thermus* (18), was confirmed as a member of *T. thermophilus* because the 16S rDNA sequence of this strain is identical to that of "*T. flavus*." Therefore, *T. thermophilus* seems to be a valid species that is widely distributed in thermal areas around the world.

[illegible]

TABLE 4. Sequences of the 16S rDNAs of *T. aquaticus*, *T. thermophilus*, *T. filiformis*, and *T. Brockianus* strains in the species-specific regions of helices 6 and 10

Strain or taxon	Sequence in the species-specific region of ^a :	
	Helix 6 ^b	Helix 10 ^c
<i>T. aquaticus</i>		
YT1 ^T	CGTgggg---taTCTCACg	AcACATccTGTggggGTgTTTaaagggTTTTGCC
YS25	CGTgggg---taTCTCACg	AcACATccTGTggggGTgTTTaaagggTTTTGCC
YS52	CGTgggg---taTCTCACg	AcACATccTGTggggGTgTTTaaagggTTTTGCC
<i>T. thermophilus</i>		
HB8 ^T	CGCggggTTTtaCTCCGTg	AcCCGCccCTTggggGTgTCCaaagggCTTTGCC
HB27	CGCggggTTTtaCTCCGTg	AcCCGCccCTTggggGTgTCCaaagggCTTTGCC
" <i>T. flavus</i> "	CGCggggTTTtaCTCCGTg	AcCCGCccCTTggggGTgTCCaaagggCTTTGCC
HSA1	CGCggggTTTtaCTCTGCg * *	GcCCGCccCTTggggGTgTCCaaagggCTTTGCC * *
<i>T. filiformis</i>		
Wai33A1 ^T	TGCggggTTTtaCTCCGTg	TcGTGCccTTTggggTGCgATTaaagggTGAAGAG
Tok20A1	CGCggggTTTtaCTTCGCg	TcGTGCccTTTggggTGCgATTaaagggTGAAGAG
OK6A1	TGCggggTTTtaCTTCGCg	TcGTGCccTTTggggTGCgATTaaagggTGAAGAG
RT41A	TGCggggTTTtaCTTCGCg	TcGTGCccTTTggggTGCgATTaaagggTGAAGAG
T351	TGCggggTTTtaCTTCGCg	TcGTGCccTTTggggTGCgATTaaagggTGAAGAG
Tok8A1	CGCggggTTTtaCTTCGCg * *	TcGTGCccTTTggggTGCgATTaaagggTGAAGAG
<i>T. Brockianus</i>		
YS38 ^T	CATggggTTTtaCTCCGTg	TcATGTccTGTggggCATgATTaaagggCGAGAGT
ZHG1A1	CATggggTTTtaCTCCGTg	TcATGTccTGTggggCATgATTaaagggCGAGAGT

^a The lowercase letters indicate residues that are identical in all species. The uppercase letters indicate residues that are species specific. The asterisks indicate residues that vary within a species.

^b Sequence beginning at position 51 (numbering of Saul et al. [40]).

^c Sequence beginning at position 158 (numbering of Saul et al. [40]).

The levels of DNA homology reported in Tables 2 and 3 show that the cluster 2 Yellowstone isolates form a DNA homology group that is distinct from both *T. aquaticus* and *T. thermophilus*. We propose that this group should be called *Thermus Brockianus* in honor of T. D. Brock.

There is only one difference between the 16S rDNA sequence of *T. Brockianus* YS38^T from North America and the 16S rDNA sequence of strain ZHG1A1 from Iceland (16, 40). This indicates that strain ZHG1A1 is a *T. Brockianus* strain, and this is further evidence that the geographic spread of *Thermus* species is wider than has often been thought. A description of *T. Brockianus* sp. nov. is given below.

Thermus Brockianus sp. nov. *Thermus Brockianus* (Brock' i.a.nus. N.L. adj. *Brockianus*, referring to T. D. Brock, who initiated a new era of thermophile microbiology and described the genus *Thermus* on the basis of isolates obtained from Yellowstone National Park). Aerobic, heterotrophic, thermophilic, short rods that are gram negative. Paler yellow pigmentation than *T. aquaticus*. Distinctive spreading colonial morphology occurs on tryptone-yeast extract plates at 70°C. The peptidoglycan has ornithine cross-links with glycine in the interpeptide bridge. The respiratory quinone is menaquinone 8. The G+C content of the DNA is 61 to 63 mol%. Most strains grow on fructose, galactose, and trehalose, but only a

TABLE 5. Phenotypic characteristics that distinguish type strains and clusters of *Thermus* strains

Taxon or group	Pale spreading colonies	Growth ^a on:					Hydrolysis ^a of:		
		Glucose	Fructose	Galactose	Mannose	Trehalose	Casein	Gelatin	Starch
<i>T. aquaticus</i>									
YT1 ^T	—	—	—	—	+	—	+	+	+
11 strains ^b	0	18	9	18	27	27	64	64	55
29 strains ^c	0	17	10	48	31	35	90	83	83
<i>T. Brockianus</i>									
YS38 ^T	+	—	+	+	—	+	—	—	—
8 strains ^b	87	25	87	63	25	75	0	25	25
13 strains ^c	92	23	85	69	38	77	0	15	46
<i>T. thermophilus</i>									
HB8 ^T	+	+	+	+	—	+	—	—	—
3 strains ^d	0	100	100	100	100	33	0	0	0

^a —, negative; +, positive. The numbers are the percentages of positive strains for each group (see below).

^b Strains used in DNA-DNA homology tests.

^c Strains used in phenotypic tests (21).

^d Strains HB8^T, B, and AT62.

minority of the strains grow on glucose or mannose. None of the strains hydrolyzes casein in plate assays, although a minority hydrolyze clear gelatin and starch. The type strain is strain YS38, which was isolated by R. J. Sharp from Pine Spring in Yellowstone National Park (24). Phenotypic characteristics which distinguish *T. brockianus* from *T. aquaticus* and *T. thermophilus* are shown in Table 5.

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