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

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We examined aerobic, thermophilic, gram-negative bacteria that were isolated from hot springs in Portugal and were identified as *Thermus* strains and placed in phenetic groups on the basis of their phenotypic characteristics. We determined the composition of the peptidoglycan, identified the respiratory quinones, and determined the mean base composition of the DNA, and the levels of DNA-DNA homology were determined by both the filter hybridization and reassociation rate methods. *Thermus aquaticus*, *Thermus brockianus*, and *Thermus filiformis* were not detected in this collection of organisms, although three *Thermus thermophilus* strains were identified. We propose that the isolates that belonged to phenetic clusters E and F are members of a new species, *Thermus oshimai*; the type strain of *T. oshimai* is strain SPS17.

Members of the thermophilic genus *Thermus* and its type species, *Thermus aquaticus*, are aerobic, nonsporulating, heterotrophic rods that have optimum pH values between 7.0 and 8.0 and an optimum temperature of 70°C (4). The Gram reaction is negative, and the cell wall has an outer layer that balloons out from the inner peptidoglycan at regular intervals (3, 49). *Thermus* strains have been isolated from hot springs and other thermal environments in the United States (2, 4, 5, 27, 35, 44, 53), Iceland (1, 7, 18, 21–23, 32, 42), Belgium (8), Britain (31), Portugal (26, 39, 47, 48), the Czech Republic (34), Germany (15), the former USSR (24, 25), Japan (28, 29, 37, 45, 46), New Zealand (16, 17), Thailand (20), and Kenya (51). *Thermus* isolates are usually yellow pigmented because they contain carotenoids, but colorless strains have been isolated from sources sheltered from light (2, 22, 31, 35, 39, 44). The *Thermus* species that have been described previously are *Thermus aquaticus*, *Thermus filiformis*, *Thermus thermophilus*, *Thermus scotoductus*, *Thermus brockianus*, and the red-pigmented organism *Thermus ruber*. *Thermus filiformis* has a unique filamentous habit and was validly described despite the fact that only a single isolate was available (17); however, several non-filamentous isolates from New Zealand exhibit high levels of

DNA-DNA homology with this strain (11). Strain HB8^T (T = type strain) was isolated in Japan (28) and was validly described as a *Thermus thermophilus* strain (29). Although the name *Thermus thermophilus* was not included in the Approved Lists of Bacterial Names (43), it has been proposed (26, 49–53) that this taxon should be reestablished and should include the invalid species “*Thermus flavus*” (37) and “*Thermus caldophilus*” (45). Recently, pale strain X1 (35), which turns brownish in older cultures, and similar Icelandic isolates were described as members of the species *Thermus scotoductus* (22). The species *Thermus brockianus*, which coexists with *Thermus aquaticus* in the hot springs of Yellowstone National Park, was also described recently (53). *Thermus ruber*, which contains red carotenoids and grows optimally at 60°C, has been validly described (24, 25) but is easily distinguished from the other species mentioned above phenotypically and by its lower optimum temperature; this organism exhibits very low levels of DNA-DNA homology with other *Thermus* species (42). *Thermus silvanus* and *Thermus chliarophilus* are species that are related to *Thermus ruber* and have low optimum growth temperatures (55° and 50°C, respectively) (47).

For a valid taxonomy to be established, large collections of

TABLE 1. Sources of the *Thermus* isolates used

Strain(s)	Other designation(s)	Source
<i>Thermus aquaticus</i> YT1 ^T	ATCC 25104 ^T , DSM 625 ^T , NCIB 11243 ^T	ATCC ^a
<i>Thermus filiformis</i> Wai33A1 ^T	ATCC 42380 ^T , NCIB 12588 ^T	ATCC
<i>Thermus brockianus</i> YS38 ^T	NCIB 12588	R. J. Sharp
<i>Thermus thermophilus</i> HB8 ^T	ATCC 27624 ^T , DSM 579 ^T , NCIB 11244 ^T	T. Oshima
<i>Thermus thermophilus</i> B	NCIB 11247	R. A. D. Williams
<i>Thermus thermophilus</i> IB21		G. Alfredsson
<i>Thermus scotoductus</i> X1		R. Ramaley
<i>Thermus oshimai</i> SPS7, SPS8, SPS10, SPS11, SPS12, SPS14, SPS17 ^T , and SPS18		M. S. da Costa (Sao Pedro do Sul)
<i>Thermus oshimai</i> AC2, AS1, AS2, AS3, and CB1		M. S. da Costa (Azores)
<i>Thermus oshimai</i> JK66 and JK91		J. Kristjansson (Iceland)

^a ATCC, American Type Culture Collection, Rockville, Md.

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TABLE 2. Levels of DNA-DNA homology of *Thermus* strains as determined by filter hybridization

Source of DNA on filter	% Homology with filter-bound labeled DNA from:																				
	Phenotypic cluster E and F strains ^a										Phenotypic cluster A through D strains ^a										
	YTI ^T	YS38 ^T	HB8 ^T	B	RQ1	Wai33A1 ^T	X1	SPS8	SPS14	SPS17 ^T	AC2	AS3	CBI	JK66	JK91	Vi3a	Vi5b	Vi7a	CG2	FQ1	LFC1
<i>Thermus aquaticus</i> YTI ^T <i>Thermus brockianus</i> YS38 ^T <i>Thermus thermophilus</i> strains	100	42	44	42	48	37	32	31	48	42	49	52	45	37	46	28	36	36	48	47	46
	48	100	30	31	39	38	28	45	41	39	40	46	45	38	33	29	36	29	42	40	39
	38	28	100	86	87	37	28	39	33	31	39	41	50	36	43	31	28	31	51	45	49
	30	29	89	100	81	41		37	39	32	32			32	47	30	24	28	37	42	42
<i>Thermus filiformis</i> Wai33A1 ^T <i>Thermus scotoductus</i> X1 Phenotypic cluster E and F strains	38	42	99	100	100	29		37	42	32	52	47	51	32	47	49	46	41	52	45	52
	26	28	92	94	98	31				32	43	55	43		43	51	53	53	48	49	54
	38	24	39		34	100															
	21	30	30	27			100														
Strains from Iceland that resemble phenotypic cluster E and F strains	42	51	57	52		38			99		92			95	93	38	40	40	50		52
	39	38	47	48		45	40	100	94	97	85	93	83	82	91	53	50	50	50	45	52
	49	54	54	54		43	39	91	98	100				88	100	44			49	48	52
	44	49						90	97	81				86	90	51	54	54		37	37
	45	52	51			55		100		97				95	90	46	48	48	39		37
	43	39	50	44		35		81	100	99				96	85	36	48	48	39	44	44
	32	41	52		43	45	40	93	80	100	80	85	84	96	89	50	46	48	54	46	52
	50	53	53	51		35	35	86	100	91	87	88	90	82	100	43	46	46	48	54	54
	39	50	50		38	49	34	94		75	100	95	95		89	40	40	41	51	50	52
	44	42	50		42	57	55	99		76	84	91		91	87	44	40	40	44	45	48
	46	40	46		38	54		89		77	80	94		94	93	30	35	35	36	40	44
	49	42	55		36	44	40	100		80	93	100	90	90	94		38	38	50	42	52
	31	38	53		43	49		77		72	83	88	100		76		33	33	56	50	54
Strains from Iceland that resemble phenotypic cluster E and F strains	40	48	55		28	57		80	79	79				100	83	38		36	38		42
	42	53	51			55		89	85	86				85	100			44	42		34
Phenotypic cluster A through D strains from Vizela and the Azores																					
	Vi1a	32	52					46			50			34		91		83			85
	Vi2a	35	54		50			49			48			36		90	88	88			89
	Vi2b	36	53	43	56			53		46	43			42	37	92	85	80	100		99
	Vi3a	39	58	50	53		43	54						49	49	100	87	92	88		87
	Vi4a	34	53	53	39	44		49		34	50	47	43		44	100	80	88	85	100	97
	Vi5b	42	53		45		42	52		35				35	35	81	100	100			
	Vi7a	38	54	45	40		41	52		35				34	44	90	86	86	85		90
	Vi7b	43	50	43	52		40	54		35				43	42	96	100	100	97		
	Vi8							49		33				31	39	87	78	79			
	CB2	46	48	51		41	51	52			47		53		44	80	83	83	100		96
	CG2	32	46	47		42	40		39		46		49			88	85	100			96
	EG1	45	43	36		47	32		58		48	56	53		51	71	74	74	97		92
	FQ1	37	36	47		42	46		49		39	47	46		37	70	70	74	88	100	100
	LFC1	39	48	46		34	34		56		39	40	59	44	40	74		86	78		100
	LFF1	38	47	53		43	33		48			48	50	46	42		84	84	97		100
LFF2	43					32			39	45		42						75		89	

^a Phenotypic clusters as defined by Santos et al. (39).

TABLE 3. Levels of DNA-DNA homology of *Thermus* strains as determined by spectrometry

Strain	% DNA-DNA homology with:											
	YT1 ^T	Wai33A1 ^T	HB8 ^T	YS38 ^T	X1	SPS11	SPS14	SPS17 ^T	JK66	JK90	Vi7a	Vi13
<i>Thermus aquaticus</i> YT1 ^T	100	35	42	41	52			38		50	42	50
<i>Thermus filiformis</i> Wai33A1 ^T		100	36	21	52			34		53	40	41
<i>Thermus thermophilus</i> HB8 ^T			100	36				35		22	29	42
<i>Thermus Brockianus</i> YS38 ^T				100				40			36	
<i>Thermus scotoductus</i> X1					100	33	39		43	51		
Phenotypic cluster E and F strains ^a												
SPS11						100		80	82			
SPS14							100	82	69	78	35	
SPS17 ^T								100	69	81	31	
JK66									100	70		
JK90										100		41
Phenotypic cluster A through D strains ^a												
Vi7a											100	97
Vi13												100

^a Phenotypic clusters as defined by Santos et al. (39).

isolates must be examined in detail, and data for significant cell constituents, particularly the nucleic acids, must be included. In this paper we present DNA-DNA homology data, 16S ribosomal DNA (rDNA) sequence data (10, 40), and phenotypic property data (39) for a collection of strains: our data support creation of a new species, *Thermus oshimai*. Strain SPS17 is the type strain of this new species.

MATERIALS AND METHODS

Strains and culture conditions. The *Thermus* strains which we used and their sources are shown in Table 1. Cells were grown at 65°C, harvested, and stored at -20°C as described previously (42, 53).

Respiratory quinone. A cell paste was freeze-dried, extracted, and chromatographed on preparative Keisegel 60F thin-layer plates. Individual quinones were eluted from the plates and then rechromatographed on RP18F₂₅₄ plates (6, 53) to determine the lengths of the side chains. The R_f values of the quinones in extracts of *Thermus* strains were compared with the R_f values of the menaquinones of *Bacillus stearothermophilus* and *Thermus aquaticus* YT1^T. UV absorption spectra of isooctane extracts from the preparative plates were recorded at wavelengths between 230 and 350 nm.

Peptidoglycan preparation and analysis. Cells were extracted by the rapid peptidoglycan preparation method (41) and then were hydrolyzed with 4 M HCl; the hydrolysate was separated by thin-layer chromatography on cellulose plates and tested for the presence of ornithine (13) and the absence of diaminopimelic acid (36) as described previously (53). The amino acids and amino sugars in some samples were analyzed quantitatively with an LKB model 4000 amino acid analyzer.

DNA purification and mean base composition. Cells were lysed and DNA was purified as described previously (38, 42, 53), and the mean base composition was determined by thermal denaturation in 0.1× standard saline citrate (SSC; 1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) with either a Gilford Response spectrophotometer or a Pye-Unicam model 8800 spectrophotometer equipped with a model SPX876 programmed heating accessory. The mean base composition was calculated by using reference DNA from *Escherichia coli* DSM 2840 (G+C content, 52 mol%) and the following formula: G+C content = 52 + 2.08(T_m - T_m of DSM 2840 DNA) (30), where T_m is the melting temperature.

DNA-DNA homology. Hybridization of sheared fragments of [³H]DNA with single-stranded DNA bound to 5-mm nitrocellulose discs was performed as described previously (42, 53). Levels of DNA-DNA homology were also obtained by determining the reassociation rates of sheared fragments of denatured DNA samples (9, 12) under optimum conditions with a Gilford Response spectrophotometer (53). The final sodium concentration used for renaturation (0.515 M) gave an estimated T_m of 101°C when the following equation was used: $T_m = 0.41 \times \text{G+C content} + 78.7 + 13.3 \log[\text{Na}^+]$ (9). The renaturation temperature used was 25°C below the estimated T_m , and 33 µl of 5 M sodium chloride at the same temperature was rapidly mixed into each cuvette. The decrease in A_{260} at this temperature was monitored for 30 min.

RESULTS AND DISCUSSION

The mean base compositions of the DNAs of the 30 strains examined ranged from 60 to 66 mol% G+C, which is consis-

tent with the values obtained for other species belonging to the genus *Thermus*. The respiratory quinone was determined to be a menaquinone on the basis of its R_f value on the preparative thin-layer plate examined and its UV absorption spectrum. The R_f value for the menaquinones of all strains on a reverse-phase plate was identical to the R_f value for strain YT1^T. Thus, menaquinone 8 was identified as the menaquinone in all strains of the new species, including type strain SPS17.

A qualitative analysis revealed that ornithine was the major dibasic amino acid in the peptidoglycan of the new species, as it is in all other *Thermus* strains that have been tested (15, 33). Lysine was present as a minor substituent.

Strains that were identified in a previous phenotypic study as members of clusters A to D and were isolated by M. S. da Costa from hot springs at Caldas da Vizela (Vi strains) in mainland Portugal and similar isolates from Sao Miguel Island form a homogeneous DNA homology group and have been placed in *Thermus scotoductus* (48). Clusters E and F that were identified in the same numerical analysis of phenotypic properties (39) comprise strains that were isolated by M. S. da Costa from hot springs at Sao Pedro do Sul in mainland Portugal (SPS strains) and Sao Miguel Island in the Azores. These two phenotypic clusters belong to one distinct DNA-DNA homology group (Tables 2 and 3), and members of these clusters exhibit high levels of homology with other members of the group but low levels of homology with *Thermus aquaticus*, *Thermus Brockianus*, *Thermus thermophilus*, *Thermus scotoductus*, and *Thermus filiformis*. This agreement between the results of a numerical taxonomic analysis on the one hand and chemical and genetic characteristics of the cells on the other supports the hypothesis that the new species described in this paper is valid. Two strains isolated by J. Kristjansson in Iceland (strains JK66 and JK91) also appear to belong to the same genospecies. Although this species occurs in the Azores and in Iceland, as well as at Sao Pedro do Sul in Portugal, another species appears to be the predominant organism at Vizela, which is only about 75 km from Sao Pedro do Sul. This indicates that factors other than mere geographical distance may contribute to the colonization of hot springs by different *Thermus* species.

The cataloging of oligonucleotides derived from hydrolysis of 16S rRNA revealed that there is a distinct, if remote, relationship between *Thermus aquaticus* and *Thermus ruber* on the one hand and the genus *Deinococcus* on the other (15). Alignment of the 16S rRNA gene sequence of *Thermus thermophilus*

TABLE 4. Sequences of 16S rDNAs of yellow-pigmented and colorless *Thermus* strains in regions where species-specific differences are significant

Organism	Sequence in:		
	Helix 6 region	Helix 10 region	Helix 18 region
<i>Thermus aquaticus</i>			
YT1 ^a	tctgTgcgggCC-GTgggg---taTCTACAGGTCag	gACACATCctTctgGGGTGtTTaaagGGTtTTT--gCcCgc	gaAaCcccCGATGAggggActga
YS025	tctgTgcgggCCGCTgggg---taTCTACAGGTCag	gACACATCctTctgGGGTGtTTaaagGGTtTTT--gCcCgc	gaAaCcccCGATGAggggActga
YS052	tctgTgcgggCCGCTgggg---taTCTACAGGTCag	gACACATCctTctgGGGTGtTTaaagGGTtTTT--gCcCgc	gaAaCcccCGATGAggggActga
<i>Thermus thermophilus</i>			
HSA1	tctgTgcgggCC-GCgggggttttaCTCTCGCGTCag	gACCCGCCCCtTtggGGGTGtTCCaaagGGCTTTT--gCcCgc	gaAaCcccCGATGAggggActga
HB8 ^T	tctgTgcgggCC-GCgggggttttaCTCTCGCGTCag	gACCCGCCCCtTtggGGGTGtTCCaaagGGCTTTT--gCcCgc	gaAaCcccCGATGAggggActga
HB27	tctgTgcgggCC-GCgggggttttaCTCTCGCGTCag	gACCCGCCCCtTtggGGGTGtTCCaaagGGCTTTT--gCcCgc	gaAaCcccCGATGAggggActga
<i>Thermus flavus</i>			
<i>Thermus filiformis</i>			
Wai33A1 ^T	tctgTgcgggCT-GCgggggttttaCTCTCGCGTCag	gtcGTGCCCCtTtggGGGTGtTCCaaagGGTGAAGAGCccCgc	gaAaTcccTGATGAggggGctga
Tok20A1	tctgTgcgggCC-GCgggggttttaCTCTCGCGTCag	gtcGTGCCCCtTtggGGGTGtTCCaaagGGTGAAGAGCccCgc	gaAaTcccTGATGAggggGctga
OK6A1	tctgTgcgggCT-GCgggggttttaCTCTCGCGTCag	gtcGTGCCCCtTtggGGGTGtTCCaaagGGTGAAGAGCccCgc	gaAaTcccTGATGAggggGctga
RT41A	tctgTgcgggCT-GCgggggttttaCTCTCGCGTCag	gtcGTGCCCCtTtggGGGTGtTCCaaagGGTGAAGAGCccCgc	gaAaTcccTGATGAggggGctga
T351	tctgTgcgggCT-GCgggggttttaCTCTCGCGTCag	gtcGTGCCCCtTtggGGGTGtTCCaaagGGTGAAGAGCccCgc	gaAaTcccTGATGAggggGctga
Tok8A1	tctgTgcgggCC-GCgggggttttaCTCTCGCGTCag	gtcGTGCCCCtTtggGGGTGtTCCaaagGGTGAAGAGCccCgc	gaAaTcccTGATGAggggGctga
<i>Thermus brockianus</i>			
YS38 ^T	tctgGcggggCC-ATgggggttttaCTCTCGGTGTCag	gtcATGTCTCtTtggGGGTGtTCCaaagGGCGA--GAGTcCgc	gaAaGccccCGATGAggggGatga
ZHG1A1	tctgGcggggCC-ATgggggttttaCTCTCGGTGTCag	gtcATGTCTCtTtggGGGTGtTCCaaagGGCGA--GAGTcCgc	gaAaGccccCGATGAggggGatga
NMX2A1	tctgAGcgggGC-AGg-----tttaTACC--TGTTCag	gtcCTGTCTCtTtggGGGTGtTCCaaagGGTGAAGAGCccCgc	gaAaGccccCGATGAggggGatga
Vi7a	tctgAGcgggGC-AGg-----tttaTACC--TGTTCag	gtcCTGTCTCtTtggGGGTGtTCCaaagGGTGAAGAGCccCgc	gaAaGccccCGATGAggggGatga
SPS14	tctgTgcgggGT-GG-----TTCGCCACCCag	gtcCGGCCCCc-tggGGCGGTGtTCCaaagGGCGA--Ag--cCgc	gaAaAccccCGATGAggggGatga

^a The lowercase letters indicate nucleotides that are identical in all strains, and the uppercase letters indicate nucleotides that differ in the various taxa. A dash indicates a space that was introduced to maximize the alignment similarity (i.e., a deletion in the 16S rDNA).

HB8^T (14) with other 16S rRNA sequences revealed that the genus *Thermus* is related to the green nonsulfur bacteria, the second deepest bacterial branch point after the *Thermotoga maritima* branch point. Many 16S rRNA and 16S rDNA sequences of *Thermus* strains are now available (10, 40, 52) (GenBank accession numbers L09660 to L09672). The sequences of the 16S rDNA genes of *Thermus ruber*, *Thermus brockianus* YS38^T, and strains Vi7 and SPS14 (10) confirmed the conclusion based on DNA-DNA homology data that the yellow-pigmented *Thermus* strains are closely related to each other but not to *Thermus ruber* (42). The lower "thermophilic bias" that was evident in the sequence of the *Thermus ruber* gene, which was associated with the lower growth temperature of this organism compared with the yellow-pigmented strains, was important in confirming the relationship between the thermophilic genus *Thermus* and the mesophilic genus *Deinococcus* (10).

The sequences of the 16S rDNA genes of *Thermus aquaticus* YT1^T, "*Thermus flavus*" AT62, *Thermus* sp. strains HSA1, OK6A1, Rt41A, T351, Tok8A1, Tok20A1, and ZHG1A1, *Thermus filiformis* Wai33A1^T, and *Thermus ruber* were determined by Saul et al. (40) and have been deposited in the GenBank data base. When these sequences were aligned with those of *Thermus aquaticus* YS25 and YS52 (unpublished sequences determined at the Deutsche Sammlung von Mikroorganismen for R. J. Sharp), *Thermus thermophilus* HB8^T (14, 15) and HB27 (GenBank accession number L09659), *Thermus brockianus* YS38^T, and strains Vi7 and SPS17^T (10), it was found that the regions that include helices 6, 10, and 18 (Table 4) vary from species to species. The variations show that 16S rRNA sequences may be used to identify species. These data support the hypothesis that the geographical distribution of *Thermus* species may not be as restricted as has been thought. The sequence of strain HSA1 (isolated in the South Island of New Zealand [16]) is very similar to the sequences of *Thermus thermophilus* HB8^T and "*Thermus flavus*" from Japan. *Thermus thermophilus* strains are known to occur in widely separated areas, including Icelandic terrestrial (47) and marine hot springs (23) and Sao Miguel in the Azores. The 16S rDNA sequence of ZHG1A1, which was isolated in Iceland (18), indicates that this strain belongs to the species *Thermus brockianus*, which was found (53) among strains obtained from Yellowstone National Park in the United States (27). Similarly, the sequence of the 16S rDNA gene of isolate NMX2A1 from New Mexico (19) indicates that this organism is similar to the Vizela strains from Portugal and Sao Miguel Island in the Azores, which themselves belong to a species whose strains have been isolated from sources in Britain, the United States, and Iceland. The concept of a circumscribed geographical distribution of *Thermus* species may therefore be more apparent than real. Clearly, there may be unknown chemical and physical reasons why a particular species may thrive in one spring but not in another. It seems likely that the use of 16S rDNA sequences and species-specific probes for the 16S rDNA gene with large collections of isolates should throw light on the true distribution of *Thermus* species and also should allow the occurrence of these organisms to be correlated with the results of physical and chemical analyses of hot spring waters. The 16S rDNA sequences currently available for *Thermus* species have recently been tabulated to assist in the design of species-specific probes (52).

Description of *Thermus oshimai* sp. nov. *Thermus oshimai* (osh'i.ma.i. N.L. adj. *oshimai*, referring to T. Oshima, a prolific researcher who has studied the molecular biology of the genus *Thermus*). Aerobic, heterotrophic, thermophilic short rods that are gram negative. *Thermus oshimai* forms a yellow pigment

like *Thermus brockianus*, but does not exhibit the distinctive spreading colonial morphology of *Thermus brockianus* on tryptone-yeast extract plates at 70°C. Peptidoglycan has ornithine cross-links with glycine in the interpeptide bridge; the respiratory quinone is menaquinone 8; and the DNA G+C content is 62 to 65 mol% (63 mol% in SPS17). More than 85% of the strains grow anaerobically in the presence of nitrate and form nitrite (39). Most strains hydrolyze fibrin, casein, and hide-azure in plate assays, and most strains grow on glutamate and asparagine, fructose, mannose, and galactose. All strains grow on sucrose, maltose, and trehalose. Unlike the strains of the four other phenetic groups (39), *Thermus oshimai* strains grow on lactose (and form β -galactosidase) and on melibiose (and form α -galactosidase). About one-half of the strains grow on glucose. The type strain is strain SPS17, which was isolated by M. S. da Costa from a sample obtained from Sao Pedro do Sul, Portugal (39); this strain has been deposited in the National Collection of Industrial and Marine Bacteria as strain NCIMB 13400.

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