Isolation and Preliminary Taxonomic Studies of *Thermus* Strains Isolated from Yellowstone National Park, USA

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Forty-eight strains of *Thermus* isolated from hot springs in Yellowstone National Park, Wyoming, USA, and eight reference strains were subjected to a numerical taxonomic analysis using Gower's coefficient (S_G) with single and average linkage clustering. Two major groups were distinguished, which could be differentiated by colony morphology, ability to reduce nitrate and proteolytic activity. Cluster 1 contained *Thermus aquaticus* YT-1, the type strain of the species, and cluster 2 contained authentic strains of '*T. flavus*' and '*T. thermophilus*'. *T. ruber* was recovered as a single member cluster. The mol % G + C of DNA from representative strains from each cluster was 64.4 to 66.8 for cluster 1, 62.2 to 67.1 for cluster 2 and 62.5 for *T. ruber*.

INTRODUCTION

Thermus aquaticus was first isolated from hot springs in Yellowstone National Park, Wyoming, USA, by Brock & Freeze (1969). Strain YT-1, which became the designated type strain, was described as an aerobic, yellow pigmented, Gram-negative, heterotrophic bacterium with an optimum growth temperature of about 70 °C, and a pH optimum of 7.0 to 8.0. Since this first description, *Thermus* strains have been recovered from thermal regions in Japan (Oshima & Imahori, 1974), the USSR (Loginova & Egorova, 1975), Iceland (Pask-Hughes & Williams, 1977) and New Zealand (Cowan *et al.*, 1984). Other sources of strains include streams receiving thermal effluent (Ramaley & Hixson, 1970) and hot water systems (Brock & Boylen, 1973; Pask-Hughes & Williams, 1975).

This paper describes the isolation of 48 strains of *Thermus* from thermal areas in Yellowstone National Park, and a numerical analysis of these strains and of recognised reference strains including *T. aquaticus* YT-1, '*T. thermophilus*', '*T. flavus*' and *T. ruber*.

METHODS

Strains, sampling and media. Sixty-five hot springs and effluent channels were examined in four major thermal areas [Mud Volcano, Potts Basin, the Lower Geyser Basin (White Creek area) and Norris Geyser Basin] of Yellowstone National Park, Wyoming, USA. The temperature and pH of the pools or effluent channels were recorded at the time of sampling (Table 1). Liquid samples were taken using sterile 10 ml pipettes and transported in 5 ml cryotubes (Sterilin). The samples were in transit for approximately 7 to 10 d before examination in the laboratory. The biomass was resuspended in the sample tubes and 0.5 ml transferred to 10 ml of *Thermus* medium (Brock, 1978). The samples were incubated for 24–48 h at 70 °C and tubes showing evidence of growth were inoculated onto *Thermus* agar (Brock, 1978) and incubated for 24 h at 70 °C. Following three serial single colony transfers, a collection of 48 independent isolates was established (Table 1). Reference strains used were *T. aquaticus* strains YT-1 and Y-VII-51B (Brock & Freeze, 1969), *Thermus* sp. B (Williams, 1975), *Thermus* sp. X-1 (Ramaley & Hixson, 1970), *Thermus* sp. T2 (ATTC 27737), '*T. flavus*' (Saiki *et al.*, 1972), '*T. thermophilus*' (Oshima & Imahori, 1974) and *T. ruber* (a gift from Dr R. A. D. Williams, London Hospital Medical School, London, UK). All strains were maintained as frozen suspensions in 10% (v/v) glycerol at –80 °C. For routine culturing, *Thermus* liquid medium and *Thermus* agar were used (Brock, 1978).

Table 1. *Origin of Thermus strains isolated from thermal areas in Yellowstone National Park, USA*

Strain reference no.	Sample site	Properties of sample site	
		Temp. (°C)	pH
Cluster 1a			
YS 001	Firecracker Spring*	76.5	9
YS 005	Firecracker Spring*	80.5	9
YS 012, YS 013, YS 014	Unnamed pool J†	76	8.2
YS 015, YS 016, YS 017, YS 018	Unnamed pool K†	75	6.95
YS 020	Unnamed pool L†	72	5.9
YS 023, YS 024, YS 049, YS 052	Area of YW 135‡	73.3	8.5
YS 025, YS 026, YS 027	Area of YW 134‡	70.2	8.5
YS 028, YS 029	Area of Verdant Spring‡	62.2	9.3
YS 031, YS 032, YS 033	Pine Spring/Five Sisters 2‡	72.4	8.8
YS 034, YS 035	Pine Spring/Five Sisters 3‡	72	7.8
YS 039, YS 041	Pine Spring/Five Sisters 4‡	55	10.5
YS 048	Unnamed pool E†	42.9	6
Cluster 1b			
YS 007, YS 008	Unnamed pool G†	67.5	9
YS 009, YS 010	Unnamed pool I†	66	9.3
Cluster 2			
YS 002	Pine Spring/Five Sisters 4‡	55	10.5
YS 011	Unnamed pool J†	76	8.2
YS 019	Unnamed pool L†	72	5.9
YS 021, YS 022	Area of YW 137‡	68.7	7.4
YS 030	Pine Spring/Five Sisters 1‡	54.8	8.2
YS 037, YS 038, YS 051	Pine Spring/Five Sisters 3‡	72	7.8
YS 040	Pine Spring/Five Sisters 4‡	55	10.5
YS 044, YS 045	Mary's Bay, Lake Yellowstone	NT	NT
YS 047	Unnamed pool J†	76	8.2
Unassigned strains			
YS 003, YS 004	Firecracker Spring*	76.5	9
YS 036	Pine Spring/Five Sisters 3‡	72	7.8
YS 050	Unnamed pool I†	66	9.3

NT, Not tested.

* Lower Back Basin in Norris Geyser Basin.

† Potts Hot Spring Basin, West Thumb area.

‡ White Creek, Lower Geyser Basin. Spring nomenclature according to Brock (1978).

Numerical phenetic survey. Growth on single substrates was examined using basal *Thermus* medium modified by replacing tryptone and yeast extract with 2 mM-(NH₄)₂SO₄ and by the addition of the selected carbon source at a concentration of 0.2% (w/v). Carbon sources used were erythritol, adonitol, arabinose, xylose, rhamnose, aesculin, dulcitol, galactose, glucose, inositol, fructose, mannitol, mannose, salicin, sorbitol, lactose, maltose, raffinose, sucrose, trehalose, dextrin, glycogen, inulin, starch, glycerol and the sodium salts of glutamate, acetate, citrate, formate, malate, propionate, pyruvate and succinate. Growth was examined by streaking a single colony onto a supplemented *Thermus* agar plate and incubating at 70 °C overnight.

Antibiotic sensitivity was examined using cells from an overnight plate culture suspended in 2 ml *Thermus* medium. The OD₅₄₀ was adjusted to 1.0 with medium to standardize cell density and 0.1 ml of the suspension was then spread over the surface of an agar plate and allowed to dry. Oxoid antibiotic sensitivity discs were applied to the agar and plates incubated at 70 °C for 16–20 h before measuring the zones of inhibition.

Resistance to heavy metals was determined using 6 mm discs cut from a Whatman no. 1 filter paper, each disc impregnated with 20 µl of heavy metal salts solution. The metals and concentrations used were sodium arsenate (2 M), antimony potassium tartrate (0.05 M), bismuth trichloride (0.01 M) and sodium arsenite (1 M). The discs were applied to seeded agar plates which were incubated for 16–20 h before zones of inhibition were measured.

Colony morphology was examined on *Thermus* agar plates; the degree of motility was dependent upon the time and temperature of incubation. The degree of 'spreading' of colonies was recorded after 2 d incubation at 70 °C.

Reduction of nitrate and nitrite was examined using the method of Cowan (1974) but using supplemented

Thermus medium. Duplicate bottles of nitrate and nitrite broth were inoculated with single colonies, one bottle from each pair being examined after 1 and 3 d incubation at 70 °C.

Oxidase (cytochrome *c* oxidase) and catalase activity were examined using overnight cultures grown on *Thermus* medium and examined using the methods of Skerman (1967) and Kovacs (1956).

Sensitivity to NaCl was examined using *Thermus* medium supplemented with 3% (w/v) NaCl; pH studies were done using the same medium adjusted to pH 5.1 and 6.1. Growth was examined following 1 and 3 d incubation at 70 °C.

Anaerobic growth of broth cultures (10 ml) was examined following 1 and 3 d incubation at 70 °C in a BBL-Gas Pak anaerobic jar with a CO₂/H₂ atmosphere.

Gelatin hydrolysis was studied using gelatin discs (Oxoid) added to 15 ml *Thermus* medium and inoculated with a single colony from an overnight plate culture. Cultures were examined after 1 and 3 d incubation at 70 °C.

Casein hydrolysis was examined using a modification of the method of Gordon *et al.* (1973). Casein agar (5 ml) was overlaid onto *Thermus* agar. After inoculation, plates were incubated for 24 h at 70 °C and examined for evidence of hydrolysis.

Starch hydrolysis was examined on starch agar plates (Cowan, 1974) prepared using *Thermus* medium as a base. Plates were examined for hydrolysis after 1 and 3 d incubation at 70 °C. Hydrolysis was determined using iodine and recorded as either restricted (hydrolysis in the immediate vicinity of the colony) or diffuse (hydrolysis of starch in the area surrounding the colony).

The phenetic data, comprising 71 characters for 48 fresh isolates and eight reference strains, were coded using multistate numerical coding and similarity coefficients were calculated using the coefficient of Gower (1971). The similarity coefficients were sorted using single linkage analysis and unweighted average linkage analysis (UPGMA) (Sneath & Sokal, 1973).

Computations were done using an Acorn system 4 microcomputer with a 48K memory interface with a RICOH PP1600 printer and Watanabe MILOT XI plotter. All programs were written in BASIC by J. R. Woodrow.

DNA analysis. A 200 ml overnight culture of *Thermus* was harvested by centrifugation (3700 *g* for 10 min) and washed twice in 10 mM-CaCl₂. The bacterial pellet was resuspended in 10 ml standard saline citrate (SSC) (Marmur & Doty, 1962). Lysozyme was added to a final concentration of 0.2 mg ml⁻¹, and the mixture incubated for 5 min at 37 °C; 2 ml 10% SDS were then added and mixed in quickly and gently. An equal volume of buffered (SSC) phenol was added and mixed with the lysate to form an emulsion. The mixture was centrifuged at 3000 *g* for 5 min and the upper aqueous layer collected in a separating funnel. Traces of phenol were removed by washing the aqueous layer twice with ether. The lower layer from the funnel was centrifuged for 20 min at 12000 *g* to remove any residual protein. The supernatant was collected and heated to 60 °C for 10 min to remove any traces of ether. DNA was precipitated by the addition of 2 vols ice-cold ethanol and spooled onto glass rods. The DNA was washed three times in ice-cold ethanol and dried in a stream of nitrogen. The DNA was further purified by isopycnic centrifugation in caesium chloride (Munster, 1984). The DNA was finally resuspended in a small volume of sterile SSC to give a final DNA concentration of approximately 0.1 mg ml⁻¹.

The mol % G + C was determined using the method of Marmur & Doty (1962). The DNA melting temperature was measured using a Pye Unicam SP8 100 spectrophotometer fitted with an electrically heated cell holder and an SPX 876 series 2 temperature programme controller.

RESULTS AND DISCUSSION

The majority of the *Thermus* strains were isolated from the alkaline environments Potts Hot Spring Basin and White Creek (Table 1). The pH and temperature ranges from which *Thermus* strains were isolated varied from pH 6.0 to pH 10.5 and from 55 °C to 80.5 °C. A temperature gradient existed across the pools and samples taken from the centre, where the temperature varied from 85 °C to 93 °C, did not yield any *Thermus* strains. The isolation of strains from springs with pH values as high as 10.5 was unexpected, since Brock (1978) has reported optimum growth of strains at pH 7.5–7.8 with no growth above pH 9.5. However, strains have also been isolated from hot springs in Iceland at pH 10.0 (Kristjansson & Alfredsson, 1983). Seven isolates grew well in the laboratory at pH 5.1 although the pH range of their natural environment ranged from 7.4 to 10.5. Of the seven strains isolated from environments below pH 7.0, three did not grow at pH 5.1 in the laboratory and four grew only weakly. It appears that the pH of the natural environment does not necessarily condition the optimum pH for growth in laboratory media.

At 37 °C growth was either absent or very weak, and at 55 °C only nine strains grew strongly. The optimum growth temperature appeared to be in the region of 70 °C, except for *T. ruber*, which did not grow satisfactorily at 70 °C. *T. ruber* appears to have a growth temperature

Fig. 1. Dendrogram depicting relationships between *Thermus* strains following analysis using the S_G coefficient and UPGMA.

optimum considerably lower than that of the yellow and cream pigmented strains of *Thermus* and grew strongly at 45 °C. Similar studies on pink pigmented strains of *T. ruber* from Iceland (R. J. Sharp & M. Parsons, unpublished observations) also indicate a temperature optimum in the region of 50–60 °C.

T. aquaticus YT-1 was described as an aerobic organism (Brock & Freeze, 1969); however, in this study 14 strains were found to grow as facultative anaerobes in the presence of nitrate. These strains were considered to produce nitrate reductase which enabled them to use nitrate as a terminal H^+ acceptor.

Studies of growth on single carbon sources showed that the majority of strains grew well on

Table 2. *Differential properties of the clusters*

Character	Cluster . . . No. of strains . . .	Percentage of positive reactions		
		1a 27	1b 4	2 19
Spreading colonies		4	9	100
Starch hydrolysis		82	100	35
Casein hydrolysis		100	0	6
Gelatin hydrolysis		100	0	24
Reduction of NO ₃ to NO ₂		7	0	71
Reduction of NO ₂ to NO		4	0	71
Growth in anaerobic broth		25	100	82

sodium pyruvate, sodium glutamate and sodium acetate, and that a few strains exhibited strong growth with lactose, galactose, maltose, sucrose, trehalose and sodium succinate. Growth on arabinose, mannitol and sodium citrate was either absent or only slight. Strain YS 036 was unique in exhibiting growth on all but three (erythritol, glycogen and starch) of the 33 carbon sources examined.

Analysis of the data by the S_G coefficient and UPGMA analysis indicated that the majority of strains (51) were allocated to two major clusters, defined at the 82% similarity level (Fig. 1). Confidence could be placed in the numerical classification since the average probability (P) of an erroneous result was 2.56% when calculated on the basis of results for the 71 characters on 10 duplicate strains. This was well within the range of error generally considered acceptable (Sneath & Johnson, 1972). Cluster 1 contained 32 strains, which were typified by extracellular amylase and protease production. The colonies were discrete, showing no evidence of cell motility. The cluster could be divided into subclusters 1a and 1b at the 85% similarity level (Fig. 1). *T. aquaticus* YT-1 clustered with the other strains in cluster 1 at the 83% similarity level. Cluster 2, defined at the 82% similarity level, contained 19 strains, all with spreading colony morphology indicating motility; 82% of the strains grew anaerobically in the presence of nitrate and 71% reduced nitrate to nitrite under aerobic conditions. The majority of the strains in cluster 2 grew at pH 5.1, and all seven strains which grew strongly at pH 5.1 were members of this cluster. *T. aquaticus* Y-VII-51B, *Thermus* spp. T2, X-1 and B, '*T. flavus*', and '*T. thermophilus*' were all members of cluster 2. Four isolates and *T. ruber* did not group in either cluster. However, three of these isolates, strains YS 036 and YS 003, which clustered at the 89% similarity level, and YS 004, which clustered with YS 036 and YS 003 at the 81.5% similarity level, formed a third distinct cluster. These three strains exhibited the widest range of carbon source utilization of all the isolates examined.

Analysis of the data by single linkage analysis again indicated that four strains in cluster 1 formed a subcluster (1b), clustering with the 27 strains of subcluster 1a at the 90% similarity level. These four strains differed from strains of subcluster 1a in being unable to hydrolyse gelatin and casein and in growing anaerobically in the presence of nitrate (Table 2).

The mol % G + C of representative strains from the various clusters are presented in Table 3. These results do not suggest the existence of more than one distinct species, although the low mol % G + C for *T. ruber* may be significant, and it would be relevant to examine other pink pigmented strains.

These data indicate the existence of two major taxa, which may be distinguished primarily on colony morphology and to a lesser degree on protease production, anaerobic growth, nitrate reduction and growth at pH 5.1. Three other minor groups were identified but their significance is not yet apparent. Cluster 1b, which was evident from both single linkage and UPGMA analysis, differed from cluster 1a in proteolytic activity and anaerobic respiration. In most other respects, it resembled cluster 1a and may be a subgroup of cluster 1. Strains YS 036, YS 003 and YS 004 may be the nucleus of a third taxonomic group. *T. ruber* showed little affinity to the other strains examined; it had a lower optimum growth temperature and its mol % G + C differed markedly from that of the yellow, white and cream pigmented strains.

Table 3. *DNA base composition of selected strains of Thermus*

Values of T_m are means $\pm$ SD, and of mol % G + C are means, of three determinations.

Cluster	Strain	T_m ($^{\circ}$ C)	Mol % G + C
1a	YS 013	95.7 $\pm$ 0.24	64.4
1a	YS 026	95.7 $\pm$ 0.21	64.4
1	<i>T. aquaticus</i> YT-1	96.7 $\pm$ 0.21	66.8
1b	YS 007	96.1 $\pm$ 0.14	65.4
1b	YS 010	96.1 $\pm$ 0.09	65.4
2	YS 019	95.9 $\pm$ 0.09	64.9
2	YS 040	96.3 $\pm$ 0.38	65.8
2	B	96.8 $\pm$ 0.24	67.1
2	T2	96.7 $\pm$ 0.24	66.8
2	X-1	96.0 $\pm$ 0.01	65.1
2	<i>T. aquaticus</i> Y-VII-51B	94.8 $\pm$ 0.24	62.2
2	' <i>T. thermophilus</i> '	96.2 $\pm$ 0.62	65.6
2	' <i>T. flavus</i> '	96.2 $\pm$ 0.24	65.6
-	YS 036	96.1 $\pm$ 0.09	65.4
-	<i>T. ruber</i>	94.9 $\pm$ 0.09	62.5

The designation of '*T. flavus*' and '*T. thermophilus*' as separate taxa may need to be reconsidered since both appeared in cluster 2 along with *T. aquaticus* Y-VII-51B and *Thermus* spp. B, T2 and X-1. *T. aquaticus* YT-1, which is designated as the type strain, did not cluster with the other *T. aquaticus* reference strain included in the study. Studies of a larger group of strains, including isolates from geographically separate environments, should clarify, if not answer, some of the questions raised in this study.

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