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Properties of *Thermus ruber* Strains Isolated from Icelandic Hot Springs and DNA:DNA Homology of *Thermus ruber* and *Thermus aquaticus*

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Seventeen pink-pigmented strains of the genus *Thermus* were isolated from samples collected from thermal areas of Iceland. The strains were examined by using phenotypic characterization and DNA:DNA homology and were compared with recognized strains. Visually, the strains could be divided into three groups based on their pigmentation; however, spectroscopic studies of the pigments indicated little difference among them. Most strains required a vitamin supplement for growth and used fructose, maltose, mannose, or sucrose as the sole carbon source. In the presence of nitrate, two strains were able to grow under anaerobic conditions. The optimum growth temperature was 60°C; growth did not occur at 30 or 70°C.

Pink-pigmented, thermophilic gram-negative rods were originally isolated from hot springs (70 to 93°C, pH 6.5 to 7.3) in the Kamchatka Peninsula, USSR, and named *Thermus ruber* (13). The name was revived by Loginova et al. (14) to designate the type strain described in the original paper. The cells were described as gram-negative nonmotile rods 3 to 6 μm in length and 0.5 to 0.8 μm in diameter, with a minimum growth temperature of 35 to 40°C and a maximum growth temperature of 70°C. In this paper we describe pink-pigmented strains isolated from samples collected from hot pools in Hveragerthi, Iceland. The strains were compared with the type strain of *T. ruber* isolated in the USSR to determine whether the red-pigmented *Thermus* isolates vary with different geographical locations, as has been suggested for yellow-pigmented strains (10). Phenotypic characterization was carried out by the method of Munster et al. (15) and by DNA-DNA reassociation with the type strains of *T. ruber* and *Thermus aquaticus*, using the filter hybridization technique of Dent and Williams (7).

MATERIALS AND METHODS

Strains, sampling, and media. *Thermus* strains are listed in Table 1. In October 1984, samples of mud and water were collected in 1-oz (ca. 28.35-g) bottles from eight thermal pools in the center of Hveragerthi in Iceland. The samples were transported to London without refrigeration and then were stored for 2 weeks at 4°C before isolation. The sample tubes were shaken, and 0.5 ml of each sample was transferred to 10 ml of *Thermus* medium (2) and incubated for 48 h at 60°C. Samples from tubes showing evidence of growth were inoculated onto *Thermus* agar (2) and incubated in sealed bags for 24 h at 60°C. The plates were examined for pink colonies, and after three serial transfers of single colonies, a collection of 17 independent isolates were established (Table 1). Comparative studies were made with *T. ruber* (13, 14). All the strains were maintained as frozen suspensions in 10% (vol/vol) glycerol at −80°C. *Thermus* liquid medium and *Thermus* agar (2) were used for routine culturing.

The reference strains used and their sources were as follows: *T. aquaticus* YT1 (NCMIB 11243, ATCC 27634, DSM 625), from the American Type Culture Collection, Rockville, Md.; *T. ruber* BKMB-1258 (NCMIB 11269, DSM 1279), furnished by L. G. Loginova; *Lactobacillus murinus* CNRZ 220 (heterologous DNA for hybridizations), furnished by D. Hemme; and *Escherichia coli* B DSM 2840 (standard DNA for percent G+C determination), from the Deutsche Sammlung von Mikroorganismen, Göttingen, Federal Republic of Germany.

Physiological test methods. Growth on single substrates was determined by using basal *Thermus* mineral medium containing 2 mM $(NH_4)_2SO_4$, 1% stock vitamin solution (4 μg/ml each of thiamine, riboflavin, pyridoxine, biotin, choline, folic acid, inositol, nicotinic acid, pantothenic acid, and aminobenzoic acid), and 1% selected carbon source. The carbon sources used were esculin, L-(+)-arabinose, dextrin, dulcitol, erythritol, D-(+)-galactose, *meso*-inositol, lactose, glycerol, D-(−)-fructose, maltose, mannitol, D-(−)-mannose, raffinose, L-(+)-rhamnose, salicin, sorbitol, sucrose, and D-(+)-xylose and the sodium salts of acetate, citrate, malate, propionate, pyruvate, and succinate. Growth was examined in single colonies which had been streaked onto supplemented *Thermus* agar and incubated at 60°C overnight.

Growth temperature studies were carried out with *Thermus* agar slants incubated at 30, 37, 60, and 70°C. Strains were examined for sensitivity to NaCl on *Thermus* media supplemented with 1 or 2% NaCl, and growth was also tested on *Thermus* media adjusted to pH 4, 5, 6, 8.5, 9, 10, or 11 at 60°C.

Strains were tested for anaerobic growth on *Thermus* agar and in *Thermus* liquid medium supplemented with 0.1% KNO_3 following incubation for 2 days at 60°C in GasPak anaerobic jars (BBL Microbiology Systems, Cockeysville, Md.) with a CO_2-H_2 atmosphere generated by BBL sachets.

The reduction of nitrate and nitrite by strains was tested by the method of Cowan (5) but on supplemented *Thermus* medium. Duplicate bottles of nitrate and nitrite broth were inoculated with single colonies, and one bottle from each pair was examined after 1 and 3 days of incubation at 60°C.

Arbutin degradation was detected by the method of Baird-Parker (1). Cultures were examined for tellurite reduction by

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TABLE 1. Red-pigmented *Thermus* isolates from Icelandic hot springs

Strain[a]	Characteristic of spring water	
	pH	Temp (°C)
16059	7.8	50–70
16060	7.8	50–70
16061	7.8	50–70
16062	8.8	77
16063	7.6	48–60
16064	8.8	77
16066	7.8	61–80
16067	7.8	61–80
16068	7.8	61–80
16102	7.8	50–70
16103	7.8	50–70
16106	8.0	45–50
16107	8.8	77
16109	7.6	48
16110	7.8	61–80
16111	7.8	61–80
16149	7.8	61–80

[a] All strains were isolated from samples collected near Hverabakeri, Hveragerthi, except 16106, which was isolated from a sample collected near the Horticultural School, Hveragerthi.

the method of Wilkinson and Jones (24), with 0.01% (wt/vol) potassium tellurite. Strains were tested for casein and starch hydrolysis by the methods of Munster et al. (15). Gelatin hydrolysis was studied by adding gelatin disks (Oxoid Ltd., Basingstoke, United Kingdom) to 15-ml volumes of *Thermus* medium which had each been inoculated with a single colony from an overnight plate culture. The cultures were examined after 1 and 3 days of incubation at 60°C. The hydrolysis of elastin, fibrin, and hide powder azure was measured by the methods of Hudson et al. (10). DNase activity was tested by the method of Jeffries et al. (11). After 48 h, the cells were washed from the plates, which were then flooded with 0.1 M HCl. A zone of clearing around or underneath the area of growth was scored as a positive result. The hydrolysis of Tween 80 was examined by the method of Degryse et al. (6). Oxidase (cytochrome *c* oxidase) and catalase activities were examined by using overnight cultures grown on *Thermus* medium by the methods of Skerman (22) and Kovacs (12).

The antibiotic sensitivities were examined (Table 2) by using cells from overnight plates suspended in 2 ml of *Thermus* medium. The optical density at 540 nm was adjusted to 1.0 with sterile 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. Antibiotic sensitivity disks (Oxoid) were applied to the agar, and the plates were incubated at 60°C for 16 to 20 h before the zones of growth inhibition were measured.

Spectrophotometric comparison of pigments. Cells were scraped from plates of *Thermus* agar which had been incubated at 60°C overnight and freeze-dried. The residue was suspended in methanol and centrifuged at 5,000 × *g* for 10 min. Clear extracts were scanned in 1-cm-light-path quartz cuvettes against a methanol blank with a Pye Unicam SP1800 spectrophotometer set between 360 and 700 nm.

Isolation of DNA and determination of mean base composition. Strains were grown in *Thermus* medium overnight in shake flask culture at 60°C (*T. ruber*) or 65°C (other *Thermus* isolates). Cells were centrifuged at 8,000 × *g* and stored at −20°C. The frozen cells were suspended in distilled water and incubated with 30 mg of protease (Sigma Chemical Co., Poole, United Kingdom) per 100 ml and 30 mg of lysozyme (Sigma) per 100 ml for 1 h at 37°C. The protease was preincubated at 37°C for 1 h to destroy nucleases. Lysis was completed by adding 1/10 of a volume of 4% sodium dodecyl sulfate to the suspension, which was then shaken for 15 min with one-half its volume of chloroform containing 4% isopentanol. After centrifugation for 10 min at 10,000 × *g*, the viscous aqueous supernatant was removed, and DNA was spooled out on glass rods with 2 volumes of cold ethanol. The DNA was redissolved in 0.1× standard saline citrate (SSC; 1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and incubated with 5 mg of RNase per 100 ml at 37°C for 1 h. The RNase was pretreated by heating at 80°C for 10 min to inactivate any DNase. After further treatment with predigested protease, 1/10 of a volume of 2% deoxycholate was added, and the DNA solution was incubated for 1 h further at 37°C and overnight at 4°C. The DNA was purified by centrifugation at 45,000 × *g* for 40 h in cesium chloride (1

TABLE 2. Antibiotic sensitivity of strains of *T. ruber*

Antibiotic (μg/disk)	Zone of growth inhibition (mm) for strain[a]:																	
	16059	16060	16061	16062	16063	16064	16066	16067	16068	16102	16103	16106	16107	16109	16110	16111	16149	BKMB-1258
Methicillin (10)	7.5	13	8	13	14.5	16.5	14.5	16.5	10.5	13	9	19	16	12.5	13	15	15	5
Cloxacillin (5)	14	14.5	12	13.5	15	18	6	16	12	12.5	15	13	17	13.5	10	12	13	10
Ampicillin (2)	13.5	14	13	14	13.5	16.5	7	16	15.5	15	15.5	17	14.5	10	13	13	16	5
Penicillin G (5)[b]	14.5	17.5	12.5	18	18.5	18	15	20	14	16.5	16	19	7	14	16	13.5	19.5	14
Tetracycline (30)	10	11.5	10.5	12	10	10	10	9.5	10	11.5	9	10.5	11.5	11	7.5	8.5	12.5	8
Tetracycline (50)	11	13	11	12	10	10	10.5	10	10.5	11	11.5	11.5	11	11.5	10	10.5	13.5	10
Streptomycin (25)	9.5	9.5	8.0	9.6	8.5	7.0	7.5	9.0	8.5	9	7	8	9	8	6.5	8.5	8.5	9
Colistin sulfate (25)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
Fusidic acid (10)	2	3.5	4	3	0.5	4	3	3.5	1	5.5	1.5	4.5	4.0	2.5	5.0	0	4	4
Novobiocin (5)	9	7	7.5	6.5	10	8.5	5.5	8	6	7.5	9.5	7.5	7.0	6.5	7	7	8	7
Chloramphenicol (50)	17	10	10.5	12.5	11.5	11	12	12	10	11	12.5	13	12	12	10	12	13.5	10
Neomycin (10)	2.5	3.5	3	4.5	1.5	4.5	3	2.5	2	2	2.5	4	2	2.5	2	2	3	
Erythromycin (30)	12	13.5	14	15	14	12	12	14.5	13.5	14.5	14.5	14	13.5	12	12.5	10	17.5	12

[a] No sensitivity to sulfafurazole (500 μg), nalidixic acid (30 μg), kanamycin (5 μg), or trimethoprim (5 μg) was observed.
[b] Measured in units, rather than micrograms.

TABLE 3. DNA:DNA homology between *Thermus* strains

Strain	T_m	% G+C[a]	% Relatedness to strain:				
			YT1	BKMB-1258	16059	16066	16107
T. aquaticus YT1	80.4	64	100	14	7	9	11
T. ruber							
BKMB-1258	79.2	61	15	100	95	92	90
16059	78.2	59		103	100	99	101
16060	79.1	61		105	81	84	87
16061	79.6	62		100	85	96	98
16062	78.6	60		106	101	102	95
16063	79.5	62		110	89	102	98
16064	79.3	62		102	89	92	84
16066	79.6	62		105	98	100	101
16067	79.7	62		103	94	99	96
16068	79.0	61		96	85	87	96
16102	79.2	61		101	81	75	90
16103	79.4	62		103	78	83	93
16104	79.7	62		101	92	89	84
16106	79.7	62		92	86	99	96
16107	79.1	61		104	88	94	100
16109	79.5	62		108	101	105	92
16110	79.0	61		102	87	94	94
16111	79.8	63		101	93	96	95
16149	79.0	61		108	102	93	90
L. murinus[b]	68.8	40.0	8	3	2	2	4

[a] G+C = 52.0 + 2.08(T_m − 74.7), where 74.7 is the midpoint temperature (T_m) and 52.0 is the % G+C of *E. coli* B (DSM 2840) (18).
[b] *L. murinus* was used as a reference strain.

g/ml of DNA solution) and ethidium bromide (0.2 mg/ml). The DNA band was removed and dissolved in 0.1× SSC, and the ethidium was extracted with butan-2-ol. The purified DNA was dialyzed against 0.1× SSC, and the DNA mean base composition was determined by thermal denaturation in a Pye Unicam 8800 spectrophotometer and an SPX876 series 2 temperature programmer. The mean base composition was calculated from the formula G+C = 52.0 + 2.08(T_m − T_m of *E. coli* B DNA), where T_m is the midpoint temperature, with DNA prepared from *E. coli* DSM 2840 as an internal standard.

DNA-DNA hybridization on nitrocellulose filters. DNA homology was determined as described previously by Dent and Williams (7). The DNA was labeled with [^{3}H]dCTP by nick translation (kit TRK700; Amersham International, Amersham, United Kingdom). The specific activities ranged between 10^5 and 10^6 cpm/g of DNA.

DNA (600 μg) was denatured at pH 14 for 20 min at room temperature, carefully neutralized in an ice bath, loaded onto nitrocellulose filters (Sartorius type SM52), washed with 3× SSC, and dried in a vacuum. The filters were baked at 80°C for 4 h and multiple 5-mm disks were cut from each 50-mm filter. The disks, bearing 7 to 12 μg each of DNA, were incubated for 4 h with 500 μl of Denhardt solution at 60°C; then the Denhardt solution was aspirated and replaced with 200 μl of 3× SSC containing 0.05 μg of [^{3}H]DNA and 23% formamide. Hybridizations were allowed to proceed for 20 h at 60°C. The samples were then cooled in ice, the liquid was aspirated, and the filters were washed twice with 500 μl of ice-cold 3× SSC and dried at 80°C. The bound radioactivity was measured in 3 ml of toluene cocktail O (British Drug Houses, Poole, United Kingdom) with an SL 3000 liquid scintillation spectrometer (Intertechnique, Dover, N.J.). Homologous hybridization count rates were equated with 100% binding homology, and the heterologous count rates were expressed as a proportion of the homologous reactions. The results in Table 3 are the means of triplicate hybridizations.

RESULTS AND DISCUSSION

The ability of strains of *T. ruber* to grow on carbohydrates, carboxylic acids, and sugar alcohols varies considerably (Table 4). The most metabolically active strains in the absence of vitamin supplements were 16106 and 16109. Strains 16060, 16061, and 16062 were also able to grow on some substrates without vitamins, but the others were unable to grow. The addition of a vitamin supplement to the medium enabled these less active strains to grow on a range of substrates. Most strains grew well on fructose, maltose, mannose, and sucrose as the sole carbon source, unlike the yellow-pigmented strains previously described (10, 15), but growth was poor or absent on acetate and pyruvate. Strains 16110 and 16111 were the least metabolically active and grew only with fructose as the sole carbon source. For most strains, proteolysis was detectable against all substrates used except elastin, and the hydrolysis of Tween 80 and DNA was common. Only two strains showed measurable zones of hydrolysis on starch plates (Table 5), despite the fact that most strains grew on dextrin as a substrate (Table 4). Studies by Munster et al. (15) indicated that over 60% of the yellow-pigmented *Thermus* species hydrolyzed starch.

All strains grew in 0.3% tryptone–0.1% yeast extract (pH 7.8) at 37 and 60°C but not at 30 or 70°C. At 60°C, none of the strains grew at pH 4.0, but all grew sparsely at pH 11.0. Most grew better at 60°C and pH 10.0, and only one strain (16109) did not grow at pH 5.0. Enrichment cultures incubated at 70°C with source material from the United States, Kenya, Portugal, and Iceland have always failed to produce red-pigmented strains. All the isolates grew in both 1 and 2% sodium chloride. None grew anaerobically on tryptone-yeast extract plates, but two strains (16060 and 16062) did grow

TABLE 4. Growth of *T. ruber* isolates in *Thermus* basal medium supplemented with vitamins and carbon sources

Substrate	Growth of strain[a]																
	16059	16060	16061	16062	16063	16064	16066	16067	16068	16102	16103	16106	16107	16109	16110	16111	16149
Acetate	−	−	−	−	−	−	−	−	−	−	+	−	+	+	−	−	−
L-(+)-Arabinose	−	−	−	−	−	−	−	−	−	−	−	−	−	++	−	−	−
Dextrin	+	+	+	+	++	++	+	+	+	+	+	++	++	++	−	−	+
Dulcitol	−	+	+	−	−	−	+	+	−	−	−	−	−	−	−	−	−
Erythritol	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
D-(+)-Galactose	+	+	+	+	++	+	+	+	+	++	++	+++	+++	+++	−	−	++
Glycerol	+	+	+	−	−	−	−	−	−	−	−	+	++	+	−	−	−
meso-Inositol	−	−	−	−	−	−	−	−	−	+	+	+	+	++	−	−	+
Lactose	++	+	+	+	+	+	−	−	−	++	++	++	+++	+++	−	−	+
D-(−)-Fructose	++	+	++	+	++	++	+	+	−	+++	+++	++	+++	+++	+	+	++
Maltose	+	++	+	+	++	+	+	+	−	+	+	+++	+++	+++	−	−	+
Mannitol	+	−	+	−	+	+	+	+	−	−	+	+	++	+	−	−	−
D-(−)-Mannose	+	−	+	+	+	+	+	++	+	+	++	++	+++	+++	−	−	+
Pyruvate	−	−	−	−	−	−	−	−	−	−	−	+	+	++	−	−	−
Raffinose	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−
L-(+)-Rhamnose	+	+	+	−	−	−	−	−	−	−	−	−	+	+	−	−	−
Salicin	+	+	+	−	−	−	−	−	−	−	−	+	++	++	−	−	−
Sorbitol	−	−	−	−	+	+	+	+	−	+	+	+	+	+	−	−	+
Succinate	−	+	+	−	−	−	−	−	−	−	−	−	+	++	−	−	−
Sucrose	+	+	+	+	++	++	+	++	+	+	++	++	+++	+++	−	−	+
D-(+)-Xylose	+	+	+	+	+	+	+	+	+	+	+	++	++	++	−	−	+

[a] None of the strains grew on citrate, malate, propionate, or esculin. −, No growth; +++, strong growth; ++ or +, intermediate growth.

anaerobically in the presence of nitrate. These strains, like some of the yellow-pigmented strains (15), were believed to produce nitrate reductase, which enabled them to use nitrate as a terminal electron acceptor. All strains were catalase positive and oxidase negative after 2 days, and all of them hydrolzsed arbutin and reduced potassium tellurite in 2 days. The type strain of *T. ruber* showed the same reactions in all of these tests as the Icelandic isolates.

Unlike the yellow-pigmented *Thermus* strains previously described by Munster et al. (15), all of the pink-pigmented strains we examined were resistant to kanamycin at a concentration of 5 μg/ml. Resistance to nalidixic acid and sulfafurazole was observed in all strains. Previous studies (15) showed a variation in susceptibility. As in previous studies (15), all strains we studied were resistant to nalidixic acid. Susceptibility to β-lactam antibiotics was similar to that described in previous reports (9, 10, 15). In contrast to the strains examined by Munster et al. (15), all of the strains we studied were resistant to colistin sulfate.

Despite some visible differences in pigmentation, the results of spectroscopy of the extracted carotenoids for all strains were similar (Table 6) to that of the *T. ruber* type strain. The spectra of acetone extracts of the *T. ruber* type strain and strain H3 (9) show similar spectral features. These subjective color differences may be due to variations in the concentration of the carotenoid in the cells, or there may be labile pigments that do not survive in the solvent extracts. There is no correlation between colony color and the optimal temperature for the strains or their source temperatures (Table 1). Hensel et al. (9) observed similar spectra for acetone extracts of strains H1 and H3, which were described as having orange and cherry-red colonies, respectively.

The moles percent G+C in the DNA of all the pink-pigmented strains was in the range of 59 to 63. This is slightly lower than the moles percent G+C generally found in the yellow *Thermus* strains.

Neither the three Icelandic red-pigmented strains used for preparing labeled DNA nor the type strain of *T. ruber* (BKMB-1258) showed significant DNA homology with the type strain of *T. aquaticus*. The Icelandic isolates showed high DNA:DNA homology with the representative test strains and with the *T. ruber* type strain (BKMB-1258).

TABLE 5. Hydrolysis of macromolecules by strains of *T. ruber*

Substrate	Growth of strain[a]																	
	16059	16060	16061	16062	16063	16064	16066	16067	16068	16102	16103	16106	16107	16109	16110	16111	16149	BKMB-1258
Casein	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+
DNA (salmon sperm)	W+	−	+	W+	W+	−	+	+	+	W+	−	W+	W+	W+	W+	+	+	−
Fibrin	W+	W+	+	+	W+	−	W+	W+	+	−	−	+	+	W+	W+	W+	W+	+
Gelatin	+	+	+	+	+	+	+	+	+	NT	+	+	+	+	+	+	+	+
Hide powder azure	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+
Starch	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−	−	−	−
Tween 80	+	+	W+	−	W+	−	W+	+	+	+	−	−	−	+	+	+	+	−

[a] All strains failed to hydrolyze elastin. +, Hydrolysis; −, no hydrolysis; NT, not tested; W, weak hydrolysis.

TABLE 6. Spectroscopy of methanol extracts of red-pigmented *T. ruber* strains

Strain	Color of colony	Wavelength (nm) of spectral feature		
		Shoulder 1	Peak	Shoulder 2
BKMB-1258	Red	452	474	495
16059	Orange	452	474	496
16060	Orange	452	472	492
16061	Orange	452	470	492
16062	Red	452	473	496
16063	Red	452	475	496
16064	Red	452	472	492
16066	Orange	450	474	495
16067	Red	452	470	492
16068	Orange	452	474	496
16102	Orange-red	452	474	498
16103	Orange-red	451	474	496
16106	Orange-red	452	474	496
16107	Orange-red	454	476	496
16109	Orange-red	452	474	496
16110	Orange	451	473	496
16111	Orange	452	473	494
16149	Orange-red	452	470	494

The evidence indicates that the Icelandic isolates were a relatively homogeneous group of strains and, although isolated from distant geographical locations, closely resembled the type strain. The data from DNA-DNA hybridization indicate considerable differences between the type strain of *T. aquaticus* and the *T. ruber* strains.

Thermus strains stain as unambiguously gram-negative bacteria (3, 4, 8, 16, 17, 19–21, 23), and the ultrastructure of the cell wall with its corrugated external layer supports the inclusion of the genus among gram-negative taxa. However, there are many features which indicate that *Thermus* spp. have gram-positive affiliations, e.g., the inclusion of menaquinones rather than ubiquinones as respiratory chain components, sensitivity to some antibiotics, the presence of branched fatty acids in the membranes, and the presence of ornithine peptidoglycan (9). Furthermore, a distant relationship to *Deinococcus* species has been suggested on the basis of partial oligonucleotide catalogs of the 16S rRNA (9). Despite this suggestion, it seems prudent to regard *Thermus* spp. as of uncertain affiliations until more evidence is available, rather than attempt to redefine the taxonomic position of the whole genus.

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