Characterization of halotolerant *Thermus* isolates from shallow marine hot springs on S. Miguel, Azores

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Fifteen yellow-pigmented halotolerant *Thermus* strains from shallow marine hot springs on a beach on S. Miguel, Azores, several isolates from terrestrial hot springs on S. Miguel and mainland Portugal, and four reference strains were subjected to a numerical taxonomy study based on 61 characters by unweighted average linkage analysis (UPGMA) using the simple matching (S_{SM}) coefficient. This analysis produced four clusters at the 75 % similarity level. Two clusters, A and B, were composed of halotolerant strains from the beach hot springs and one strain isolated inland, all of which also grew at 80 °C; cluster C was composed of strains from one hot spring in mainland Portugal, while cluster D was composed of terrestrial strains from the Furnas area of S. Miguel. '*T. thermophilus*' HB-8 and *Thermus* B were also halotolerant, also grew at 80 °C, and formed a large group at the 61 % similarity level with the strains of clusters A and B.

Introduction

Terrestrial hydrothermal areas are common geological features throughout the world, but shallow hydrothermally heated marine sea floors are very rare. Most of the organisms reported from these marine environments are extremely thermophilic anaerobic archaeobacteria (Fiala & Stetter, 1986; Zillig *et al.*, 1987; 1990) and eubacteria of the genus *Thermotoga* (Huber *et al.*, 1986; Jannasch *et al.*, 1988). Other, more moderate thermophilic eubacteria of the genus *Thermus* (Kristjansson *et al.*, 1986; Hjorleifsdottir *et al.*, 1989; Hudson *et al.*, 1989) and *Rhodothermus marinus* (Alfredsson *et al.*, 1988) have also been isolated from neutral to alkaline shallow marine hot springs where they may constitute major aerobic populations.

The high-temperature strains of the genus *Thermus*, with optimum growth temperatures between 70 °C and 75 °C, have a worldwide distribution in neutral to alkaline terrestrial hot springs (Munster *et al.*, 1986; Hudson *et al.*, 1989; Santos *et al.*, 1989) and artificial environments (Pask-Hughes & Williams, 1975; Stramer & Starzyk, 1981). The small number of marine isolates examined are markedly more halotolerant than the vast majority of high-temperature *Thermus*, but share several phenotypic characteristics with the terrestrial organisms. The relationship of the marine strains to *T. aquaticus* (Brock & Freeze, 1969) and *T. filiformis* (Hudson *et al.*,

1987), or to other, possibly unrecognized species, is unknown.

In this study we describe the phenotypic characteristics of several *Thermus* isolates from the coastal marine vents at Ribeira Quente, S. Miguel. These strains are compared to terrestrial strains isolated nearby, to isolates from mainland Portugal and to several reference strains.

Methods

Sources and isolation of strains. Water samples were collected from small depressions dug in the sand during low tide from four sites within an area of about 500 m² at the sandy beach at Ribeira Quente on S. Miguel, Azores. Water samples were also collected from terrestrial hot springs about 10 km inland in the Furnas area of S. Miguel, and at S. Pedro do Sul in central mainland Portugal. Temperature, pH and salinity (Yellow Spring Inst. Co., model 33) were measured at the time of sampling (Table 1).

Water samples were filtered within 6 h, through Gelman type GN-6 (0.45 µm pore size) membrane filters, placed on *Thermus* agar (Brock, 1978) and incubated for 3 d at 70 °C in plastic bags. Representative yellow-pigmented colonies were isolated, transferred to *Thermus* agar for purification and stored at –80 °C in *Thermus* medium with 15% (v/v) glycerol.

T. aquaticus YT-1 (ATCC 25104), '*T. thermophilus*' HB-8 (ATCC 27634) and *T. filiformis* (ATCC 43280) were obtained from the American Type Culture Collection. Strain B (NCIMB 11247) was kindly donated by R. A. D. Williams; strains SPS-17 and RQ-1 were described previously (Santos *et al.*, 1989; Williams, 1989).

Characters tested. Most phenotypic characteristics were examined as applied by Hudson *et al.* (1986) and Santos *et al.* (1989) in *Thermus* medium (5.0 ml) or *Thermus* agar incubated at 70 °C for up to 7 d.

Table 1. Origin of isolates arranged by cluster

Cluster	Strain	pH	°C	Salinity (%)	Sample location
A	PRQ-14	7.2	72	21	Beach, Ribeira Quente, S. Miguel
A	PRQ-16	7.2	72	21	Beach, Ribeira Quente, S. Miguel
A	PRQ-21	7.1	74.5	29	Beach, Ribeira Quente, S. Miguel
A	PRQ-24	7.0	55	29	Beach, Ribeira Quente, S. Miguel
A	PRQ-26	7.0	55	29	Beach, Ribeira Quente, S. Miguel
A	PRQ-25	7.0	55	29	Beach, Ribeira Quente, S. Miguel
A	PRQ-23	7.0	55	29	Beach, Ribeira Quente, S. Miguel
A	PRQ-27	7.0	55	29	Beach, Ribeira Quente, S. Miguel
B	PRQ-15	7.2	72	21	Beach, Ribeira Quente, S. Miguel
B	PRQ-30	6.8	40	30	Beach, Ribeira Quente, S. Miguel
B	PRQ-31	6.8	40	30	Beach, Ribeira Quente, S. Miguel
B	RQ-1	NA	70	NA	Cliff, Ribeira Quente, S. Miguel
B	PRQ-17	7.1	74.5	29	Beach, Ribeira Quente, S. Miguel
B	PRQ-18	7.1	74.5	29	Beach, Ribeira Quente, S. Miguel
B	PRQ-28	6.8	40	30	Beach, Ribeira Quente, S. Miguel
B	PRQ-19	7.1	74.5	29	Beach, Ribeira Quente, S. Miguel
-	B	8.9	82	NA	Hruni, Iceland
-	<i>T. thermophilus</i> HB-8	6.3	80	NA	Mine, Japan
-	<i>T. aquaticus</i> YT-1	NA	71.5	NA	Yellowstone Park, USA
C	SPS-17	8.5	50	NA	S. Pedro do Sul
C	SPS-21	8.3	70.5	NA	S. Pedro do Sul
C	SPS-20	8.3	70.5	NA	S. Pedro do Sul
-	<i>T. filiformis</i>	7.3	82	NA	North Island, New Zealand
D	ECG-1	8.5	75	NA	Runoff, Caldeira Grande, S. Miguel
D	CB-4	7.5	90	NA	Caldeira da Barreira, S. Miguel
D	ECG-2	8.5	75	NA	Runoff, Caldeira Grande, S. Miguel
D	CB-3	7.5	90	NA	Caldeira da Barreira, S. Miguel
D	ECB-6	8.4	75	NA	Runoff, Caldeira da Barreira, S. Miguel

NA, Not available.

Some tests were modified as follows: Casein hydrolysis was performed with 5.0% (w/v) skim milk powder (Difco) and 1.5% (w/v) agar overlaid on *Thermus* agar which had been allowed to solidify (R. Sharp, personal communication). The hydrolysis of gelatin was tested in *Thermus* agar with 0.4% gelatin (Difco). Cultures were flooded with HgCl₂-HCl (Smibert & Krieg, 1981) after incubation of replicate plates for 1 d or 3 d. The degradation of arbutin was examined in *Thermus* agar containing 0.5% filter-sterilized arbutin and 5.0 mg l⁻¹ ammonium ferric citrate. The degradation of aesculin was tested in *Thermus* agar supplemented with 0.01% aesculin and 5.0 mg l⁻¹ ferric citrate. Nitrate reduction was performed in *Thermus* medium containing only 1.0 g l⁻¹ KNO₃ and 0.25% agar. Starch hydrolysis was examined in *Thermus* agar in which tryptone was replaced by NH₄Cl (0.05%) and containing 10.0 g l⁻¹ soluble starch. Plates were flooded with Lugol's iodine after 5 d incubation.

Single carbon source utilization was tested in 96-well round bottom culture clusters (Costar). The double strength basal medium consisted of CMD salts (Castenholtz, 1969) adjusted to pH 8.2 and sterilized by autoclaving. Filter-sterilized (NH₄)₂SO₄ (0.5 g l⁻¹ final concentration) was added to cooled medium. Since several strains did not grow or exhibited poor growth in the basal medium a growth factor supplement and an amino acid solution were added to the basal salts-(NH₄)₂SO₄ medium (R. Sharp, personal communication). The growth factor supplement, consisted of thiamine, riboflavin, pyridoxine, biotin, folic acid, inositol, nicotinic acid, pantothenic acid, *p*-aminobenzoic acid, cyanocobalamin, adenine, thymine, cytidine, uracil and inosine, each at a final concentration of 40.0 µg l⁻¹. The amino acid solution contained the following L-amino acids: aspartic acid, tyrosine, valine, phenylalanine, lysine, glutamic acid, alanine, asparagine, arginine,

tryptophan, cysteine, cystine, serine, leucine, threonine, isoleucine, proline, methionine and histidine, each at a final concentration of 3.5 mg l⁻¹. The inoculum (1 ml) with a turbidity equal to the standard No. 2 of McFarland was added to 15 ml medium and 200 µl of this suspension was added to duplicate wells containing 5 µl of the appropriate filter-sterilized carbon source at a final concentration of 2.5 g l⁻¹. Controls with no added carbon source, as well as controls in *Thermus* medium were also included. Incubation was at 65 °C for 3 d (Santos *et al.*, 1989).

Calculation of test error. Five random strains were prepared in duplicate at the beginning of the study, assigned different numbers and tested as separate isolates (Sneath & Johnson, 1972).

Analysis of results. The phenetic data, consisting of 61 characters scored as plus (+) or minus (-) were analysed using the simple matching (*S*_{SM}) coefficient (Legendre & Legendre, 1984). Clustering was accomplished by unweighted average linkage analysis (UPGMA) using the Numerical Taxonomy and Multivariate Analysis Program (NTSYS-pc) developed by Rolf (1987).

Results

Clustering of isolates

Analysis of the *S*_{SM}/UPGMA results indicated that four clusters, labelled A to D, could be defined at the 75% similarity level (Fig. 1). Error based on mismatches of

Fig. 1. Dendrogram representing the relationships of the *Thermus* isolates resulting from the UPGMA analysis based on the S_{SM} coefficient. The clusters, labelled A to D, were formed at the 75% level.

results for the duplicate strains was estimated at 4.6%. In addition, *T. aquaticus* YT-1, strain B, '*T. thermophilus*' HB-8 and *T. filiformis* did not cluster, at this level, with any of the other organisms.

Clusters A and B were composed of isolates from the beach at Ribeira Quente. Strain RQ-1, isolated from a hot spring about 5 km inland, was included in cluster B. Cluster C was composed of the S. Pedro do Sul isolates, while cluster D comprised all isolates from the Furnas area of S. Miguel.

At a lower similarity level (61%) two large groups were formed; one included all marine isolates and the terrestrial strains RQ-1, B and '*T. thermophilus*' HB-8 while the other comprised only terrestrial strains.

Characterization of the isolates

All *Thermus* strains formed yellow-pigmented colonies, nevertheless the marine isolates of cluster A were distinguished from all other organisms by their deep yellow-orange pigmentation.

All strains from the beach at Ribeira Quente and strains RQ-1, B and HB-8 were halotolerant and grew at 80 °C. These organisms grew in medium with at least 3% (w/v) NaCl, and most also grew in 5% (w/v) NaCl-containing medium (Table 2). The halotolerance of these

organisms was associated with their overall tolerance to KCl, CaCl₂, MgCl₂ and SrCl₂.

The halotolerant organisms of clusters A and B were generally less proteolytic than the non-halotolerant terrestrial *Thermus* strains. In contrast, strains of clusters A and B degraded *p*-nitrophenyl laurate within 7 h, while most in clusters C and D did not. Arbutin was degraded by most strains in clusters B, C, D but not by the isolates in cluster A.

Except for the members of cluster D, most strains examined here reduced nitrate and nitrite. These results correlated well with the ability of these strains to grow anaerobically in presence of nitrate. None of the strains grew anaerobically in *Thermus* medium without NO₃.

The pattern of single-carbon-source assimilation was essentially similar in all strains. Several carbon sources, which included proline, glutamine, and acetate, were used by all strains. Others such as acetamide, rhamnose and L-arabinose were not utilized at all. Melibiose, raffinose and sucrose were used by the strains of the cluster C, but most other organisms were negative for these sugars. Three strains utilized glycerol, PRQ-14 utilized glycerol and mannitol, and '*T. thermophilus*' HB-8 alone used D-xylose as single carbon source. Table 3 lists the most important characters for distinguishing the clusters from one another.

Of 29 other strains from world-wide terrestrial sources, only the four strains capable of growth at 80 °C also grew in 2% (w/v) NaCl. Only two of these, '*T. flavus*' AT-62 and '*T. caldophilus*' GK-24 grew in 3% (w/v) NaCl.

Discussion

Most high-temperature *Thermus* strains have been isolated from terrestrial hot springs and artificial thermal environments. These organisms constitute one of the major aerobic heterotrophic populations at sites with pH > 5 and temperatures between 55 °C and 85 °C (Kristjansson & Aldredsson, 1983; Munster *et al.*, 1985).

Several strains have also been described from shallow marine hot springs venting fresh water in Iceland (Kristjansson *et al.*, 1986) and one strain was isolated from a site on a beach on Fiji (Hudson *et al.*, 1989). The marine strains from Iceland were, based on the phenotypic characteristics examined, similar to terrestrial strains isolated nearby, except that the former were halotolerant.

This study demonstrates a relationship, at a low similarity level, between the marine strains from the beach at Ribeira Quente and the terrestrial halotolerant strains '*T. thermophilus*' HB-8 and B. Strain RQ-1 was isolated from a hot spring about 5 km upstream from the beach sites (Santos *et al.*, 1989), and is clearly similar to

Table 2. Percentage of positive results for the clustered strains derived from S_{SM} /UPGMA analysis and phenotypic characteristics of *T. aquaticus* YT-1, '*T. thermophilus*' HB-8, *T. filiformis* and *Thermus* strain B

All strains were positive for growth at 45 °C, 75 °C, the utilization of fructose, maltose, acetate, pyruvate, asparagine, glutamine, glutamate, proline and for the presence of alkaline phosphatase. All strains were negative for the utilization of L-arabinose, rhamnose, α -ketoglutarate, acetamide and for the presence of acid phosphatase.

Character	Reference strains				Clusters (No. in clusters)			
	YT-1	HB-8	B	<i>Thermus filiformis</i>	A(8)	B(8)	C(3)	D(5)
Filamentous	—	—	—	+	0	0	0	0
Growth at/in:								
80 °C	—	+	+	—	100	100	0	0
1% NaCl	+	+	+	—	100	100	100	60
3% NaCl	—	+	+	—	100	100	0	0
5% NaCl	—	—	+	—	63	75	0	0
2% KCl	—	+	+	—	100	75	0	0
2% MgCl ₂	—	—	+	—	100	100	0	0
3% CaCl ₂	—	+	+	—	100	100	0	0
3% SrCl ₂	—	+	+	—	100	100	0	0
3.5% Tryptone	+	+	+	—	13	63	100	0
pH 5.5	+	+	+	+	100	100	33	60
pH 9.5	+	+	+	—	13	63	0	0
Anaerobic growth with nitrate	—	—	+	—	100	88	100	0
Production of nitrite:								
1 d	—	—	+	—	100	88	100	0
7 d	—	—	—	—	0	13	0	20
Hydrolysis of:								
Elastin (72 h)	+	—	—	—	0	0	33	20
Fibrin (72 h)	+	—	+	—	13	0	67	40
Hyde powder azure (24 h)	+	+	+	+	75	25	67	60
Hyde powder azure (72 h)	+	+	+	+	100	75	100	100
Casein (24 h)	+	—	+	—	13	0	33	20
Casein (72 h)	+	—	+	—	88	13	67	60
Gelatine (24 h)	+	+	+	+	38	38	67	60
Gelatine (72 h)	+	+	+	+	88	50	100	100
Starch (5 d)	+	—	—	—	50	0	33	20
Degradation of:								
Arbutin	—	+	+	+	0	100	100	100
Aesculin	—	—	+	+	0	63	100	60
Browning with L-tyrosine	+	+	—	—	13	0	100	60
Triple sugar iron reddening	+	+	+	+	100	88	0	20
Reduction of tellurite (0.05%)	+	+	+	+	88	50	100	20
Presence of:								
DNAase (72 h)	+	+	+	—	100	38	33	80
α -Galactosidase	+	+	+	+	13	100	100	40
β -Galactosidase	—	+	+	+	0	100	100	20
Degradation of <i>p</i> -nitrophenyl substrates:								
α -Glucopyranoside Cells (5 h)	+	+	—	+	100	100	100	20
β -Glucopyranoside Cells (20 h)	—	+	+	—	0	100	67	0
Palmitate CFS (20 h)	—	—	—	—	25	50	0	0
Laurate CFS (7 h)	—	—	—	—	100	100	33	0
Antibiotics MICs (μ g ml ⁻¹)								
Phosphomycin MIC > 100	—	+	—	—	13	50	100	80
Kanamycin MIC > 1.25	+	+	+	—	63	63	100	20
Spectinomycin MIC > 400	—	+	+	—	100	100	67	40
D-Cycloserine MIC > 25	+	—	+	—	13	88	100	100
Utilization of:								
Xylose	—	+	—	—	0	0	0	0
Glucose	+	—	+	—	100	88	33	100
Galactose	+	—	—	+	50	75	100	60
Mannose	+	+	+	—	100	100	100	100
Cellobiose	+	+	+	—	0	100	67	40
Lactose	+	—	+	+	0	13	100	0
Sucrose	+	+	—	+	100	100	100	100
Trehalose	+	+	—	+	100	100	100	100
Melibiose	+	+	—	+	0	12	100	0

Table 2. (continued)

Character	Reference strains				Clusters (No. in clusters)			
	YT-1	HB-8	B	<i>Thermus filiformis</i>	A(8)	B(8)	C(3)	D(5)
Raffinose	+	—	—	+	0	12	100	0
Dextrin	—	—	—	—	13	25	0	0
Salicin	—	—	+	+	0	100	100	80
Glycerol	—	—	+	—	13	13	0	0
Mannitol	—	—	—	—	13	0	0	0
Citrate	—	—	+	—	38	25	33	0
Lactate	—	+	+	—	88	75	33	80
Malate	+	—	+	+	100	100	100	80
Succinate	+	—	+	+	100	100	100	100
Arginine	—	—	—	—	50	38	67	40
Ornithine	+	—	+	+	100	87	100	80
Serine	+	+	+	—	100	100	33	100

+, Positive result or growth; —, negative result or no growth; CFS, cell-free supernatant.

Table 3. Differential characteristics of clusters

Character	Cluster			
	A	B	C	D
Growth at 80 °C	+	+	—	—
Growth in 3% NaCl	+	+	—	—
Growth in 2% MgCl ₂	+	+	—	—
Production of nitrite (1 d)	+	+	+	—
Degradation of arbutin	—	+	+	+
β -Galactosidase	—	+	+	—
Utilization of melibiose	—	—	+	—
Utilization of raffinose	—	—	+	—

—, <20% positive; +, >80% positive.

the marine strains of cluster B. Other strains from hot springs at Furnas, about 7 km upstream, were not halotolerant and formed a separate cluster. In an earlier study, two large groups based on phenotypic characteristics were also formed; one was composed of strains from terrestrial hot springs from mainland Portugal and S. Miguel, none of which were halotolerant, and the other of the halotolerant strains '*T. thermophilus*' HB-8, RQ-1 and RQ-3 (Santos *et al.*, 1989).

The large degree of phenotypic diversity observed previously was affirmed here also. Geographical separation of the hot springs may explain the phenotypic diversity of clusters C and D, but the presence of two distinct populations at the sites on the beach at Ribeira Quente must be due to other factors. The variability within the high-temperature strains of *Thermus* has led others to propose the existence of several species based on phenetic characteristics (Hudson *et al.*, 1989), and

genospecies based on DNA:DNA hybridization studies (Williams, 1989). It is noteworthy that '*T. thermophilus*' HB-8, strain B and RQ-1, among others, share a very high DNA homology and may form a separate genospecies (Williams, 1989), although they clustered separately in this study.

Exchange of these halotolerant strains between nearby marine and terrestrial hot springs is probably frequent and may account for the presence of similar organisms at the beach at Ribeira Quente and a few kilometers upstream at the hot springs on the cliff by Ribeira Quente. It can be argued that terrestrial halotolerant strains would have a selective advantage in colonizing marine environments of moderate to high salinity (depending on the origin of thermal water and the degree of mixing with sea water) and would therefore predominate. Dispersion in the reverse direction would also permit the halotolerant strains to gain a foothold, all else being equal, in fresh water hot springs and establish long term populations.

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