Extremely Thermophilic Gram-negative Bacteria from Hot Tap Water

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SUMMARY

Two strains of heterotrophic, non-motile, Gram-negative extreme thermophiles have been isolated from hot tap water. These strains (NH and DI) have been characterized and compared with strains of the genus *Thermus*. Few of the single organic compounds tested supported growth in the presence of ammonium salts, and, like *Thermus* strains, growth on undefined media was restricted to dilute tryptone-yeast extract-mineral salt solutions. Nutrient agar and similar common laboratory media did not support growth. The growth rate was similar to that of *Thermus* strains, as was the unusual pattern of antibiotic resistance. Mean base composition of NH was 61.4 % guanine plus cytosine (G+C), and of DI was 62.0 to 62.2 % G+C, which are several per cent lower than the G+C contents of other strains so far described.

INTRODUCTION

The interest in bacteria which grow at high temperatures was given new impetus by the description of *Thermus aquaticus* (Brock & Freeze, 1969), an aerobic, yellow-pigmented, Gram-negative, heterotrophic bacterium with an optimum growth temperature of 70 °C. The original isolates were from hot springs in Yellowstone National Park, Montana, and in California, U.S.A., but it was observed that similar isolates could be obtained from hot tap water and a spring receiving thermal pollution, but not from cold tap water or cold springs. Ramaley & Hixon (1970) isolated a strain which lacked yellow pigment, but which otherwise resembled *Thermus aquaticus*, from a stream receiving hot water effluent in Bloomington, Indiana, U.S.A. They proposed that the pigment protected the cells in hot springs against sunlight, and that non-pigmented strains, if they grew faster than pigmented ones, would have a selective advantage in the dark environment of hot water tanks. Many similar strains, mostly non-pigmented, were subsequently purified from the water tanks of laundries and homes in Madison, Wisconsin, U.S.A. (Brock & Boylen, 1973). A yellow-pigmented strain, originally called *Flavobacterium thermophilum* (Oshima & Imahori, 1971) and isolated from the Mine Hot Spring, Shizuoka, Japan, has been compared with *Thermus aquaticus* and renamed *Thermus thermophilus* (Oshima & Imahori, 1974). A pink-pigmented isolate from Yellowstone National Park which was morphologically distinct from *Thermus*, and had an alkaline optimum pH for growth, has recently been allotted to another new genus, *Thermomicrobium* (Jackson, Ramaley & Meinschein, 1973).

This paper forms part of a study of thermophilic bacteria as a source of thermostable enzymes, and describes two cultures enriched from the hot tap-water of two separate hot water systems at The London Hospital.

METHODS

Strains used. Hot tap water (100 ml) was mixed with 100 ml medium containing final concentrations of 0.1 % (w/v) tryptone (Difco) and 0.1 % (w/v) yeast extract (Difco), with 10 % (v/v) of mineral salts (Ramaley & Hixon, 1970). This mixture was incubated 3 days at 70 °C as a shallow layer in a bottle lying on its side. The turbid cultures were streaked on the above medium plus agar 2 % (w/v). The Petri dishes were incubated overnight sealed in polythene bags to prevent drying out. Individual colonies were restreaked repeatedly, and the purified cultures freeze-dried in brain-heart infusion broth plus serum.

Strain YT₁, the type strain of *Thermus aquaticus* (Brock & Freeze, 1969) was supplied by the A.T.C.C. (strain No. 25104). Strain X₁ (Ramaley & Hixon, 1970) was kindly donated by Dr R. Ramaley.

Growth on single substrates. Agar plates (2 %, w/v) were prepared containing 0.2 % (w/v) substrate, together with 0.05 % (w/v) (NH₄)₂SO₄ and 10 % (v/v) mineral salts solution (Ramaley & Hixon, 1970). Each strain was streaked on a whole plate for each substrate. Plates were incubated at 70 °C and examined on the 3rd and 5th days for growth. If every time that growth was tested it was sparse and limited to the primary inoculum streak, this was attributed to carry-over of nutrients from the inoculum and scored as negative growth.

Determination of growth rate. A thermal gradient incubator (Scientific Industries International Inc. (U.K.) Ltd., Loughborough) was used with tubes containing 20 ml of growth medium (GM), comprising final concentrations of 0.3 % (w/v) yeast extract, 0.3 % (w/v) tryptone, together with 10 % (v/v) mineral salts solution. The tubes were shaken at 45 oscillations per minute so that a bubble traversed the length of the culture tube at each oscillation. Growth was measured turbidimetrically with an EEL 222 colorimeter.

Antibiotic resistance. Plates containing GM plus 2 % (w/v) agar were flooded with a culture of each strain and air-dried until no free fluid was evident on the agar surface. Oxoid 'Multodiscs' were then applied to the plates, which were incubated in polythene bags at 70 °C. The zone size, from the edge of a disc to the edge of bacterial growth, was measured after 5 days.

Bulk growth of bacteria. Portions (500 ml) of GM were used in 2 l Erlenmeyer flasks fitted with baffles to increase aeration. Cultures were incubated at 70 °C in a Gallenkamp IH620 rotary shaking incubator at 150 rev./min. Bacteria were harvested by centrifugation (5700 g) for 15 min, washed three times with distilled water and stored at -20 °C.

Spectrophotometry of pigments. Approximately 6 g wet wt bacteria were dispersed in 10 ml boiling methanol and the pigments allowed to extract for 30 min. The spectrum of the clear supernatant after centrifugation was recorded between 310 and 510 nm using a Perkin-Elmer 402 spectrophotometer.

Preparation of extracts. Ice-cold bacterial suspensions were disrupted with a sonic disintegrator, or by pressing in a Biox Press (Nacka, Sweden). Unbroken bacteria and debris were removed by centrifugation at 170000 g for 2.5 h, and the supernatant was used immediately or stored at -20 °C.

Spectrophotometry of cytochrome. A difference spectrum of the extract versus the same reduced with freshly prepared sodium dithionite solution was scanned between 390 and 590 nm by using a Perkin-Elmer 402 spectrophotometer.

Preparation and analysis of DNA. Washed whole bacteria were treated with trypsin (0.2 mg/ml in 0.2 M-phosphate buffer pH 8.0) at 37 °C for 3 h and then recovered by centrifugation (5700 g, 15 min.). Lysis was carried out with 2 % (w/v) sodium laurylsulphate at

70 °C for 15 min. DNA was isolated by the method of Marmur (1961). Where DNA was not precipitated in a form that could 'be spooled' up on a glass rod, the preparation was left at 4 °C and the nucleic acid removed by centrifugation (1400 g, 10 min). Pure DNA solution was dialysed for 3 days at 4 °C against 500 vols. of the appropriate buffer, and the hyperchromicity due to thermal denaturation and the melting temperature determined with a Gilford model 240 spectrophotometer. The cell was heated by circulating ethylene glycol, and the temperature measured with a thermistor probe in a metal pocket inserted through the cap of the cell. The temperature was read with a thermistor thermometer (Grant Instruments Developments, Ltd, Barrington, Cambs.) which was checked against a mercury thermometer with a certificate of calibration (British Standards Institute). Both standard saline-citrate (Marmur & Doty, 1962) and $0.1 \times$ standard saline-citrate (Shildkraut & Lifson, 1965) were used. DNA prepared from *T. aquaticus* YT₁ was used as a standard.

Assay and heat inactivation of enzymes. Malate dehydrogenase was assayed by measuring the increase in extinction at 340 nm due to the reduction of NAD by malate. The assay mixture comprised 25 µl of suitably diluted bacteria-free extract in a 3 ml cuvette, and contained 30 µmol-malate, 1 µmol-NAD, 45 µmol-EDTA and 450 µmol-tris at pH 9.5. Iso citrate dehydrogenase was assayed similarly with 25 µl of diluted extract in a 3 ml cuvette, the extract containing 1 µmol-isocitrate, 10 µmol-MgCl₂, 0.5 µmol-NADP and 112 µmol-sodium diethylbarbiturate at pH 8.5. Assays were performed at 45 °C. Heat inactivation was studied by sealing 0.3 ml of extract diluted in the appropriate buffer in a glass ampoule and totally immersing these in an oil bath at 90, 95 or 100 °C. Multiple ampoules were used and one was withdrawn at each time interval, cooled in ice-water, and maintained at 4 °C until assayed for enzyme activity. Buffer solutions used for inactivation experiments were 50 mM-phosphate pH 7.6 for malate dehydrogenase, and 40 mM-diethylbarbiturate pH 8.5 for isocitrate dehydrogenase.

RESULTS

Isolation of strains

After 3 days' incubation all enrichment media became turbid. Of ten cultures of hot tap water, from this Institution and private houses, all contained Gram-positive bacteria but two also had substantial growth of Gram-negative bacteria. One Gram-negative strain was purified from each of these two cultures by restreaking well-isolated colonies on yeast extract-tryptone plates. After two days at 70 °C the colonies were buff-coloured, compact, smooth and with entire margins. The two strains, designated NH and DI, originated in separate hot water systems.

Both strains were aerobic, non-motile, non-sporulating rods which sometimes formed filaments. Growth occurred on the dilute GM in liquid culture and on plates, but not on commonly used bacteriological media such as nutrient agar, blood agar, Rogosa agar and MacConkey agar.

Growth on single substrates

Like the *Thermus* strains already described (Brock & Freeze, 1969; Ramaley & Hixon, 1970), strains DI and NH grew on few single organic compounds (Table 1) with ammonium sulphate as a nitrogen source. When distinct growth occurred the colonies were smaller than they would have been on yeast extract-tryptone, although the colonial appearance was similar. Strains NH, YT₁ and X₁ grew significantly on both glycerol and glutamate. Strain DI grew sparsely and inconsistently on glycerol, but not on glutamate or any other substrate tested.

Table 1. *Growth on agar plates containing single substrates*

Substrate (0.2 %, w/v)	Strain			
	YT ₁	X ₁	NH	DI
Glucose	—	—	—	—
Fructose	—	—	—	—
Arabinose	—	—	—	—
Xylose	—	—	—	—
Sucrose	±	—	+	—
Starch	—	—	—	—
Succinate	±	±	—	—
Citrate	±	—	—	—
Acetate	+	+	—	—
Malate	—	—	—	—
Formate	—	±	—	—
Mannitol	—	+	—	—
Glycerol	+	+	+	±
Alanine	—	—	—	—
Glycine	—	—	—	—
Glutamate	+	+	+	—

+, Distinct growth; ±, growth slight and irregular and sometimes limited to the primary inoculum streak; —, no growth.

Growth rate on liquid media

The growth curve for *T. aquaticus* YT₁, determined in a thermal gradient incubator (Fig. 1), resembles in shape that published by Brock & Freeze (1969) for shake flask cultures, but in our experiments the optimum growth temperature was 69 rather than 70 °C. The shape of the growth curve for isolate DI was similar to that for YT₁, with the optimum at 69 °C. Strain NH grew rapidly in a broader range of temperature, the optimum being at 70 °C. At 55 °C the mean doubling time for NH was 114 min, almost exactly twice the value at the optimum temperature. By contrast the corresponding difference in mean doubling time was threefold for DI.

Antibiotic sensitivity

The patterns of resistance to antibiotics in four strains of Gram-negative thermophiles (Table 2) were similar. Notable is the general sensitivity of all strains to penicillin, except that YT₁ was less sensitive than the other strains to ampicillin. Strains NH and DI differed from those previously described in their resistance to sulphafurazole.

Pigments and cytochrome

The packed bacteria and colonies of NH and DI appear to have little pigmentation. Illumination of growing bacteria on agar or in liquid medium did not stimulate pigmentation. Methanol extracts of YT₁ and DI gave a distinct peak at 400 nm which was absent in similar extracts from strain NH. Neither NH nor DI showed any trace of the prominent pigment peaks at 450 and 475 nm observed in *T. aquaticus* YT₁. Aqueous extracts of both new strains, when reduced with sodium dithionite, showed prominent peaks in the regions 420, 520 to 525, and 555 to 560 nm, characteristic of *c*-type cytochromes. Fig. 2 shows the difference spectrum of NH, in which α and β peaks for cytochrome *c* itself (518 and 550 nm) are evident. Pronounced peaks at 524 and 557 nm may have been due to cytochrome *c*₁ but the shoulder at 530 nm and the peak at 557 nm may have been due to cytochrome *b*. It is also possible that type *a* cytochromes contributed to the peak at 550 nm, but no shoulder at 430 to 450 nm was observed in the difference spectrum.

Fig. 1

Fig. 1. Growth temperature profile of *T. aquaticus* YT₁. ●, Data of Brock & Freeze (1969) for shake flask cultures of YT₁; ○, determinations with the thermal gradient incubator with YT₁; □, strain DI; △, strain NH.

Fig. 2

Fig. 2. Difference spectrum of oxidized versus reduced cytochromes in extract of strain NH.

Table 2. Resistance to antibiotics in Oxoid Multodiscs

Antibiotic	Quantity in disc (μg)	Strain			
		YT ₁	X ₁	NH	DI
Fusidate	10	+++	+++	+++	+++
Colistin	50	++	++	++	+
Kanamycin	5	+++	+	+	+
Neomycin	10	—	—	—	—
Novobiocin	5	++	++	++	++
Cloxacillin	5	—	—	—	—
Ampicillin	2	++	—	—	—
Methicillin	10	—	—	—	—
Penicillin	5 (units)	—	—	—	—
Chloramphenicol	50	—	—	—	—
Erythromycin	50	—	—	—	—
Sulphafurazole	500	±	—	+++	++
Streptomycin	25	—	—	—	—
Tetracycline	50	++	—	++	++

+++ , No zone of inhibition; ++ , zone less than 6 mm from edge of disc; + , zone greater than 6 mm from edge of disc; — , zone of inhibition too large to read; ± , result sometimes + , sometimes — .

Table 3. Mean base composition of DNA of Gram-negative thermophiles

Strain	% G + C, determined by		
	Density-gradient centrifugation	Thermal denaturation in:	
		SSC	0.1 × SSC
<i>Thermus aquaticus</i>			
YT ₁	67.4†	65.4	64.4
YT ₁ -VII-5614C	65.4†	—	—
YT-VII-51D	65.4†	—	—
YT-VII-563A	65.4†	—	—
<i>Thermus</i> sp. X ₁	64–65‡	—	—
<i>Thermomicrobium roseum</i>	64.3‡	—	—
<i>Thermus thermophilus</i> HB8	68.4*	68.8*	66.9*
Strain NH	—	61.4	NT
Strain DI	—	62.2	62.0

SSC, standard saline-citrate; NT, not tested.

* Oshima (1972).

† Brock & Freeze (1969).

‡ Jackson *et al.* (1973).

Table 4. Heat inactivation of enzymes

Strain	Time taken to reduce enzyme activity by half (min)					
	Malate dehydrogenase			Isocitrate dehydrogenase		
	90 °C	95 °C	100 °C	90 °C	95 °C	100 °C
YT ₁	230	40	5.5	17.5	5.8	3.0
NH	194	30	4.5	11.5	2.4	1.6
DI	180	30	4.5	19.0	3.1	1.3

DNA base composition

Strains NH and DI have DNA mean base compositions of 61.4 and 62.2 % by thermal denaturation in standard saline-citrate (Table 3), which places these strains at the lower end of the range of % guanine plus cytosine (G + C) amongst Gram-negative extreme thermophiles. In the same solvent the mean base composition of YT₁ DNA was estimated to be 2 % G + C lower than the value reported by Brock & Freeze (1969).

Heat inactivation of enzymes

The stability of both malate dehydrogenase and isocitrate dehydrogenase is greater in strain YT₁ than in NH or DI, and malate dehydrogenase is the more stable enzyme in all strains and at all temperatures (Table 4). The time course of decay of active enzyme in the sealed tubes was always sigmoid, but less so at 90 °C than at 100 °C. The increase in activity of isocitrate dehydrogenase, particularly from YT₁, over the first part of the heat treatment was due to reversal of cold inactivation of enzyme stored in the refrigerator. The times of half inactivation were all calculated from the activities at time zero.

DISCUSSION

The two isolates described seem similar to the non-pigmented Gram-negative extreme thermophiles found growing in laundry heaters by Brock & Boylen (1973), and are probably strains of the genus *Thermus*. Isolates NH and DI grew well on dilute tryptone-yeast extract broth with mineral salts under aerobic conditions, but failed to grow on the more concentrated media, such as nutrient agar, that support the growth of spore-forming thermophiles. Streaking on such plates has therefore been used routinely to check for contamination with bacilli. No spores or Gram-positive bacteria have been observed in cultures over a period of two years.

Strain YT₁ was found to grow, sometimes sparsely, on sucrose, succinate, citrate, acetate and glutamate in agar plates. These results agree with those of Brock & Freeze (1969), but moderate growth was also observed on glycerol which supported the growth of all four strains. Isolate DI grew less well than the other strains upon glycerol, but it failed to grow on any other substrate. The exacting nutritional requirements of NH and DI, which are presumably reflected in their restricted ability to grow on single substrates, offers little hope for the use of these strains in physiological and genetic studies which require defined media.

The shape of the curve of growth rate versus temperature for NH resembled that of *T. thermophilus* HB8 (Oshima & Imahori, 1974) rather than that of *T. aquaticus* YT₁ (Brock & Freeze, 1969). It may be that in water tanks the fluctuation in temperature as water is used and reheated may tend to select thermophilic strains capable of growth in a broad temperature band. The temperature of tap water from the systems in question lies in the range 55 to 68 °C, hence there may be little selective pressure on the upper temperature limit for growth in these strains.

The unusual pattern of antibiotic resistance amongst the Gram-negative extreme thermophiles has been noted for *T. aquaticus* YT₁ (Brock & Freeze, 1969), *Thermus* sp. X₁, *Thermomicrobium roseum* (Jackson *et al.* 1973) and *T. thermophilus* (Oshima & Imahori, 1974). The present strains broadly conform to this pattern. All the Gram-negative extreme thermophiles are very sensitive to the penicillins, as well as to neomycin, streptomycin, chloramphenicol and erythromycin.

Spectrophotometry of a methanol extract of cells of NH and DI did not reveal any indications of the presence of the carotenoid pigments which are such an obvious feature of several other Gram-negative extreme thermophiles (Brock & Freeze, 1969; Heinen *et al.* 1971; Oshima, 1972; Jackson *et al.* 1973). The isolates of Brock & Boylen (1973) either were not pigmented or were very pale yellow, and these authors suggested that carotenoid pigments might confer an advantage on bacteria growing in sites exposed to sunlight. There would be no selective pressure to retain such pigments in bacteria growing for prolonged periods in dark situations. The suggestion that loss of pigment might confer a selective advantage over hypothetical pigmented ancestral strains, in that the growth rate might be thus improved, cannot be tested. However, Brock & Boylen (1973) have not reported growth rates for their strains, and our isolates grew slightly less rapidly than *T. aquaticus* YT₁ and much less rapidly than another pigmented strain, *T. thermophilus* HB8. It therefore seems unlikely that loss of pigment is associated with such a selective advantage.

Difference spectra of the soluble fraction showed marked peaks for *c*-type cytochromes together with *c*₁ or *b* cytochromes. No unequivocal evidence of *a* cytochrome is presented, however. McFeters & Ulrich (1972), using difference spectra and low temperature spectra, detected cytochromes *a*, *a*₃, *b* and *c* in *T. aquaticus* YT₁ preparations, and it seems likely that the electron transport chains in the Gram-negative thermophiles may prove to be similar.

The mean base composition of DNA from *T. aquaticus* YT₁ was estimated to be 67.4 % G+C by caesium chloride density gradient centrifugation. However, our own estimate by thermal denaturation in standard saline citrate was 65.4 % G+C. Such differences are commonly found between results obtained in different laboratories by different methods. However, when results obtained in our own laboratory are compared, the two new strains have 3.2 and 4.0 % less G+C than does YT₁. Thus the present strains have the lowest %G+C of all the *Thermus*-like isolates so far tested and it would be of great interest to compare mean composition of their DNA with that of other non-pigmented strains.

The present isolates, while interesting strains of bacteria, show no advantages over *T. aquaticus* YT₁ as sources of thermostable malate dehydrogenase or isocitrate dehydrogenase. The growth rates, optimum temperatures for growth, and growth yields on tryptone-yeast extract are similar, and the enzymes in extracts of YT₁ are somewhat more resistant to thermal inactivation than those of the new strains. The inability of NH and DI to grow on substrates likely to increase the specific activity of these enzymes in the bacteria also means that improvement of enzyme yield is unlikely to be attained by the use of these strains. The sole advantage which they seem to possess is freedom from pigment; the carotenoids of *T. aquaticus*, together with a slime which it produces, do cause some problems in protein purification.

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