Thermomicrobium, a New Genus of Extremely Thermophilic Bacteria

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A new, extremely thermophilic bacterium from an alkaline hot spring in Yellowstone National Park is described. The organism is gram negative and occurs as pleomorphic rods, often appearing in pairs. It is an obligate aerobe and grows optimally at 70 to 75 C at a pH of 8.2 to 8.5 with a generation time of 5.5 h. When grown in culture, the organism has a very pronounced pink color due to cellular carotenoid pigments. The deoxyribonucleic acid base ratio as determined by cesium chloride density gradient ultracentrifugation was found to be 64.3 mol% guanine plus cytosine. Nutritional studies with the new organism demonstrated a more restricted growth in comparison to organisms belonging to the genus *Thermus*. In studies involving defined carbon sources, it was shown that the new organism grew well only on sucrose or glycerol when glutamate served as an additional nutritional source. The organism is regarded as belonging to a new genus, for which the name *Thermomicrobium* is proposed. This genus is placed in the family *Achromobacteraceae*. The type species of the new genus is *Thermomicrobium roseum* sp. nov., of which ATCC 27502 is the type strain.

Extremely thermophilic bacteria represent an adaptation not only to high temperature but also frequently to several other extreme environmental factors. Hot springs, which constitute a natural thermal environment, are typically low in organic matter (5) and high in dissolved solids (9), and many hot springs are exposed to extremely high light intensity (9). These latter factors impose further constraints on microbial life in hot springs. In addition, the pH of a hot spring affects the thermophilic microbial flora (5). Characterization of various thermophiles may provide insight into the common mechanism of microbial adaptation to extreme environments. Recent studies of alkaline thermal environments have led to the isolation and characterization of distinctly different isolates in the genus *Thermus* (7, 14) and of a new thermophilic photosynthetic gliding bacterium (13). In the present paper, we report the isolation and some of the characteristics of a thermophilic bacterium from an alkaline hot spring in Yellowstone National

Park and compare it to the extreme thermophiles *Thermus aquaticus* strain YT-1 and *Thermus* sp. strain X-1, which are the predominant isolates at 70 C from the various alkaline or neutral thermal springs studied to date.

MATERIALS AND METHODS

Bacterial strains. The new thermophile described herein was isolated from Toadstool Spring in Yellowstone National Park. The isolation and source of this strain are described below in detail. This strain has been deposited in the American Type Culture Collection (ATCC), Rockville, Md., under the number 27502. *Thermus aquaticus* strain YT-1 (= ATCC 25104) was received from T.D. Brock, who isolated it from a hot spring in Yellowstone National Park. *Thermus* sp. strain X-1 was isolated from a stream directly behind Jordon Hall on the Indiana University campus in Bloomington, Ind.; this stream receives the heated effluent from distillation towers in Jordon Hall, and the temperature at the point where the sample was taken was 26 C (14).

Media. Initial isolation of the new thermophile was made by enrichment with 0.1% yeast extract plus 0.1% tryptone in a basal salts medium with ammonia as the source of inorganic nitrogen (1). This salts medium (referred to as Allen's salts) had the following composition (in milligrams per liter of deionized distilled water): (NH₄)₂SO₄, 1,300; KH₂PO₄, 280;

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MgSO₄·7H₂O, 247; CaCl₂·2H₂O, 74; FeCl₃·6 H₂O, 19; MnCl₂·4H₂O, 1.8; Na₂B₄O₇·10H₂O, 4.4; ZnSO₄·7H₂O, 0.22; CuCl₂·H₂O, 0.05; Na₂MoO₄·2H₂O, 0.03; and VCl₂, 0.03; the pH was adjusted to 2.0 with H₂SO₄ during storage. In growth studies, various concentrations of yeast extract plus tryptone in a basal salts medium with nitrate as the source of inorganic nitrogen (referred to as Castenholz's salts) (14) were used.

Growth conditions. Cultures were incubated in either Temp-Blok heaters or in a Napco incubator at 70 C. For growth studies under maximum aeration, cultures were grown in a Fermentation Design shaking water bath at 70 C. Growth was measured turbidimetrically with a Klett-Summerson photometer (no. 66 filter) with 13-mm tubes with Morton closures or 250-ml Bellco side-arm flasks. For studies of growth on solid media, agar plates were wrapped in Reynolon plastic wrap (Reynolds food service film) to prevent drying and were incubated at 70 C in a Napco incubator.

Antibiotic and inhibitor sensitivity. Table 2 shows the antibiotic sensitivities of *Thermus aquaticus* YT-1, *Thermus* X-1, and the new thermophile. Six-millimeter paper disks containing the indicated amounts of antibiotic (sensitivity disks for antibiotics, Difco Laboratories) were placed on plates containing 0.5% yeast extract plus 0.5% tryptone plus Castenholz's salts with 3% agar at pH 8.5, streaked with the indicated organism, and incubated at 70 C. The plates were incubated 3 days for the new thermophile and 2 days for the two *Thermus* strains. The antibiotics used in Table 3 were dissolved in distilled-deionized water, added to the above-mentioned liquid medium, without agar, to give the appropriate concentration, and were incubated 5 days at 70 C.

Electron microscopy. The cells were grown to mid-log phase in 0.5% yeast extract plus 0.5% tryptone plus Castenholz's salts at pH 8.5 and 70 C. The cells were then chilled, centrifuged, and prepared by method one of Brock and Edwards (6).

DNA base ratio. Deoxyribonucleic acid (DNA) was isolated by the method of Marmur (12). The buoyant density of the DNA was determined by cesium chloride density gradient centrifugation in a Beckman model E ultracentrifuge, and the DNA base ratio was calculated by the method of Schildkraut et al. (15). DNA from *Sarcina lutea* and bacteriophage T-2 were used as standards.

Chemicals. D-Fructose, D-glucose, sodium azide, glycerol, and sodium lauryl sulfate were obtained from the Fisher Scientific Co. L-Glutamic acid (monosodium salt), succinic acid (free acid), and actinomycin D were obtained from Sigma Chemical Co. Mannitol, sucrose, and sodium chloride were obtained from the Mallinkrodt Chemical Works. Sodium acetate was obtained from Matheson, Coleman, and Bell, and sodium citrate was from the Baker Chemical Co. Peptone, brain heart infusion, nutrient broth, tryptone, and yeast extract were obtained from Difco; casein hydrolysate was from Nutritional Biochemicals Corp.; and Trypticase soy broth was from BBL. Rifampin was obtained from Schwarz/Mann, lot W3045.

RESULTS AND DISCUSSION

Enrichment and isolation. Samples of bacterial mats and water from various springs in Yellowstone National Park were used as inocula for a medium composed of Allen's salts plus 0.1% yeast extract and 0.1% tryptone at pH 7.8. The enrichments, made at 70, 75, 80, and 85 C, were streaked into 0.1% yeast extract plus 0.1% tryptone plus Castenholz's salts plus 3% agar. Only the sample from Toadstool Spring, enriched at 70 C and incubated 1 week on plates at 70 C, gave compact pink colonies; the other isolates generally were of the genus *Thermus*. Pure cultures were obtained by restreaking and incubating for 5 days. Transfer of the pink isolate was facilitated if the pH was increased to 8.5 and the concentration of both yeast extract and tryptone increased to 0.5%.

Inoculum source. The pink isolate was obtained from a sample taken near the source of spring, unofficially called Toadstool Spring (pH 8-9), at 74 C. The temperature at the source of the spring is 91 C. Toadstool Spring is located 2 m north of Mushroom Spring in the Lower Geyser Basin of Yellowstone National Park (4). The sample was a small orangish-red bacterial mat attached to the silica substrate under a piece of waxed paper. The silica substrate was otherwise white, which indicates a general lack of abundant bacterial growth. Experiments in various alkaline springs have shown that there is bacterial growth at the upper ends of these thermal gradients even though the bacteria have not been cultured. Growth is indicated by attachment of the bacteria to glass slides and by an increase in their numbers (3). We believe this isolate may represent one of the previously uncultured organisms growing near the source of these thermal waters. The waxed paper may have provided a concentration of the organism by shielding from the high light intensity and rapid water flow which otherwise would restrict growth of the bacterial mat.

Morphology. Cells of the pink isolate are usually pleomorphic with the forms present as short, irregular-shaped rods, occurring singly or in pairs, and dumbbell-shaped rods. Cultures grown in unshaken tubes form a pink pellicle. No spores have ever been observed by phase-contrast microscopy of cells of the pink thermophile. Longitudinal sections of the organism show the layered cell wall of a gram-negative bacterium (8) (Fig. 1a and 1b), with the outermost layer appearing to be a regular repeating unit arranged in an array over the cell surface. Figures 1c to 1e show a variety of

FIG. 1. Transverse, longitudinal, and oblique views of cells of *Thermomicrobium roseum*. (a) Longitudinal view, $\times 20,500$; (b) longitudinal and transverse views, $\times 14,800$; (c) random views, $\times 8,500$; (d) random views, $\times 11,400$; (e) view of pleomorphic cell, $\times 15,400$; (f) transverse view showing mesosome-like invaginations $\times 32,500$.

forms seen in this pleomorphic rod. Figures 1b and 1c show the organism during division, whereas Fig. 1a is after division. Some of the cells display mesosome-like invaginations that are probably responsible for the internal structure in Fig. 1f. One of the most interesting

features made visual by electron microscopy was the appearance of electron-dense hexagonal structures (Fig. 2), which appear to represent bacteriophage particles.

Physiological and nutritional characteristics. The nutritional studies employed both basal

FIG. 2. Longitudinal view showing electron-dense hexagonal structures within the cell which appear to be bacteriophage. $\times 44,000$.

salts media. Good growth of the pink isolate was obtained with 0.1 up to 0.7% yeast extract with an equal concentration of tryptone. Very slight growth was obtained with 1.0% yeast extract plus tryptone, with no growth observed at higher concentrations. Cell yield increased as the percentage of yeast extract plus tryptone was increased until the cell yield maximized at 0.5%. Maximum growth rate was obtained with 0.3% yeast extract plus tryptone. Table 1 shows the carbon sources supporting growth of the new thermophile.

In media containing defined carbon sources, no growth was obtained with the new organism unless glutamate was present, and then only in Castenholz's basal salts containing nitrate rather than ammonia as the source of inorganic nitrogen. Using complex media, the organism grew better in Allen's salts. Some of the negative results in Allen's salts are due to the lowered pH, caused by the loss of ammonia from the medium at 70 C, which inhibited further growth of the organism.

An apparent lack of growth is also obtained in cultures containing a carbon source, such as glucose or fructose, that is rapidly metabolized to organic acids causing a decrease in pH. We

also have reason to believe that some of the negative results with YT-1 compared with X-1 in Table 1 are due to the higher pH employed and that YT-1 is more sensitive to higher pH than X-1.

However, appreciable growth of the new thermophile in a defined medium (glutamate, glutamate plus glycerol, or glutamate plus succinate) does seem to indicate the possibility of this organism's use for general physiological and genetic studies, although no present claim is being made that this organism might not have some vitamin requirement.

The optimum temperature for growth is 70 to 75 C, and the maximum temperature is 85 C. The generation time at the optimum growth temperature is 5 to 5.5 h. The pH optimum for growth is between pH 7.5 and 8.7, with the maximum growth occurring between 8.2 and 8.5.

The pink pigment of the new organism appears to be a carotenoid. The acetone extract of *T. roseum* (Fig. 3) has absorbance maxima similar to those of the red carotenoid pigments, torulene (11) and 3,4-dehydrolycopene (10). These maxima also resemble maxima observed in spectra of the extracts of pink thermophilic

TABLE 1. *Nutrition of various extreme thermophiles (pH 8.5, 70 C)*

Carbon source	Growth conditions ^a	<i>Thermus aquaticus</i> YT-1		<i>Thermus</i> sp. X-1		<i>Thermomicrobium roseum</i>	
		Type of inorganic nitrogen supplied					
		NO ₃ ^{-b}	NH ₄ ^{+c}	NO ₃ ⁻	NH ₄ ⁺	NO ₃ ⁻	NH ₄ ⁺
D-Fructose	A	—	—	—	—	—	—
	B	—	—	—	—	—	—
D-Glucose	A	—	—	—	—	—	—
	B	—	—	—	—	—	—
Glycerol	A	—	—	±	±	—	—
	B	+	+	+	+	+	—
Succinic acid (free acid)	A	—	—	—	—	—	—
	B	—	+	—	—	—	—
Sodium succinate	A	—	—	—	—	—	—
	B	—	+	+	—	—	—
Mannitol	A	—	—	+	—	—	—
	B	+	+	+	+	—	—
Sucrose	A	—	—	—	—	—	—
	B	—	+	+	±	+	—
Sodium acetate	A	±	—	+	—	—	—
	B	—	±	+	—	—	—
Sodium citrate	A	—	+	—	—	—	—
	B	+	+	—	—	—	—
Sodium glutamate	A	+	+	+	±	±	—
Peptone	A	+	+	+	+	—	+
Casein hydrolysate	A	—	±	+	+	±	+
Brain heart infusion	A	++	+	+	+	—	+
Nutrient broth	A	+	+	—	+	+	+
Trypticase soy broth	A	+	+	—	+	—	+
Tryptone	A	—	+	+	+	—	+
Yeast extract	A	+	+	+	+	+	+

^a A = Carbon source only (0.2%); B = carbon source (0.2%) plus 0.2% glutamate, with the results indicated as: —, no growth; ±, sparse growth; +, good growth; ++, very abundant growth.

^b Castenholz's salts medium.

^c Allen's salts medium.

filamentous bacterial masses from Yellowstone National Park (2). Further characterization of the pigments is in progress.

Antibiotic and inhibitor sensitivity. The new thermophile was compared with the two strains of *Thermus* for sensitivity to various antibiotics and inhibitors (Tables 2 and 3). There are some similarities in antibiotic sensitivity among the two *Thermus* strains and the new organism; however, the latter is much more sensitive to kanamycin and neomycin and has a lower sensitivity to erythromycin and chloromycetin than the *Thermus* strains. The new organism is also much more sensitive to the inhibitor sodium azide than either of the two *Thermus* strains. Making the assumption that the antibiotics and inhibitors act in the same manner in the extreme thermophiles, Brock (5) concluded that *T. aquaticus* YT-1 has a peptidoglycan cell wall, a protein-synthesizing machinery using 70s ribosomes, an electron transport chain, and a cell membrane. These same conclusions can be made for the new thermophile.

This new organism is quite different from *T.*

FIG. 3. Absorption spectrum of the acetone extract from *Thermomicrobium roseum*.

TABLE 2. Sensitivity of various extreme thermophiles to antibiotics^a

Antibiotic	Antibiotic concn (μg/disk)	<i>Thermus aquaticus</i> YT-1	<i>Thermus</i> sp. X-1	<i>Thermomicrobium roseum</i>
Tetracycline	5	8 ^b	14	12
	30	16	20	20
Streptomycin	2	10	14	8
	10	18	26	24
Erythromycin	2	12	28	0
	15	42	46	28
Novobiocin	5	32	22	8
	30	44	36	40
Chloromycetin	5	12	16	0
	30	34	32	20
Kanamycin	5	22	10	40
	30	28	20	70
Neomycin	5	22	30	TL
	30	26	38	TL
Penicillin ^c	2	56	46	54
	10	TL	TL	TL

^a Filter-paper disks (6 mm in diameter) (Difco sensitivity disks) were placed on inoculated plates of 0.5% yeast extract plus 0.5% tryptone with Castenholz's salts and 3% agar. Incubation was 3 days for *T. roseum*; 2 days for the two *Thermus* strains, pH 8.5.

^b Results are diameter of zone, in millimeters. TL = too large to read.

^c Penicillin values are given in units per disk.

aquaticus Brock and Freeze (Fig. 4), found in the same thermal gradient. Under laboratory conditions the generation time for the new organism is 5 to 6 h compared to a generation time of 1 h for *T. aquaticus*. The cells of the new thermophile have a rose-pink color due to the carotenoid pigments instead of the bright-yellow carotenoid of *T. aquaticus* YT or the low concentration of carotenoids found in the cream-colored *Thermus* X-1. The different levels of carotenoid present in each of these organisms is of interest in view of the high light intensities present at Yellowstone National Park, which has been indicated as one of the environmental constraints on thermophilic organisms by Castenholz (9), and this supports the view that the presence of carotenoids has some ecological significance in protection of these organisms against light.

One of the most striking differences between the new organism and *T. aquaticus* is the morphology of the two organisms (compare Fig. 1 and 4): the former organism does not have the regular long, thin rod form of *T. aquaticus* (6 and unpublished observations) and appears quite small and pleomorphic. As observed by phase-contrast microscopy, the new thermophile resembles other pleomorphic organisms, such as those belonging to *Arthrobacter*, *Corynebacterium*, or *Bacteroides*. We have on occasion observed what seem to be motile cells

of the new organism. However, motile cells do not appear with sufficient regularity, and flagellated cells of this organism have not been observed with the electron microscope to date; hence, motility is not included in the description of this organism.

Thus, we regard the new organism as belonging to a new genus for which we propose the name *Thermomicrobium*. (Gr. noun *thermus* heat; Gr. adj. *micrus* small; Gr. noun *bios* life; M.L. neut. noun *Thermomicrobium* heat [-loving] microbe.) This genus is placed in the family *Achromobacteraceae*. The type species of this genus is *T. roseum* (ro'se.us. L. adj. *roseus* rose-colored), and the type strain of *T. roseum* is ATCC 27502, the only isolate of this organism to date. Because there is only a single isolate, the following description of the genus serves also as the description of the type species and of the type strain.

Thermomicrobium gen. nov.

Morphology: short, irregular-shaped rods, 1.3 to 1.8 μm in diameter and 3 to 6 μm in length. Pleomorphic forms are dumbbell-shaped or quite irregular in diameter, occurring singly or in pairs. Endospores are absent.

DNA base ratio: 64.3%.

Colony characteristics: pink, compact colonies.

FIG. 4. Longitudinal view of *Thermus aquaticus* YT-1. $\times 30,000$.

TABLE 3. Sensitivity of various extreme thermophiles to antibiotics and inhibitors

Antibiotic or inhibitor	Concn ($\mu\text{g/ml}$ of media ^a)	<i>Thermus aquaticus</i> YT-1	<i>Thermus</i> sp. X-1	<i>Thermomicrobium roseum</i>
Sodium lauryl sulfate	100	— ^b	—	—
	10	+	+	+
	1	+	+	+
Sodium chloride	2%	—	—	—
	1%	—	—	—
	0.5%	—	+	±
Sodium azide	500	+	±	—
	50	+	+	—
	5	+	+	+
Actinomycin D	10	—	—	—
	1	—	—	—
	0.1	+	+	±
	0.01	+	+	+
	0.001	+	+	+
Rifampin	100	—	—	—
	10	—	—	—
	1	+	+	+
	0.1	+	+	+
	0.01	+	+	+

^a Media used were 0.5% yeast extract plus 0.5% tryptone and Castenholz's salts medium, pH 8.5.

^b Results are indicated as in Table 1.

Liquid cultures: pink surface pellicle usually forms in unshaken liquid cultures.

Temperature characteristics: optimum growth occurs at 70 to 75 C, and the maximum temperature for growth is 85 C.

pH optimum: 8.2 to 8.5, with no growth above 9.4 or below 6.0.

Source: Hot Spring, Yellowstone National Park (74 C).

We have no evidence of any cell cycle such as that found in *Hyphomicrobium* or *Rhodomicrobium* or of nutritional control of morphology as is found with arthrobacters. Since *T. roseum* is pleomorphic, we have included several electron microscope figures to give some idea of the variety of cell morphology observed. Figure 2 shows what appears to be hexagonal particles that resemble phage particles within the *T. roseum* cell. We have no evidence of any phage-caused lysis of *T. roseum* and have not yet attempted to see if a phage can be induced from this culture.

The DNA base ratio of *T. roseum* is 64.3 mol % guanine plus cytosine (GC), and this is quite close to the DNA base ratios for the *Thermus* X-1 isolates (64-65% GC) and the *Thermus aquaticus* YT strains (65.4-67% GC) (7, 14). Cesium chloride density gradient ultracentrifugation of 5 and 50 μg of *T. roseum* DNA

did not show any satellite DNA or DNA with a base ratio other than the bulk DNA.

The determination of growth by *T. roseum* in various media compared with the growth of *Thermus aquaticus* or *Thermus* X-1 presented some problems that have already been discussed. With the more rapid generation time and ability to convert a wider range of carbon sources to cellular mass, it is easy to see why *Thermus* species have been relatively easy to obtain and why only now we are able to report the isolation of the organism described herein.

The presence and growth of bacteria has been demonstrated in situ at the upper ends of natural thermal gradients up to and above 90 C (3). However, it has been difficult to isolate and culture these organisms. Thus, the present isolation and partial characterization of a previously unreported extremely thermophilic bacterium may aid in both ecological studies of alkaline thermal environments and biochemical studies of the thermobiology of microorganisms in extreme environments.

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