Thermomicrobium fosteri sp. nov., a Hydrocarbon-Utilizing Obligate Thermophile¹

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A hydrocarbon-utilizing, obligately thermophilic bacterium was isolated from a littoral area of North Carolina, and it is herein described and named as a new species. The microorganism, designated strain PTA-1, is a gram-negative, non-sporeforming, nonmotile, pleomorphic rod that can utilize various long-chain *n*-alkanes, 1-alkenes, primary alcohols, or ketones as substrates for growth. It will not grow on nutrient broth or Trypticase soy broth. The organism is an obligate aerobe which grows most rapidly at 60 C with a generation time of 4.0 to 4.5 h at an optimum pH of 7.2 to 7.5 in minimal salts medium with 0.1% *n*-heptadecane as substrate. The temperature range for growth is 42 to 70 C. A deoxyribonucleic acid base composition of 68.8 mol% guanine plus cytosine for this organism was determined by thermal denaturation of isolated deoxyribonucleic acid. The cells contain a pink carotenoid pigment(s) that is most evident after growth at minimal temperatures with acetate as the substrate. It is proposed that this organism be placed in the genus *Thermomicrobium* as a new species, to which we give the name *Thermomicrobium fosteri*. The type strain of *T. fosteri*, PTA-1, has been deposited in the American Type Culture Collection under the number 29033.

Thermophilic bacteria have been studied since the first isolation of this kind of microorganism, from the Seine River by Miquel (15) in 1879. In the early studies, the great majority of bacteria isolated at high temperatures were gram-positive, aerobic sporeformers of the genus *Bacillus* (for a review, see reference 1). Recently (1969), Brock and Freeze (3) reported on the isolation of an extremely thermophilic, gram-negative, aerobic, nonsporeforming, rod-shaped bacterium, which they named *Thermus aquaticus*. Other species of *Thermus* have since been isolated and characterized (16, 20). In 1973, Jackson et al. (6) isolated an extremely thermophilic, gram-negative bacterium from an alkaline hot spring in Yellowstone National Park and considered it sufficiently different from the members of the currently recognized genera to propose a new genus, *Thermomicrobium*, for this microorganism. The type species of *Thermomicrobium* is *T. roseum*, of which the type strain is American Type Culture Collection (ATCC) strain 27502.

Kvasnikov et al. (10) described the physiological properties of a gram-positive sporeformer, *Bacillus circulans* subsp. *thermophilus*, which utilized *n*-alkanes as sole sources of carbon and energy. Other strains belonging to the genus *Bacillus* and capable of growth on *n*-alkanes

and paraffin at optimum temperatures of 55 to 60 C have been described (14, 17, 19). However, studies on thermophilic, gram-negative, hydrocarbon-utilizing bacteria are limited in number and involve mostly thermotolerant and thermoresistant strains of *Pseudomonas* (18). The isolation and nutritional characterization of extremely thermophilic, gram-negative bacteria (3, 6, 16, 20) have been described with substrates such as tryptone, yeast extract, sugars, and organic acids, but none of these isolates utilized hydrocarbons or related substrates. This investigation concerns the isolation and classification of an obligately thermophilic, gram-negative, hydrocarbon-utilizing bacterium.

MATERIALS AND METHODS

Bacterial strain. The bacterium utilized in this study was isolated from a mud sample obtained from a littoral area near Beaufort, N.C., and was designated strain PTA-1. This strain can be maintained in a freeze-dried state at -10 C.

Isolation and media. Initial isolation of the thermophilic bacterium was accomplished by enrichment culture at 50 C. A diluted mud sample was placed in the basal salts medium (L-salts) of Leadbetter and Foster (11), with 0.1% (vol/vol) *n*-hexadecane added as growth substrate. Since the organism does not grow readily on solid substrates, purification of the isolate was accomplished by serial dilution to extinction in L-salts containing 0.1% (vol/vol) *n*-hexadecane. Substrate specificity tests were per-

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formed in 125-ml Erlenmeyer flasks containing 30 ml of L-salts supplemented with 0.2% (vol/vol) liquid substrate or 0.2% (wt/vol) solid substrate. The utilization of gaseous hydrocarbons was determined as described previously (21). Strain PTA-1 was cultivated after isolation and purification by adding substrate to melted agar in a blender and mixing to give a stable emulsion. A 50-ml amount of the blended agar was added to a glass petri dish (20 by 100 mm). The plates were inoculated and incubated in a desiccator, the bottom of which was covered with water. Visible colonies appeared after 14 days.

Substrates. The hydrocarbon substrates employed in this study had a minimum purity of 99%. The liquid hydrocarbon substrates were sterilized by membrane filtration.

Growth conditions. Cells were grown in 2-liter Erlenmeyer flasks containing 500 ml of L-salts medium (pH 7.2) supplemented with 0.2% (wt/vol) sodium acetate or 0.1% (vol/vol) hydrocarbon as substrate. The cultures were incubated at selected temperatures on a gyratory shaker at 180 rpm. The organism was preadapted to the appropriate substrate at the test temperature before use as inoculum. Growth was measured turbidometrically at 600 nm or by dry weight of cells.

Electron microscopy. The procedures used for electron microscopy, including fixation, dehydration, and embedding of microorganisms, were described previously (8, 9).

Dipicolinic acid. Dipicolinic acid assays were by the method of Janssen et al. (7), with *Bacillus circulans* as a control.

DNA base composition. Deoxyribonucleic acid (DNA) was isolated and purified by the method of Marmur (12). The moles percent guanine plus cytosine (G+C) was determined by thermal denaturation (13) using a Beckman DUR spectrophotometer attached to a Gilford 2000 multiple-sample absorbance recorder.

Pigment(s). The pigments were extracted from PTA-1 cells by the method of Eimhjellen and Jensen (5). The absorption spectrum of the pigment(s) was determined with a Beckman Acta V recording spectrophotometer, using a cuvette of 10-mm path length.

RESULTS

Cellular morphology. The organism was a rod with singly occurring cells that had an average length of 1.5 μ m. The cells were gram negative and nonmotile. No endospores were observed in the cells by phase-contrast microscopy, and all assays for dipicolinic acid after growth under various cultural conditions were negative. An electron micrograph (Fig. 1) of ultrathin sections of strain PTA-1 demonstrated that the cells were short rods. A multi-layered cell wall characteristic of the gram-negative thermophile *Thermomicrobium roseum* (6) was observed. The cells contained an extensive network of intracellular membranes resembling mesosomes, and intracytoplasmic

hydrocarbon inclusions, similar to those described in an acinetobacter (9), are evident in cells after growth on *n*-heptadecane.

Growth characteristics. No growth was obtained with nutrient broth (Difco) or tryptic soy broth (Difco) as the substrate. The ability of strain PTA-1 to utilize various substrates was determined (Table 1). The organism can utilize long-chain *n*-alkanes, 1-alkenes, primary alcohols, and ketones, but not the shorter-chain compounds, as substrates for growth. Neither cycloparaffins nor aromatic hydrocarbons served as growth substrates. Strain PTA-1 grew with acetate (Na) as the substrate but not with citrate (Na) or succinate (Na), and it utilized both ammonia and nitrate as sources of nitrogen.

The pH range for optimum growth of strain PTA-1 was 7.2 to 7.5. No growth was observed at temperatures above 70 C or below 42 C. The effect of growth temperature on the generation time of strain PTA-1 is shown in Fig. 2. At temperatures of 50, 55, 60, and 65 C, the generation times were approximately 15.0, 9.0, 4.5, and 9.0 h, respectively.

Pigment(s). A pink, water-insoluble pigment(s) was produced by the organism when grown with sodium acetate as the substrate. Although not quantitatively determined, it appeared that strain PTA-1 produced more pigment at 50 C than at 60 or 65 C. The absorbance spectrum of the acetone-extractable pigment(s) is shown in Fig. 3. The maximum absorption occurred at 477 nm, with secondary peaks appearing at 385, 448, 507, and 550 nm.

DNA base composition. The G+C content of the DNA from strain PTA-1 was 68.8 mol%, and the ratio of the optical density at 260 and 280 nm of the purified DNA was 1.82.

DISCUSSION

The salient characteristics of various gram-negative, nonsporulating, obligate thermophiles are presented in Table 2. The G+C values for all of these organisms fall within a range of 64.3 to 69.0 mol%. The maximum and optimum growth temperatures for strain PTA-1 are somewhat lower than those for *T. aquaticus*, *T. thermophilus*, and *T. roseum*, although the minimum temperature at which each will grow is virtually the same for all of these thermophilic organisms. The optimum pH for growth of strain PTA-1 is equivalent to that for the members of the genus *Thermus* and lower than that for *T. roseum*. The higher pH required by *T. roseum* may be a reflection of the niche from which it was isolated (alkaline thermal environment).

Strain PTA-1 differs in many respects from

FIG. 1. Electron micrograph of a thin section of strain PTA-1. The total magnification in the longitudinal section (A) was $\times 85,000$ and for (B) it was $\times 88,000$. Note the hydrocarbon inclusions (C).

TABLE 1. Ability of strain PTA-1 to utilize various hydrocarbons and related compounds as growth substrates^a

Substrates ^b	Growth responses ^c
<i>n</i> -Alkanes	
C ₁₋₉	—
C ₁₀₋₂₀	+
Methylalkanes	
C ₁₉	+
1-Alkenes	
C ₇₋₁₃	—
C ₁₄₋₁₈	+
Alcohols	
C ₄₋₁₁	—
C ₁₂₋₁₇	+
Ketones	
C ₄₋₁₃	—
C ₁₄₋₁₇	+
Cycloparaffins and aromatics	
Alkynes	—
Acetate	+
Citrate	—
Succinate	—
D-Glucose	—

^a Test flasks contained L-salts with either 50:50 (vol/vol) gaseous substrate-air mixture or 0.2% (vol/vol or wt/vol) substrate added. Incubation was on a rotary shaker at 60 C for 2 weeks.

^b Substrates tested: *n*-alkanes—from C₁ to C₂₀; methylalkanes—2-methyloctadecane, 3-methyloctadecane, 4-methyloctadecane; 1-alkenes—from C₇ to C₁₈; 1-alcohols—from C₄ to C₁₇; ketones—2-butanone, 2-pentanone, 2-heptanone, 2-undecanone, 2-dodecanone, 2-tridecanone, 3-tetradecanone, 2-pentadecanone, 2-hexadecanone, 9-heptadecanone; cycloparaffins—from C₅ to C₈; aromatics—benzene, *o*-xylene, *m*-xylene, *p*-xylene; alkynes—from C₁₅ to C₁₈.

^c +, Growth in excess of 0.1 mg of dry weight per ml; —, no growth.

members of the genus *Thermus*, but it closely resembles *Thermomicrobium roseum*. The 4.0- to 4.5-h generation time for strain PTA-1 is near that for *T. roseum* and is distinctly different from those (20 to 50 min) of the known *Thermus* species. The cellular morphology of

FIG. 2. Effect of growth temperature on the generation time of strain PTA-1. Cells were grown in L-salts basal medium (pH 7.2) with 0.1% (vol/vol) *n*-heptadecane as the substrate. Incubation was accomplished on a rotary shaker at 180 rpm.

TABLE 2. Salient characteristics of gram-negative, nonsporulating, obligate thermophiles

Thermophile	G+C (mol%)	Morphology	Generation time	Carotenoids	Growth temp (C)			pH optimum	Hydrocarbon utilization
					Maximum	Optimum	Minimum		
<i>T. aquaticus</i> , 4 strains (3)	65.4-67.4 ^a	Rods, 5-10 μ m (filaments, 20-200 μ m)	50 min	Bright orange ^b	79	70	40	7.5-7.8	— ^c
<i>T. thermophilus</i> , strain 4B8 (15)	69.0 nd	Rods, 3 μ m	20 min	Yellow-orange	85	65-72	47	7.0	ND ^e
<i>T. roseum</i> ATCC 27502 (7)	64.3 ^a	Pleomorphic rods, 3-6 μ m	5.5 h	Pink	85	70-75	ND	8.2-8.5	—
<i>T. fosteri</i> PTA-1	68.8 ^f	Pleomorphic rods, 1-1.5 μ m	4.0-4.5 h	Pink	70	60	42	7.2-7.5	+ ^f

^a Determined by cesium chloride density gradient ultracentrifugation.

^b Where occurring.

^c —, No growth.

^d Determined from DNA thermal denaturation temperature.

^e ND, Not determined.

^f +, Growth.

FIG. 3. Absorbance spectrum of acetone-extractable pigment(s) produced by strain PTA-1. Cultures were grown at 50 C in L-salts basal medium with 0.2% (wt/vol) sodium acetate as the substrate.

strain PTA-1, with somewhat pleomorphic, singly occurring rods and oval-shaped cells, resembles that of *T. roseum*. The filamentous growth and "round bodies" observed in *Thermus aquaticus* (2) have not been seen in strain PTA-1. Strain PTA-1 produces a pink carotenoid pigment(s) similar to that described for *T. roseum* (6).

According to the results reported here, strain PTA-1 has properties that are much the same as those of *Thermomicrobium roseum* utilized by Jackson et al. (6) to establish the genus *Thermomicrobium* and to differentiate it from the genus *Thermus*. These properties are: (i) differences in cellular morphology, (ii) differences in type and color of the carotenoid pigment produced, and (iii) marked differences in generation time. Therefore, it is proposed that strain PTA-1 be placed in the genus *Thermomicrobium*. In *Bergey's Manual of Determinative Bacteriology* (4), this genus is placed in the section entitled "Genera of Uncertain Affiliation" under part 7, "Gram-Negative Aerobic Rods and Cocci." Moreover, strain PTA-1 is sufficiently different from *T. roseum* so as to be considered a member of a separate species (Table 2). The name proposed here for this species

is *Thermomicrobium fosteri* sp. nov. (fos'ter.i. M. L. gen. noun *fosteri* of Foster; named for the late Jackson W. Foster). Because the description of this species is based on a single isolate, the species description given herein also serves as the description of the type strain.

Thermomicrobium fosteri sp. nov.

Morphology: Somewhat pleomorphic, short, singly occurring rods and small, oval-shaped rods with an average diameter of 0.5 μ m and an average length of 1.5 μ m. Gram negative, nonsporulating, and nonmotile.

Cultural characteristics: Slow growth on solid media. Disperse growth usually occurs in liquid culture under static conditions. Cells contain a pink carotenoid pigment(s), especially when grown on acetate at lower temperatures.

Colony characteristics: Small, round, raised, smooth colonies.

Nutrition: No growth factors required. Growth on sodium acetate, long-chain *n*-alkanes, 1-alkenes, primary alcohols, and ketones in basal salts medium with either ammonia or nitrate as a nitrogen source. No growth occurs in nutrient broth or Trypticase soy broth.

Temperature characteristics: Grows optimally at 60 C; maximum growth temperature is 70 C; minimum growth temperature is 42 C.

Oxygen relationship: Strict aerobe.

pH optimum: 7.2 to 7.5.

DNA base composition: 68.8 mol% G+C.

Type strain: PTA-1; this strain has been deposited in the American Type Culture Collection (ATCC) under the number 29033.

Source: Mud sample from littoral area (near Beaufort) on coast of North Carolina. Temperature of the overlying waters was approximately 24 C.

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REPRINT REQUESTS

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