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Thermoleophilum album gen. nov. and sp. nov., a bacterium obligate for thermophily and *n*-alkane substrates*

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Abstract. Several bacterial strains that are obligate for both thermophily and hydrocarbon utilization have been isolated from a number of thermal and non-thermal environments. Mud and water samples obtained from geographic sites across the United States were subjected to enrichment procedures at 60° C with *n*-heptadecane as sole growth substrate. Organisms forming very small white colonies on agar surfaces were often evident on primary enrichment. These bacteria were Gram negative, aerobic, small, and rod-shaped. They lacked pigmentation, motility, and the ability to form endospores. Growth occurred in the temperature range from 45° C to 70° C with the optimum around 60° C and at a pH near neutrality. Only *n*-alkanes from 13 to 20 carbons in length were utilized by these organisms as growth substrate. The mol% guanine plus cytosine values for these strains were between 68 and 70%. The physiological and morphological characteristics of these organisms are distinctly different from any previously described thermophilic microbes. It is proposed that they be placed in a new genus, *Thermoleophilum* gen. nov. with the type species being *Thermoleophilum album* gen. nov., sp. nov. The type strain is ATCC 35263.

Key words: Obligately thermophilic – Hydrocarbon utilizer – *Thermoleophilum album* – Geothermal

Over the years thermophilic organisms have been described among the archaebacteria, mycoplasma, actinomycetes, cyanobacteria and other eubacteria. A majority of the early studies on bacteria that survived elevated temperatures were concerned with the Gram positive sporeforming bacilli (Allen 1953) and various other thermotolerant or thermoresistant forms. Hydrocarbon-utilization by Gram positive sporeforming bacilli has been reported (Klug and Markovetz 1967; Mateles et al. 1967; Kvasnikov et al. 1971). The report of a Gram negative thermophilic organism from thermal springs, *Thermus aquaticus*, characterized by Brock and Freeze (1969), initiated an interest in non-sporeforming obligately thermophilic bacteria and a considerable number have been described in the past 14 years. In the course of studies on obligately thermophilic microbes that grow on hydrocarbon substrates (Phillips and Perry 1976; Merkel et al. 1978a, b), it was noted that several of the newly isolated strains would not grow on any of the substrates tested except selected *n*-alkanes. Two of these strains, isolated from a hot spring in Yellowstone National Park (strains YS-3 and YS-4), were briefly described in a previous report (Merkel et al. 1978b). Further study of the hydrocarbon-utilizing thermophilic population from various environments indicated that microbes with similar characteristics were rather common. While the sources of samples for enrichment have generally been thermal areas, these organisms of narrow substrate range have also been isolated from non-thermal environments. This report describes the obligately thermophilic hydrocarbon-utilizing organisms that are confined to the utilization of *n*-alkane substrates. A taxonomic classification is also being proposed.

Materials and methods

Source of organisms

The organisms were isolated by enrichment culture techniques using mud samples taken from both thermal and non-thermal environments. Samples were obtained in the U.S. from Arkansas (Hot Springs), New Mexico (Faywood Hot Springs), Wyoming (Yellowstone National Park) and North Carolina (Roanoke Rapids and Beaufort).

Culture conditions

The substrate for enrichment was *n*-heptadecane added at 0.1% (v/v) to a mineral salts medium of the following composition: NH_4Cl, 1.0 g; $NaNO_3$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $FeSO_4 \cdot 7\ H_2O$, 1.0 mg; Na_2HPO_4, 0.21 g; NaH_2PO_4, 0.09 g; KCl, 0.04 g; $CaCl_2$, 0.015 g; Cu (as $CuSO_4 \cdot 5\ H_2O$), 5 µg; B (as H_3BO_3), 10 µg; Mn (as $MnSO_4 \cdot 5\ H_2O$), 10 µg; Zn (as $ZnSO_4 \cdot 7\ H_2O$), 70 µg; Mo (as MoO_3), 10 µg; deionized water 1 l. Incubation was at 60° C in stationary culture until turbidity was evident. After repeated transfer to an equivalent medium the organisms were obtained in axenic culture by streaking on a mineral salts agar medium. The *n*-heptadecane substrate was introduced by inverting the inoculated plate and placing a drop of the hydrocarbon in the cover. Inoculated plates were incubated at 60° C after placing them in plastic bags to prevent desiccation.

Growth studies

The optimum growth temperature was determined by comparing the dry weight of the cell mass obtained at

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various temperatures or by the incorporation of ^{14}C-heptadecane at the different incubation temperatures. To determine the amount of ^{14}C-heptadecane incorporated, cells were collected, gently rinsed with petroleum ether to remove excess hydrocarbon and resuspended in Aquasol-2 (New England Nuclear, Boston, MA, USA). Radioactivity was measured on a Packard Tri-Carb Liquid Scintillation Counter (Model 3330). Optimum pH for growth was estimated by comparing the turbidity of cultures (595 nm) or the dry weight of cells obtained following growth in media at differing pH's. Mono- and dibasic sodium phosphates were used to adjust the pH of the mineral salts medium. The generation time of the organisms was estimated by following growth turbidimetrically at 595 nm.

The capacity of various compounds to serve as sole source of carbon and energy was determined in a liquid medium at 60° C. Hydrocarbon substrates were added at 0.1% (v/v) and other substrates were added at 0.2%. The substrates were sterilized separately and added aseptically to the mineral salts medium. Gaseous hydrocarbons were added as described elsewhere (Vestal and Perry 1969). Growth was determined by the presence of a visible turbidity when compared with an equivalent inoculated control without added substrate.

The ability to utilize various compounds as a source of nitrogen was determined by replacing the nitrogen in the mineral salts medium at equivalent levels. Results are reported on the basis of growth after 1 week incubation at 60° C.

Catalase was determined by adding a 3% solution of hydrogen peroxide to colonies grown on a solid substrate. Antibiotic sensitivities were determined in liquid culture as these organisms grow poorly on agar surfaces. Antibiotic solutions were sterilized separately and added at various concentrations to the routine growth medium. Inhibition of growth was determined by the lack of visible turbidity after incubation for 7 days at 60° C. Results are reported as the minimum concentration (μg/ml) which inhibited growth.

Electron microscopy

Cells were fixed in 3% glutaraldehyde, 0.1 M phosphate buffer, pH 7.3, for 1 h at 4° C. They were then washed in phosphate buffer (3 times, 20 min) and collected on Nuclepore filters. The filters with cells attached were dehydrated through a graded ethanol series and into Freon 113. The filters were then dried using freon in a Bowmar critical point drying apparatus. Samples were sputter coated with gold-palladium in a Hummer V sputter coater (Technics, Inc.) and examined in a JEOL T-200 Scanning Electron Microscope operating at 15 kV.

DNA base composition

DNA was isolated from cells and purified as described (Kloos and Wolfshohl 1979), except that cells were lysed with lysozyme (Merkel et al. 1978a) and the final precipitation was with isopropanol (Marmur 1961). The mol% G+C was calculated according to the Tm method described by Boháček et al. (1967) using 10^{-2} M sodium phosphate (pH 7) – 10^{-3} M EDTA as the solvent. DNA melting curves were obtained employing a Beckman monochromator (Model DU) equipped with a Gilford photometer (Model 252), quartz microcuvette assembly, thermoprogrammer (Model 2527) and analogue multiplexer (Model 6046).

Table 1. Environments from which bacterial strains obligate for *n*-alkane substrates were isolated[a]

Inoculum source	Temperature at source	Strain designation	ATCC[b] number
Hot Springs, AR	61° C	HS-5	35263
Faywood Hot Springs, NM	undetermined	NM	35266
Yellowstone National Park, WY	60° C	YS-3	35264
Yellowstone National Park, WY	63° C	YS-4	35265
Roanoke Rapids, NC	ambient	RR-D	35267
Beaufort, NC	ambient	PTA-1	35268

[a] Enrichment was performed at 60° C with *n*-heptadecane as growth substrate in mineral salts medium.
[b] American Type Culture Collection

Peptidoglycan analysis

Peptidoglycan was isolated, purified and analyzed as previously described (Merkel et al. 1978a).

Results

Selection of hydrocarbon-utilizing organisms based on colony morphology yielded several different axenic cultures. Generally the organisms grew on alkane or complex substrates while several cultures would grow solely on the alkane substrates. Cultures of this type were selected for further study (Table 1).

All of these strains were obligately thermophilic, aerobic, Gram negative rods (Fig. 1) and formed very small pale (translucent to white) colonies on the agar surface. No motility has been observed and apparently no growth factors are required. In stationary liquid culture at 60° C with *n*-heptadecane as substrate, the cell yields range from 0.3 to 0.6 g/l. Aeration did not increase the growth yield.

The optimum temperature for growth was 60° C at a pH near neutrality. Generation times are estimated to be a minimum of 6 h. Growth occurred in a temperature range from 45 to 70° C (Table 2).

All strains were capable of growth on a limited range of *n*-alkanes (Table 2). No growth was observed with any other hydrocarbons tested, including longer and shorter chain *n*-alkanes, 1-alkenes from 12 to 19 carbons in length, cyclohexane and cycloheptane, alcohols from 12 to 18 carbons in length, or ketones from 13 to 17 and 19 carbons in length. A previous report (Merkel et al. 1978b) suggested that growth occurred in strains YS-3 and YS-4 with 1-alkenes as substrate; chromatographic analysis indicated that the homologous *n*-alkane was present in the substrates tested. The following substrates did not support growth: arabinose, cellobiose, fructose, galactose, glucose, lactose,

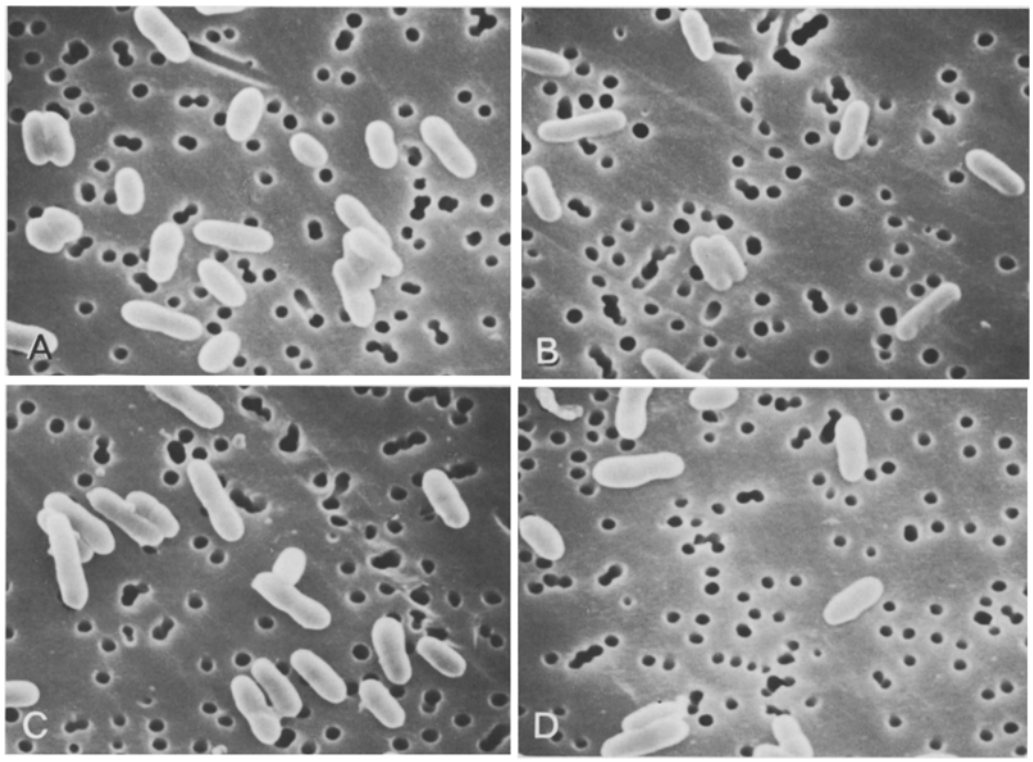

Fig. 1. Electron micrographs of HS-5 **A**, RR-D **B**, PTA-1 **C** and NM **D**; cells were grown on *n*-heptadecane at 60°C. Magnification is 9.420×; 1 μm

Table 2. Properties of the *n*-alkane utilizing strains[a]

	HS-5	NM	YS-3	YS-4	RR-D	PTA-1
Morphology	rod 0.9 μm[b]	rod 1.0 μm	rod[c] 0.7 μm	rod[c] 1.5 μm	rod 1.0 μm	rod 1.1 μm
Optimum growth temperature (°C)	58–62	55–60	60[c]	60[c]	58–62	60–65
pH optimum	6.5–7.5	7.0–7.5	6.5–7.5[c]	6.0–7.0[c]	6.5–7.5	ND[e]
Generation time (minimum, hours)	6.5	9.0	6+[c]	6+[c]	7.5	7.5
Substrate range	$C_{13}-C_{20}$[d]	$C_{14}-C_{20}$	$C_{13}-C_{20}$	$C_{13}-C_{20}$	$C_{14}-C_{20}$	$C_{14}-C_{19}$
mol% G+C	70.4	68.8	69.0	70.0	70.0	68.8

[a] All data (except substrate specificity) obtained from cells grown on *n*-heptadecane at 60°C
[b] Dimensions calculated from scanning electron micrographs
[c] Data from Merkel et al. 1978a
[d] Represents carbon length of *n*-alkanes
[e] Not determined

maltose, mannose, melibiose, rhamnose, ribose, sorbose, sucrose, trehalose, xylose, glycerol, mannitol, sorbitol, acetate, butyrate, propionate, citrate, pyruvate, succinate, acetone, nutrient broth, peptone, yeast extract or tryptone-yeast extract.

All of the strains utilized ammonium chloride as a nitrogen source. HS-5, NM and RR-D could also utilize sodium nitrate. Only HS-5 and NM could derive nitrogen from glycine but growth was sparse. Neither alanine nor glutamate could serve as a nitrogen source for the organisms. In no case could the amino acid tested serve as a source of both carbon and nitrogen.

Data on the sensitivities to various antibiotics are reported in Table 3, and overall they were quite similar.

The mol% G+C content of the DNA isolated from these strains was in the 68 to 70% range (Table 2).

The amino acid and amino sugar composition in the peptidoglycan of strains HS-5, NM and RR-D was determined; composition of YS-3, YS-4 and PTA-1 has been reported (Merkel et al. 1978a). Present in all strains were muramic acid, glutamic acid, diaminopimelic acid, alanine, glucosamine and galactosamine. Lysine was the other dibasic amino acid in strain HS-5, YS-3 and PTA-1, whereas ornithine was present in strains NM, RR-D and YS-4.

Table 3. Sensitivity of the obligate thermophiles to antibiotics

	HS-5	NM	YS-4	RR-D	PTA-1
Chlortetra-cycline	50[a]	50	50	25	50
Strepto-mycin	50	5	50	10	50
Kanamycin	5	1	10	10	50
Erythro-mycin	5	5	50	10	50
Neomycin	5	5	5	10	5
Chlor-amphenicol	10	5	5	5	5
Penicillin	10	10	5	5	25
Novobiocin	1	1	10	5	5

[a] Value represents minimum concentration (μg/ml) which inhibited growth in mineral salts + *n*-heptadecane incubated at 60° C. Growth or inhibition of growth was determined on the basis of visible turbidity

Table 4. A comparison of characteristics among *Thermoleophilum* strains, *Thermus* species and *Thermomicrobium roseum*[a]

	Thermo-leophilum sp.[b]	*Thermus* sp.[c]	*Thermomicro-bium roseum*
Cell morphology	short rod 0.7–1.5 μm	rod 5–10 μm	pleiomorphic rod 3–6 μm
Pigment	none	cream yellow orange pink	pink
Growth on complex media[d]	–	+	+
Growth on hydrocarbons[e]	+	–	–
Optimum growth temperature (° C)	60	60–70	70–75
Optimum pH	6.5–7.5	7.0–7.8	8.2–8.5
Generation time	6–10 h	0.5–1 h	5–6 h
Mol% G+C	68–70	64–69	64
Peptidoglycan[f]	DAP ORN or LYS	ORN	None

[a] Selected data from Merkel et al. 1978a and Ramaley et al. 1975
[b] Includes strains HS-5, YS-3, YS-4, NM, RR-D and PTA-1
[c] Includes *Thermus aquaticus, T. thermophilus, Thermus* sp. X-1 and the K-2 isolate
[d] Complex media = 0.1% tryptone + 0.1% yeast extract in mineral salts
[e] Hydrocarbons = 0.1% *n*-heptadecane in mineral salts
[f] Diamino acid as constituent

Discussion

The generic classification of these six strains is somewhat difficult as they have an exceedingly narrow substrate range and few hydrocarbon-utilizing thermophilic bacteria have been described. Previous publications from this laboratory (see references above) characterized several Gram negative, obligately thermophilic, hydrocarbon-utilizing organisms, including some of the strains discussed in this study, but did not suggest a generic name for the organism studied. One earlier study (Merkel et al. 1978a) did present an overall grouping of the Gram negative thermophilic bacteria based on the amino acid composition of their peptidoglycan and several other characteristics. The evidence presented previously and results from this study indicate that hydrocarbon-utilizing thermophiles do not fit into any of the genera described and are in need of further taxonomic evaluation.

Properties of the described aerobic, obligately-thermophilic, Gram negative bacteria in the genera *Thermus* and *Thermomicrobium* are compared with those of the proposed group, *Thermoleophilum,* in Table 4. The strains included in the genus *Thermus* or described as thermus-like are: *T. aquaticus* (Brock and Freeze 1969), *T. thermophilus* (Oshima and Imahori 1974), *Thermus* sp. X-1 (Ramaley and Hixson 1970), and strain K-2 (Ramaley et al. 1975). The genus *Thermomicrobium* is represented by a single species, *Thermomicrobium roseum* (Jackson et al. 1973).

The organisms obligate for both thermophily and hydrocarbon utilization share selected properties with different members of the *Thermus* group, including: (1) the optimum growth temperature around 60° C is equivalent to that for K-2; (2) the optimum pH for growth and mol% G+C as reported for *T. thermophilus*; (3) lack of pigmentation as for strain X-1; and (4) similar sensitivities to antibiotic agents. (For summary of these properties see Ramaley et al. 1975.)

However, as seen in Table 4, the dissimilarities as a group are more significant than the limited similarities between the strains. None of the hydrocarbon-utilizing thermophiles described in this report can grow on the complex media typically employed in the isolation and growth of *Thermus* species. All lack pigmentation. There is no evidence by scanning electron microscopy or routine light microscopic examination that filaments are formed as in *Thermus* (Degryse et al. 1978). The hydrocarbon-utilizers are much smaller than the other described thermophiles; the temperature and pH optima for growth are slightly lower, generation times significantly longer and mol% G+C values slightly higher than those reported for *Thermus* species. The peptidoglycan in those bacteria obligate for hydrocarbon substrates has diaminopimelic acid as the principal dibasic amino along with either ornithine (YS-3, HS-5, PTA-1) or lysine (YS-4, NM, RR-D). None of the *Thermus* species has been shown to contain diaminopimelic acid or lysine (Merkel et al. 1978a).

Thermomicrobium roseum has very little in common with these strains other than obligate thermophily (Table 4). Assignment of the hydrocarbon-utilizing bacteria described to date to this group is even less appropriate, particularly in light of its atypical cell wall composition and pleomorphism (Merkel et al. 1978a; Merkel et al. 1980).

It should be noted that PTA-1, which was previously reported (Phillips and Perry 1976) has lost selected characteristics (e. g. ability to grow on non-hydrocarbon substrates, pigmentation) and may well fit into this group. Whether the original description of PTA-1 was based on a mixed culture or routine transfer resulted in loss of selected properties remains unclear. The characteristics of PTA-1 as reported here have remained constant for several years and it is suggested that PTA-1 no longer be classified as *Thermomicrobium fosteri.* The data presented in this report

suggests that the six organisms (PTA-1, NM, YS-3, YS-4, RR-D and HS-5) probably belong in one genus. Further studies are under way to clarify this question.

Genus Thermoleophilum gen. nov.

Therm·oleo·philum. Gk. n. *thermus* heat; L. n. oleum oil; Gk. adj. *philos* loving. *Thermoleophilum* heat and oil-loving microbe.

Rod-shaped, short, Gram negative bacterium. Non-spore forming.

Aerobic. Obligately thermophilic and capable of growth only at the expense of a narrow range of *n*-alkanes.

Morphology

Cells are rod-shaped, ranging from 0.7 to 1.5 μm in length and 0.4 μm in width. Form very small translucent to white colonies on agar surfaces.

Nutrition

Aerobic. Grows only at the expense of *n*-alkanes from 13 to 20 carbons in length in a defined mineral salts medium. No growth factors required.

Growth characteristics

Growth occurs between 45 and 70° C with optimum around 60° C and within a pH range of 5.8 to 8.0 with optimum around neutrality.

DNA base composition

Mol% G+C in the range 68.8 to 70.4

Source

Can be isolated from mud and water samples, primarily from thermal environments but also found in non-thermal sources.

Thermoleophilum album sp. nov.

Al·bum. L. n. *albus*, white referring to colony color.

At present it is the only species designated in this genus and the description is as above for the genus.

Type strain is ATCC 35263, previously designated HS-5, isolated from Hot Springs, Arkansas. Its description is as follows:

Rod-shaped cells, 0.9 μm in length. Non-motile. Non-spore forming.

Aerobic. Growth occurs between 45° C to 70° C with optimum at 60° C and within pH range of 6.5 to 7.5 with optimum at pH 7.0. Only *n*-alkanes from 13 to 20 carbons in length can be used as substrate.

Mol% G+C is 70.4%.

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