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Heliobacterium modesticaldum, sp. nov., a thermophilic heliobacterium of hot springs and volcanic soils

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Abstract Enrichment cultures for heliobacteria at 50°C yielded several strains of a thermophilic heliobacterium species from Yellowstone hot spring microbial mats and volcanic soils from Iceland. The novel organisms grew optimally above 50°C, contained bacteriochlorophyll *g*, and lacked intracytoplasmic membranes. All isolates were strict anaerobes and grew best as photoheterotrophs, although chemotrophic dark growth on pyruvate was also possible. These thermophilic heliobacteria were diazotrophic and fixed N_2 up to their growth temperature limit of 56°C. Phylogenetic studies showed the new isolates to be specific relatives of *Heliobacterium gestii* and, as has been found in *H. gestii*, they produce heat-resistant endospores. The unique assemblage of properties found in these thermophilic heliobacteria implicate them as a new species of this group, and we describe them herein as a new species of the genus *Heliobacterium*, *Heliobacterium modesticaldum*.

Key words Anoxygenic phototrophic bacteria · Heliobacteria · *Heliobacterium modesticaldum* · Thermophily · Hot springs · Phylogeny

Introduction

Thermophilic representatives have been described for three of the six families of anoxygenic phototrophic bacteria. *Chloroflexus aurantiacus*, a filamentous nonsulfur green bacterium (Chloroflexaceae), can grow up to about 70°C and is widespread in neutral to alkaline hot springs worldwide (Castenholz and Pierson 1995). *Chlorobium tepidum*, a green sulfur bacterium (Chlorobiaceae), can grow up to 52°C (optimum, 48°C) and is found in acidic high sulfide New Zealand hot springs (Castenholz 1988; Castenholz et al. 1990; Wahlund et al. 1991). Among purple bacteria, only the purple sulfur bacterium *Chromatium tepidum* (Chromatiaceae) is truly thermophilic (i.e., capable of growth above 50°C; Castenholz and Pierson 1995), growing up to about 56°C with an optimum of 48–50°C (Madigan 1984, 1986). In addition to these organisms, several mildly thermophilic nonsulfur purple bacteria, such as *Rhodopseudomonas* strain GI (Resnick and Madigan 1989) and *Rhodospirillum centenum* (Favinger et al. 1989) have been isolated, but neither of these is capable of growth above 50°C (Castenholz and Pierson 1995). A similar situation exists among Ectothiorhodospiraceae, as certain extremely halophilic representatives, such as *Ectothiorhodospira halochloris*, grow optimally above 40°C, but are incapable of growth above 50°C (Imhoff and Trüper 1977).

Although most known species of Heliobacteriaceae grow optimally at around 40°C (Madigan and Ormerod 1995), until now no thermophilic species of heliobacteria has been described. Recently, a spore-forming sulfide-oxidizing heliobacterium was isolated from a Russian hot spring. However, this organism has a growth temperature optimum of 30°C and, thus, the hot spring was probably not its natural habitat (Starynin and Gorlenko 1993). In this paper, we describe four strains of a truly thermophilic heliobacterium isolated from Icelandic soils and Yellowstone hot spring microbial mats; all strains were capable of growth up to 56°C and showed growth temperature optima above 50°C. We describe these organisms herein as a new species of the genus *Heliobacterium*, *Heliobacterium modesticaldum*.

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Materials and methods

Sources of inocula

Strains of thermophilic heliobacteria were isolated from enrichment cultures started with soils obtained from the vicinity of hot springs near Reykjanes, Iceland, and from hot spring microbial

mat samples from unnamed thermal springs (45–70°C) near the Firehole River and from Octopus Spring (Brock 1978), Lower Geyser Basin, Yellowstone National Park. Immediately after inoculation, some of the enrichments were pasteurized (80°C for 20 min) before being incubated as described below. From the enrichments, four strains of thermophilic heliobacteria, designated strains Ice1, Ice2, YS5, and YS6, were obtained in pure culture. The type strain of *Heliobacterium modesticaldum* is strain Ice1 and is deposited along with strain YS6 in the American Type Culture Collection; the ATCC accession numbers are 51547 (Ice1) and 51577 (YS6).

Other heliobacteria

Heliobacterium gestii (Ormerod et al. 1990; ATCC 43375, obtained from J. Ormerod) and *Heliobacillus mobilis* (Beer-Romero and Gest 1987; ATCC 43427, obtained from H. Gest) were also used in this study.

Media and growth conditions

Thermophilic heliobacteria from Icelandic samples were enriched using a medium consisting of 0.25% Difco yeast extract. Yellowstone isolates were enriched in a modified GEMII medium (Gest et al. 1985) consisting of (per liter of deionized water): organic acid solution (sodium succinate·$6H_2O$, 40 g; sodium citrate·$2H_2O$, 16 g; DL-malic acid, 10 g; sodium acetate, 10 g; lactic acid (85%) 11.8 ml; deionized H_2O, 300 ml; pH 6.8) 15 ml; 1% disodium EDTA, 0.5 ml; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 20 mg; sodium pyruvate, 2.2 g; trace elements (see below), 1 ml; chelated iron solution (see below), 2 ml; 0.64 M KPO_4 buffer (KH_2PO_4, 20 g; K_2HPO_4, 30 g; deionized H_2O, 500 ml) 15 ml; NH_4Cl, 1 g; d-biotin (150 µg/ml ethanol), 0.1 ml; vitamin B_{12}, 20 µg.

All heliobacteria were grown routinely in medium PYE consisting of (per liter of deionized water): K_2HPO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 20 mg; $Na_2S_2O_3 \cdot 5H_2O$, 0.2 g; sodium pyruvate, 2.2 g; Difco yeast extract, 4 g; $(NH_4)_2SO_4$, 1 g; trace elements (disodium EDTA, 2.5 g; $MnCl_2 \cdot 4H_2O$, 0.2 g; H_3BO_3, 0.1 g; $NaMoO_4 \cdot 2H_2O$, 0.1 g; $ZnCl_2$, 50 mg; $NiCl_2 \cdot 6H_2O$, 50 mg; $CoCl_2 \cdot 6H_2O$, 20 mg; $CuCl_2 \cdot 2H_2O$, 5 mg; $VOSO_4 \cdot 2H_2O$, 5 mg; $Na_2WO_4 \cdot 2H_2O$, 5 mg; deionized water, 250 ml) 1 ml; chelated iron solution (disodium EDTA, 1 g; $FeCl_3 \cdot 6H_2O$, 0.5 g; concentrated HCl 1.5 ml; distilled water, 1 l), 2 ml; d-biotin (150 µg/ml ethanol), 0.1 ml; vitamin B_{12}, 20 µg. The pH was adjusted to 6.9 before autoclaving. Other media used for nutritional experiments were the following: PMS, medium PYE minus yeast extract; PMS-N, medium PMS minus $(NH_4)_2SO_4$; PMS-V, medium PMS minus vitamins; MS, medium PYE minus yeast extract and pyruvate.

Cells were grown photoheterotrophically (anaerobic/light) in stoppered culture tubes, completely filled 250 ml screw-capped bottles, or Erlenmeyer flasks as described previously (Kimble and Madigan 1992). When present, the headspace was flushed and vessels were sealed under N_2:CO_2 (95:5, v/v). Thermophilic heliobacteria were incubated at 48–52°C, while mesophilic heliobacteria were incubated at 37°C. A 1–5% volume of overnight culture was used as inoculum, and vessels were incubated in a circulating waterbath receiving 4,500–6,000 lux (incandescent illumination) in all cases.

Growth was measured as culture turbidity in a Klett Summerson photometer fitted with a no. 66 (red) filter. Cell dry weights were determined as described previously (Madigan and Gest 1979).

Absorption spectra

Spectra of intact cells were determined anoxically by centrifuging 10 ml of an overnight culture and resuspending the cell pellet in 3 ml of 30% bovine serum albumin solution containing 0.1% w/v sodium ascorbate inside an anaerobic hood. Upon removal, absorption spectra were immediately measured in a Hitachi U-2000 double-beam spectrophotometer. For determination of spectra of acetone extracts, cell pellets were extracted with acetone anoxically at 0°C in darkness for 2 h. Spectra were determined on the supernatants after centrifuging.

Isolation of DNA and determination of the mol% G+C content

The classical Marmur (1961) method was found unsatisfactory for isolation of genomic DNA from heliobacteria. Thus, the following modification of the Marmur method was employed. Forty milliliters of an overnight culture was centrifuged, and the cell pellet was resuspended in 5 ml TE (10 mM Tris-HCl, 1 mM disodium EDTA, pH 8). Cells were lysed by treatment with 50 µg/ml (final concentration) lysozyme (37°C for 30 min), followed by 0.5% (final concentration) SDS at 50°C for 5–10 min. Proteinase K was then added to a final concentration of 100 µg/ml, and the mixture incubated at 37°C for 3–6 h. The mixture was then extracted twice with equal volumes of phenol and once with an equal volume of $CHCl_3$. NaCl (1 M) was added to a final concentration of 0.1 M, and the DNA was precipitated with two volumes of cold ethanol and placed at –20°C overnight. The DNA was then centrifuged, dried, and resuspended in 5 ml TE and treated with 100 µg/ml RNase A for 3–6 h at 37°C. The DNA was then extracted once with an equal volume of phenol, and the DNA reprecipitated as described previously. The resulting DNA was dissolved in 0.1 × SSC (1 × SSC contains 150 mM NaCl plus 15 mM trisodium citrate, pH 7) to give an A_{260} of 0.2–0.4. The T_m was determined from the melting profile using an Hitachi U-2000 spectrophotometer equipped with a programmable thermoelectric cell holder. DNA purified from *Micrococcus luteus* (72% G+C), *Heliobacillus mobilis* (50.3% G+C), *Escherichia coli* (50% G+C) and *Clostridium perfringens* (28.6% G+C) were used as standards.

16S rRNA sequencing

16S ribosomal RNA was isolated from cell pellets, sequenced, and a phylogenetic tree was constructed as described previously (Wahlund et al. 1991).

In vivo nitrogenase measurements

Nitrogenase activity in intact cells was determined by acetylene reduction as described previously (Kimble and Madigan 1992), except that ethylene was quantified in a Varian model 2440 gas chromatograph fitted with a flame ionization detector (150°C) and Porapak N column (70°C).

Electron microscopy

For sectioning, cells were fixed in glutaraldehyde, followed by OsO_4, dehydrated in an ethanol series, and embedded with a 1:1 mixture of EMBed 812 and Spurrs. Sections stained with uranyl acetate and lead citrate were examined in a Hitachi H500H transmission electron microscope operating at 75 kV. For scanning electron microscopy, cells fixed in 1% glutaraldehyde, followed by OsO_4 were dehydrated in ethanol and subject to critical point drying and then examined in a Hitachi S570 scanning electron microscope operating at 20 kV.

Chemicals

All chemicals used were of reagent grade and were obtained from either Fisher, Mallinckrodt, or Sigma (all of St. Louis, Mo., USA).

Results and discussion

Enrichment, isolation and habitats

Enrichments for thermophilic heliobacteria were set up in 0.25% yeast extract medium (incubated at 50°C) with pasteurized soil samples obtained from various locations around the world (Australia, Iceland, Thailand, Japan, USA) that had previously yielded mesophilic heliobacteria (Stevenson 1993; Stevenson et al. 1993). Positive enrichments were only obtained using Icelandic soils and developed within 3–7 days; the presence of heliobacteria was signaled by dull green growth atop the soil in the bottom of a tube. Microscopic examination of these enrichments indicated that the predominant organisms were various endospore-formers, some of which were large rods morphologically identical to cells of pure cultures of thermophilic heliobacteria (see below). In addition to the Icelandic samples, crude enrichments for heliobacteria from Yellowstone hot spring mat samples were obtained from Carl Bauer (Indiana University), and several enrichments were set up in our lab with Yellowstone mat samples collected by one of us (M. T. Madigan). Mat enrichments were set up in medium GEMII or in 0.25% yeast extract media (either pasteurized or unpasteurized) and incubated at 45–50°C. Positive enrichments from mat samples developed only in GEMII medium (both pasteurized and unpasteurized) within 5 days and contained motile rod-shaped cells; endospores were only observed in the pasteurized enrichments. All primary enrichments were streaked for isolation on PYE agar plates and incubated in Gas Pak anoxic jars at 50°C. From the enrichments, four pure cultures of thermophilic heliobacteria were eventually obtained and were designated strains Ice1 and Ice2 (Icelandic isolates) and YS5 and YS6 (Yellowstone isolates).

After obtaining the Yellowstone isolates, we used medium GEMII to reexamine the soils originally tested with yeast extract as enrichment medium for the presence of thermophilic heliobacteria. However, as with the yeast extract enrichments, none of these soils yielded thermophilic heliobacteria. This indicates that thermophilic heliobacteria were not inadvertently overlooked in temperate and tropical soils and that the natural habitats of these organisms really are hot springs and volcanic soils. A further indication of this is the report by Castenholz and Pierson (1995) of the occurrence of thermophilic heliobacteria in neutral to alkaline Oregon hot springs. Thus, it is possible that these organisms inhabit hot springs worldwide whose temperatures are under 70°C.

Morphology, spore formation, and photosynthetic pigments

Cells of all strains of thermophilic heliobacteria were rod-shaped or slightly curved, measuring 0.8–1 μm wide by 2.5–9 μm in length (Fig. 1A). As observed by phase-contrast microscopy, most cells had one to two prominent dark granules (Fig. 1A), the chemical nature of which is unknown. As in all heliobacteria isolated thus far (Gest and Favinger 1983; Miller et al 1986; Madigan and Ormerod 1995), electron micrographs of thin sections of cells (Fig. 1B, 1D) revealed the absence of any type of intracytoplasmic photosynthetic membranes. In liquid cultures, strains Ice1, Ice2 and YS5 were motile, and scanning electron micrographs of strain YS5 showed cells containing polar or subpolar flagella (Fig. 1E). Strain YS6 was immotile and scanning electron micrographs (Fig. 1C) showed no sign of flagella. All strains stained gram-negatively. The cell wall of strain YS6 (Fig. 1D) appeared more rugged than that of strain Ice1, but neither strain showed evidence of a lipopolysaccharide layer typical of most other anoxygenic phototrophs.

Endospore formation has been observed in several species of mesophilic heliobacteria (Ormerod et al. 1990; Starynin and Gorlenko 1993), and we observed endospores in pure cultures of all four thermophilic heliobacteria. Although produced in low numbers, when present endospores were cylindrical in shape and located subterminally in the cell before release. Week-old cultures of all thermophilic heliobacteria were able to survive pasteurization, indicating that the spores produced are heat-resistant.

Absorption spectra of intact cells of all strains of thermophilic heliobacteria were nearly identical, with major peaks at 788, 670, and 575 nm (Fig. 2), indicating that bacteriochlorophyll *g* was the major chlorophyll pigment present (Brockman and Lipinski 1983). Absorption spectra of acetone extracts of strain Ice1 had major peaks at 760, 663, 568, 409, 436, and 469 (Fig. 2). Peaks at 436 and 469 suggest that as for mesophilic heliobacteria (van Dorssen et al. 1985), neurosporene is the major carotenoid present in thermophilic heliobacteria.

Temperature relationships

The upper temperature limit for growth of previously isolated heliobacteria was 44°C (Beer-Romero and Gest 1987; Madigan 1992). By contrast, all strains of thermophilic heliobacteria grew up to 56°C with optimal growth (generation times approximately 3 h in medium PYE) occurring at 50–52°C (Fig. 3). At 56°C, cells became elongated, indicating possible inhibition of cell division at the temperature maximum. No growth was observed below 23°C (Fig. 3). By contrast, the mesophilic species *Heliobacillus mobilis*, used as a control in these experiments, had an optimal growth temperature of about 37°C and continued to grow up to about 44°C (Fig. 3). Thus a major differentiating property between the new isolates and all previously isolated heliobacteria is thermophily; the isolated strains reported herein are the first heliobacteria to grow optimally above 50°C. Accordingly, the Icelandic and Yellowstone heliobacteria are true thermophilic phototrophs (Castenholz and Pierson 1995). We thus propose to create a new species of heliobacteria,

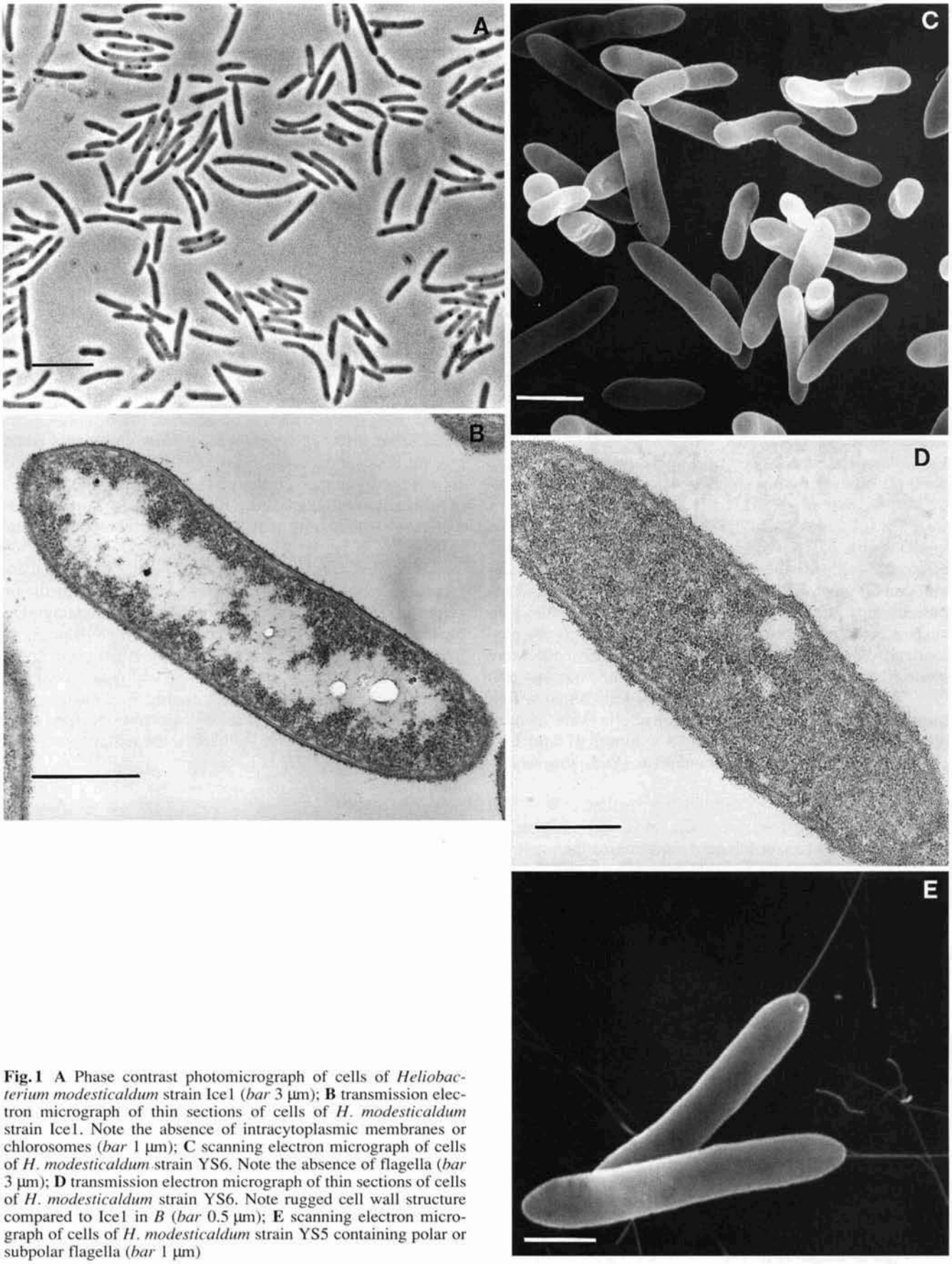

Fig. 1 **A** Phase contrast photomicrograph of cells of *Heliobacterium modesticaldum* strain Ice1 (*bar* 3 μm); **B** transmission electron micrograph of thin sections of cells of *H. modesticaldum* strain Ice1. Note the absence of intracytoplasmic membranes or chlorosomes (*bar* 1 μm); **C** scanning electron micrograph of cells of *H. modesticaldum* strain YS6. Note the absence of flagella (*bar* 3 μm); **D** transmission electron micrograph of thin sections of cells of *H. modesticaldum* strain YS6. Note rugged cell wall structure compared to Ice1 in *B* (*bar* 0.5 μm); **E** scanning electron micrograph of cells of *H. modesticaldum* strain YS5 containing polar or subpolar flagella (*bar* 1 μm)

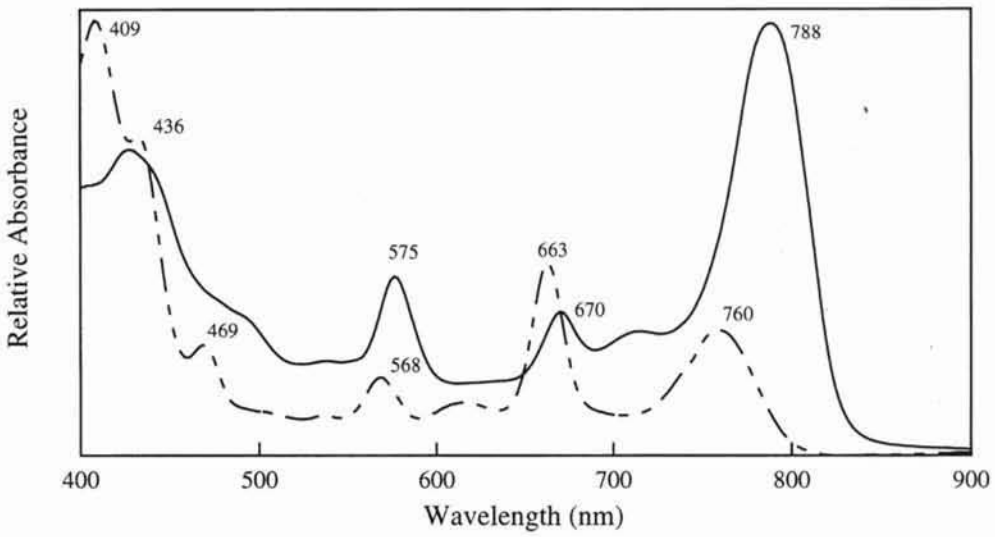

Fig. 2 Absorption spectra of intact cells (*solid line*) and acetone extracts (*dashed line*) of cells of *Heliobacterium modesticaldum* strain Ice1. All spectra were performed anoxically as described in *Materials and methods*

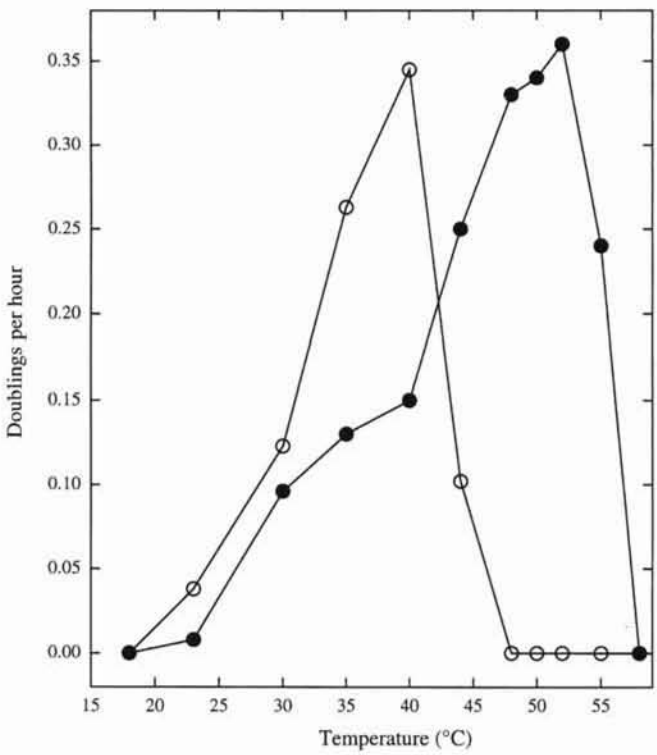

Fig. 3 Growth rate as a function of temperature for *Heliobacterium modesticaldum* strain Ice1 (*filled circles*) and *Heliobacillus mobilis* (*open circles*). Cells were grown in medium PYE at a light intensity of approximately 5,000 lux

Heliobacterium modesticaldum, to accommodate heliobacteria with properties of the thermophilic isolates.

Nutrition of *Heliobacterium modesticaldum*

Growth of *Heliobacterium modesticaldum* occurred under either photoheterotrophic or chemotrophic (anaerobic dark) conditions. All four strains required only the vitamin biotin as a growth factor. In addition, each strain also required a reduced sulfur source for biosynthetic purposes. Strains Ice1, Ice2, and YS5 utilized thiosulfate, sulfide, methionine, or cysteine as reduced sulfur sources, while strain YS6 absolutely required sulfide, methionine, or cysteine. Carbon nutritional studies of thermophilic heliobacteria showed that they are quite restricted in this regard. Of the organic compounds tested, photoheterotrophic growth of *H. modesticaldum* was observed only with pyruvate, lactate, acetate, or with yeast extract (interestingly, although not suitable as an enrichment medium, yeast extract supported good growth of pure cultures of Yellowstone strains of *H. modesticaldum*). No growth was obtained with the following carbon sources; butyrate (plus CO_2), malate, succinate, fumarate, propionate (plus CO_2), n-butanol (plus CO_2), propanol (plus CO_2), benzoate, glycerol, mannitol, ribose, fructose, glucose, sucrose, and ethanol (plus CO_2), many of which have previously been shown to be photoassimilated by one or another species of mesophilic heliobacteria (Stevenson 1993; Starynin and Gorlenko 1993; Madigan and Ormerod 1995). As described previously for mesophilic heliobacteria (Kimble et al. 1994), growth of *H. modesticaldum* also occurred under anaerobic chemotrophic conditions with pyruvate. The pH optimum for growth of *H. modesticaldum* varied somewhat with the strain. Strains Ice1, Ice2, and YS5 grew best at pH 6, while strain YS6 grew best at pH 7; the limits for growth were from pH 5.5 to about pH 8 (data not shown).

Ammonia and glutamine served as nitrogen sources for all strains of *H. modesticaldum*. However, the amino acids glutamate, aspartate, asparagine, and lysine, which served as nitrogen sources for various mesophilic heliobacteria (Stevenson 1993, Stevenson et al. 1993), were not utilized by the thermophiles. N_2 also served as a nitrogen source for *H. modesticaldum*. Nitrogenase assays of cell suspensions of strains Ice1 and YS5 showed high rates of acetylene reduction near 50°C and measurable activities at 55°C; no nitrogenase activity was present at 60°C (Table 1). Diazotrophic growth of strain Ice1 occurred just as well at 50°C as at 45°C, but only very slow growth occurred at 55°C (data not shown), the temperature limit for

Table 1 Nitrogenase activity of *Heliobacterium modesticaldum* as a function of temperature. Both strains were grown photosynthetically at 45°C on N_2 as sole nitrogen source. The doubling time of strain Ice1 was 7.4 h and of YS5, 7.2 h. *Rate* is expressed as μmol ethylene produced h^{-1} (mg bacterial dry weight)$^{-1}$; *percent activity* is that with respect to optimal nitrogenase rate obtained at 45° C (strain YS5) or 50° C (strain Ice1); *NA* no activity

Strain	Assay temperature (°C)	Rate	Percent activity
Ice1	45	3.81	99
	50	3.84	100
	55	0.53	14
	60	NA	0
YS5	45	2.18	100
	50	1.41	65
	55	0.20	9
	60	NA	0

growth on ammonia (Fig. 3). Thus, *H. modesticaldum* joins the green sulfur bacterium *Chlorobium tepidum* (Wahlund and Madigan 1993) as the only anoxygenic phototrophs known to fix N_2 above 50°C.

As for all diazotrophic bacteria, nitrogenase activity was not present in cells of *H. modesticaldum* grown on excess ammonia, presumably due to repression of nitrogenase synthesis. In addition, however, nitrogenase of *H. modesticaldum* was inactivated when ammonia was added to cell suspensions of nitrogen-fixing cultures (data not shown). This phenomenon, known as the ammonia "switch-off" effect, is widespread in anoxygenic phototrophs and has been observed previously in mesophilic heliobacteria (Kimble and Madigan 1992).

Genetic properties

The DNA base composition (measured by thermal denaturation) of DNA purified from *H. modesticaldum* strain Ice 1 was 54.6 mol% G+C, well within the range for other members of the heliobacteria (50.3–54.8%; Madigan and Ormerod 1995) and very close to that of the mesophilic species *Heliobacterium gestii* (54.8%). As a control, identical procedures were carried out on DNA isolated from cells of *Heliobacillus mobilis* and yielded a value of 50.8 mol% G+C, only slightly higher than the previously published value of 50.3% for this organism (Beer-Romero and Gest 1987).

The phylogenetic position of *H. modesticaldum* strain Ice1 was examined by 16S ribosomal RNA sequencing. Sequencing data (Table 2 and Fig. 4) clearly place the new

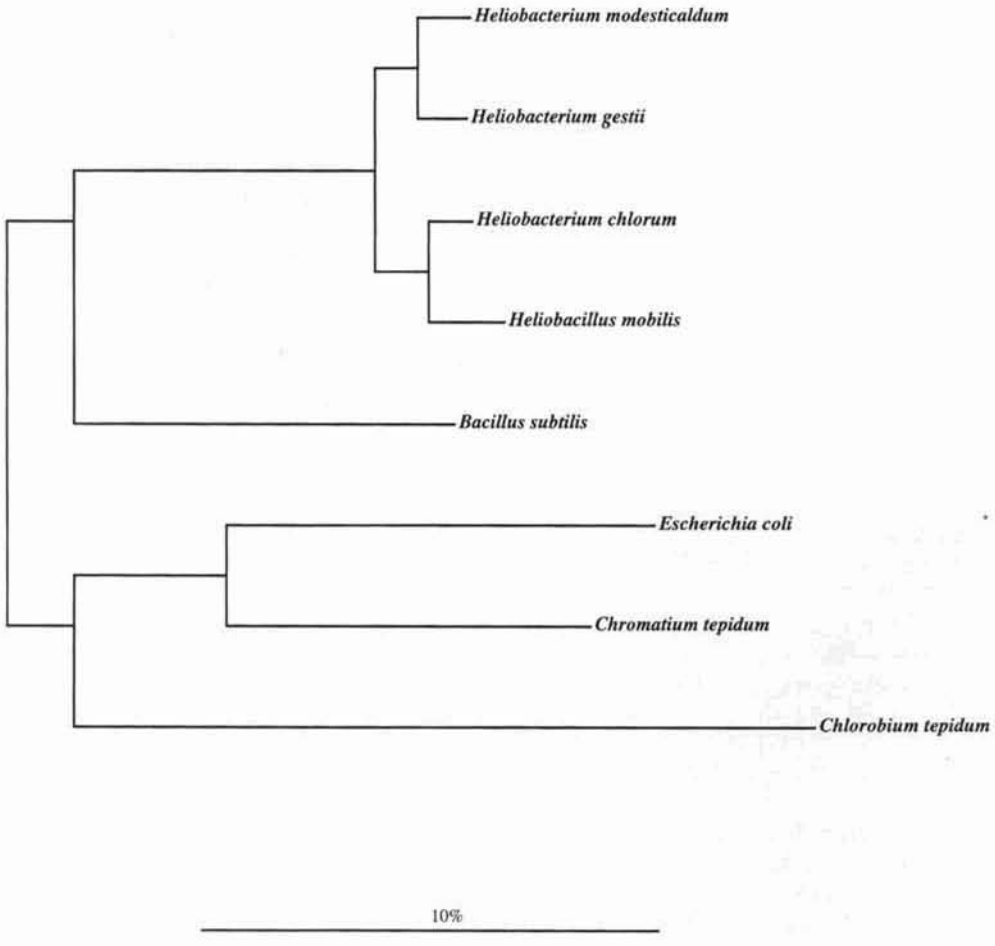

Fig. 4 Phylogenetic position of *Heliobacterium modesticaldum* among mesophilic heliobacteria species and other thermophilic anoxygenic phototrophs. *Scale* indicates distance on the tree equivalent to 10 nucleotide substitutions per 100 nucleotides in 16S rRNA. All sequences have been deposited in Genbank as follows: *Escherichia coli*, M24828; *Chlorobium tepidum*, M58468; *Chromatium tepidum*, M59150; *Bacillus subtilis*, M10606; *Heliobacillus mobilis*, U14560; *Heliobacterium chlorum*, M11212; *Heliobacterium gestii*, U14558; and *Heliobacterium modesticaldum*, U14559

Table 2 Evolutionary distance matrix (percent dissimilarity) for a collection of bacterial 16S rRNA sequences including *Heliobacterium modesticaldum* (see *Materials and methods*). Only positions represented by a known nucleotide in all sequences in the alignment are considered in the analysis

	1	2	3	4	5	6	7
1. *Heliobacterium gestii*							
2. *Heliobacterium modesticaldum*	2.2						
3. *Heliobacterium chlorum*	4.1	4.4					
4. *Heliobacillus mobilis*	4.9	4.8	2.6				
5. *Bacillus subtilis*	16.2	17.0	17.3	17.9			
6. *Chlorobium tepidum*	27.8	27.9	27.6	28.8	26.6		
7. *Chromatium tepidum*	22.5	22.5	22.1	22.8	23.8	28.0	
8. *Escherichia coli*	24.7	24.3	24.7	25.1	23.3	28.1	17.3

Table 3 Summary of properties of heliobacteria

Property	*Heliobacterium modesticaldum*[a]		*Heliobacterium chlorum*[b]	*Heliobacillus mobilis*[c]	*Heliobacterium gestii*[d]
	Ice1	YS6			
Habitat	Icelandic soil	Yellowstone hot spring(s)	Temperate soil	Tropical paddy soil	Tropical paddy soil
Morphology	Rod/curved rod	Rod	Rod	Rod	Spirillum
Dimensions	1×2.5–6.5 µm	0.8–1×3–9 µm	1×7–9 µm	1×7–10 µm	1×7–10 µm
Motility	Flagellar	None	Gliding	Peritrichous flagella	Multiple subpolar flagella
Carbon sources photometabolized	Pyruvate, lactate, acetate, yeast extract	Pyruvate, lactate, acetate, yeast extract	Pyruvate, lactate, yeast extract	Pyruvate, lactate, acetate, butyrate + CO_2, yeast extract	Pyruvate, lactate, acetate, butyrate + CO_2, ethanol + CO_2, yeast extract
Biosynthetic sulfur sources	S_2O_3, sulfide, methionine, or cysteine	Sulfide, cysteine, or methionine	SO_4^{2-}, S_2O_3, methionine, or cysteine	SO_4^{2-}, S_2O_3, methionine, or cysteine	S_2O_3, methionine, or cysteine
Endospores produced	Yes	Yes	None observed	None observed	Yes
Optimum temperature (°C)	50–52	50–52	38–42	38–42	38–42
GC content (mol%)	54.6	55	52	50.3	54.8

[a] Data from this paper
[b] Data from Gest and Favinger (1983)
[c] Data from Beer-Romero and Gest (1987)
[d] Data from Ormerod et al. (1990) and Madigan and Ormerod (1995)

organism within the low G+C gram-positive branch of the Bacteria, which includes the genera *Bacillus* and *Clostridium* (Woese 1987), but specifically in with other known heliobacteria (Woese et al. 1985). A phylogenetic tree generated from comparative sequence data showed *H. modesticaldum* strain Ice1 to be distinct from other heliobacteria and to be a specific relative of *Heliobacterium gestii*; *H. gestii* and *H. modesticaldum* form a cluster distinct from a second one containing *Heliobacterium chlorum* and *Heliobacillus mobilis* (Fig. 4). 16S rRNA sequencing of *H. modesticaldum* strain YS5 showed it to be nearly identical (>99.5% similarity) to strain Ice1, and thus the Yellowstone and Iceland isolates are likely strains of the same species. As expected, other thermophilic anoxygenic phototrophs, such as *Chlorobium tepidum* and *Chromatium tepidum*, are phylogenetically quite distant from *H. modesticaldum* (Fig. 4).

Final remarks

A summary of the major properties of heliobacteria, including the new species *H. modesticaldum*, is shown in Table 3. Major differentiating features include habitat, certain nutritional features, temperature requirements, and phylogeny. In addition, endospores are clearly produced by *H. gestii* and *H. modesticaldum*, while *H. chlorum* and *H. mobilis* have not been observed to sporulate (Table 3).

Discovery of the heliobacteria has brought a deeper understanding of the roots of photosynthesis, especially concerning Photosystem I of green plants, which closely resembles the reaction center of heliobacteria (Blankenship 1992). The discovery of a thermophilic species of heliobacteria, *Heliobacterium modesticaldum*, shows that most families of anoxygenic phototrophs contain thermophilic species and should make available a source of thermally stable bacteriochlorophyll *g* pigment-protein complexes for further biophysical research. Moreover, the strong

N_2-fixing properties of *H. modesticaldum* suggest that it may be ecologically important in hot spring microbial mats and certain types of volcanic soils. In this connection, field studies of nitrogen fixation in Yellowstone hot spring microbial mats have implicated the heterocystous cyanobacterium *Mastigocladus laminosus* as the main diazotroph present at 50–55°C (Wickstrom 1980). However, because *H. modesticaldum* inhabits these same types of mats, it is possible that a portion of the fixed nitrogen is contributed by this anoxyphototroph instead. In fact, because the anoxygenic phototroph *Chloroflexus aurantiacus*, a major component of neutral to alkaline hot spring microbial mats (Pierson and Castenholz 1974; Castenholz and Pierson 1995), is nondiazotrophic (Heda and Madigan 1986), *H. modesticaldum* may be the only anoxyphototroph contributing fixed nitrogen in these hot springs at temperatures above 47°C, the apparent upper temperature limit for nitrogen-fixing nonsulfur purple bacteria (Favinger et al. 1989; Resnick and Madigan 1989).

Description of *Heliobacterium modesticaldum*

Heliobacterium modesticaldum (modesti. caldum. L., moderately hot). Cells rods or curved rods measuring 0.8–1 μm wide by 2.5–9 μm long. Stain gram-negative. Motile by flagella or nonmotile. Contain bacteriochlorophyll *g*. Absorption spectra of intact cells show major peaks at 788, 670, and 575 nm. Neurosporene is probably the only carotenoid. Chlorosomes or intracytoplasmic membranes absent. Forms cylindrical subterminal endospores.

Obligate anaerobe can grow phototrophically by photoassimilating pyruvate, lactate, and acetate, or chemotrophically on pyruvate. Sugars and other organic acids are not utilized. Ammonia, glutamine, and N_2 serve as nitrogen sources. Biotin is required. Thiosulfate, methionine, or cysteine required as biosynthetic sulfur sources. Optimal pH 6–7. Optimum growth temperature 52°C. Maximum temperature 56°C; minimum 25–30°C. NaCl not required for growth; growth inhibited by 1% NaCl. Habitat: neutral to alkaline hot springs or volcanic soils.

DNA base ratio: 54.6–55 mol% G+C. Type strain Ice1, isolated from Icelandic soil obtained from the vicinity of hot springs, Reykjanes, Iceland. Deposited in the American Type Culture Collection as ATCC 51547.

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