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EXPERIMENTAL ARTICLES

New Thermophilic Methanotrophs of the Genus *Methylocaldum*

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Abstract—Two pure cultures of obligate methanotrophs, strains H-11 and O-12, growing in the temperature range from 30 to 61°C with a optimum at 55°C were isolated from samples of silage and manure. Based on the results of analysis of the 16S rRNA genes and genes of membrane-bound methane monooxygenase, as well as on phenotypic properties, the isolates were assigned to the genus *Methylocaldum.* Significant temperature-dependent variations in morphology and phospholipid and fatty acid composition were revealed. Both strains assimilated methane carbon via the ribulose monophosphate, serine, and ribulose bisphosphate pathways. The activity of hexulosephosphate synthase was independent of the cultivation temperature; however, the activities of hydroxypyruvate reductase and ribulose bisphosphate carboxylase were higher in cells grown at 55°C than in cells grown at 37°C, indicating the important roles of the serine and ribulose bisphosphate pathways in the thermoadaptation of the strains under study. NH_4^+ assimilation occurred through reductive amination of α-ketoglutarate and via the glutamate cycle. The relationship between the physiological and biochemical peculiarities of the isolates and their thermophilic nature is discussed.

Key words: thermophilic and thermotolerant methanotrophs, *Methylocaldum*.

Methanotrophs are a group of gram-negative bacteria that utilize methane as the major source of carbon and energy; this determines their physiological and biochemical uniqueness and their specific role in the ecological niches they occupy. Methanotrophic communities of various ecosystems form the basis of a powerful bacterial filter that decreases methane emission into the atmosphere [1]. Thus, in soil and freshwater ecotopes, methanotrophs consume up to 80% of the biologically produced methane [2]. The processes of methane oxidation in high-temperature ecosystems are far less well studied. In particular, no methane consumption was revealed in thermophilic cyano–bacterial mats in hydrothermal vents, where a trophic chain culminating in hydrogen-dependent and aceticlastic methanogenesis has been found [3]. In the last decade, persistent attempts have been made to isolate and study thermophilic methanotrophs; however, the progress in this field has not been significant [4]. So far, our knowledge of the taxonomic diversity of thermophilic methanotrophs is virtually restricted to the genera *Methylococcus* and *Methylocaldum.* The most thoroughly studied thermophilic methanotroph is *Methylococcus capsulatus*, which possesses two forms of methane monooxygenase (MMO)—soluble methane monooxygenase (sMMO) and membrane-bound methane monooxygenase (mMMO)—and is the subject of numerous molecular genetic studies. Representatives of the genus *Methylocaldum* differ from *Methylococcus capsulatus* by the absence of sMMO and by the nucleotide sequence of the 16S rRNA gene (8% divergence) [5, 6]. Recently, strain HB (conventionally assigned to a new genus, *"Methylothermus"*) has been isolated, which grows in a temperature range of 40–70°C with an optimum at 55–62°C and, like *Methylocaldum*, possesses only mMMO. It should be noted that both the phenotypic and genotypic properties of *"Methylothermus"* and *Methylocaldum* representatives have been only fragmentarily described, and the ecological role of thermophilic and thermotolerant methanotrophs, as well as the mechanisms of their thermal adaptation, has not been studied.

The aim of this work was to isolate and characterize new cultures of thermophilic methanotrophic bacteria.

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MATERIALS AND METHODS

Obtaining of enrichment cultures. Samples of silage and manure were taken in the vicinity of Pushchino in September 2001. The in situ temperatures were 60 and 15°C, respectively. To obtain enrichment cultures, 2-g sample portions were introduced into 250- or 750-ml flasks containing 50 ml of P medium; the flasks were filled with a methane–air (1 : 1) mixture [7] and incubated in a thermostat at 55°C with periodic agitation. After the medium became visibly turbid or a bacterial film appeared on its surface, 5 ml of the culture was transferred to a fresh medium and incubated under a methane-containing atmosphere at 55°C on a Clim-O-Shake rotary shaker (Switzerland) operated at 100 rpm. The development of methanotrophic bacteria in the successive subcultures was monitored by phase-contrast light microscopy.

Isolation of pure cultures. To separate cells of methanotrophic bacteria from smaller cells of heterotrophic satellites, suspensions of enrichment cultures were passed through membrane filters with a pore diameter of 0.6–1 μm (Millipore, France). The filters were placed in a small volume of fresh medium and incubated in a methane-containing atmosphere for 5–7 days. Pure cultures were obtained by serial dilutions followed by plating onto agarized P medium supplemented with the culture liquid of the enrichment culture. Visible colonies of methanotrophic bacteria appeared after 1–2 weeks. Separate colonies were transferred to fresh agarized medium. The purity of the methanotrophic cultures was judged from examinations under light and electron microscopes and from lack of growth on media containing organic compounds.

Electron microscopy. Cells were fixed, and thin sections were prepared with a Reichert ULTRACUT System ultramicrotome (Austria) and examined in a Jeol JEM 100B electron microscope (Japan) as described elsewhere [8, 9].

Physiological and biochemical studies. The potential rate of methane consumption in the samples of silage and manure was determined at 55°C by the radioisotopic method as described earlier [10]. To optimize the mineral composition of the medium, the growth rate of pure methanotroph cultures was measured in the initial P medium, diluted P medium, and P medium with the content of mineral components increased by two or three times. In further studies, the isolates were cultivated in 2P medium with the content of all mineral salts doubled as compared to P medium. To study the influence of temperature, NaCl concentration, and pH of the medium on the growth of pure cultures, the bacteria were grown under a methane–air (1 : 1) atmosphere in 250-ml flasks containing 50 ml of 2P medium. The NaCl concentrations tested were 0, 0.25, 0.5, 1.0, 2.0, 3.0, and 4.0. The pH of the medium was adjusted to required values by adding phosphate (pH 5.5–8.5) and Na carbonate (pH 9–10) buffers to a final concentration of 50 mM. The temperature range for growth of the bacteria was determined by cultivating them on a thermostatically controlled Clim-O-Shake rotary shaker (Switzerland).

To determine the ability of the isolates to utilize various organic substrates, they were grown on agarized medium at optimal pH and salt concentrations in the presence of 0.01% methanol, formaldehyde, formate, glucose, acetate, citrate, malate, acetate, pyruvate, or sucrose. The ability to grow on various nitrogen sources was tested by replacing KNO_3 in the medium with urea, yeast extract, $(NH_4)_2SO_4$, or $NaNO_2$ at a concentration of 0.1%. Autotrophic growth under an atmosphere of H_2 + O_2 + CO_2 was tested as described by Baxter *et al.* [11].

Cell extract preparation and enzyme assays were performed as described earlier [12]. Spectrophotometric analyses were conducted at 37°C on Specord UV VIS (Germany) and Shimadzu UV-160 (Japan) recording spectrophotometers. The presence of sMMO in methanotrophs was judged from the oxidation of naphthalene by cells [5]. Protein in cell extracts was measured by a modified Lowry method [13].

Chemotaxonomic characteristics. To analyze fatty acids, 3 mg of lyophilized biomass was treated with 0.4 ml of a 1 N HCl solution in methanol for 3 h at 80°C (acidic methanolysis). The resultant methyl esters of fatty acids and other lipid components were extracted with hexane with subsequent evaporation of the latter. The obtained dry residue was silylated in 20 μl of BSTFA for 15 min at 80°C and again diluted with hexane to a volume of 100 μl. A 1-μl sample of the mixture was injected into an AT-5973B gas chromatograph–mass spectrometer (Agilent Technologies, United States) equipped with a capillary quartz column (25 m × 0.25 mm) with a 0.2-μm thick layer of HP-5ms (Hewlett-Packard) as the stationary phase. Fractionation was performed at a temperature programmed to rise from 120 to 280°C at a rate of 5°/min. The injector and interface temperatures were 280°C. The data were processed using the programs supplied with the instrument. The compounds in chromatographic peaks were identified with the help of the programs of the nbs75k, NIST, and wiley275 mass spectra databases.

To analyze fatty acids in the Sherlock system, a sample of dry biomass (5 mg) was subjected to acid methanolysis. The resultant fatty acid methyl esters and dimethyl acetals were extracted with hexane, and a 2-μl portion of the mixture was injected with an automatically operated Hewlett-Packard 7673A sampler into a Sherlock system gas chromatograph for identification of the microorganisms by their fatty acid profiles (Microbial Identification System, Microbial ID Inc., Newark, Delaware, USA). Chromatographic parameters recommended in the instrument manual were employed in the analysis.

To determine phospholipids, cells were grown in the presence of ^{14}C-methane (10 μmol, 12 μCi, V/O Izotop, Russia) and collected at the midexponential phase by

Table 1. Nucleotide sequences of the primers [14] used in this work

Primer	Nucleotide sequence (5'–3')	Target gene	Annealing temperature,°C
mxaF 1003f	GCGGCACCAACTGGGGCTGGT	MDH (all methylotrophs)	59
mxaF 1561r	GGGCAGCATGAAGGGCTCCC	MDH (all methylotrophs)	59
pmoA 189f	GGNGACTGGGACTTCTGG	mMMO/AMO	56
pmoA 682r	GAASGCNGAGAAGAASGC	mMMO/AMO	56
mmoX 882f	GGCTCCAAGTTCAAGGTCGAGC	sMMO	55
mmoX 1403r	TGGCACTCGTAGCGCTCCGGCTGG	sMMO	55

Note: MDH, methanol dehydrogenase.

centrifuging at 13000 *g* for 10 min. Phospholipids were extracted from 1 g of wet biomass with 2 ml of a chloroform–methanol (1 : 2) mixture under thorough mixing for 1 h in the cold. The extract obtained was centrifuged for 10 min at 13000 g. The biomass was repeatedly extracted with 2 ml of the same mixture for 20 min. The combined supernatants, supplemented with 2 ml of chloroform and 2 ml of water, were shaken for 20 min in the cold and centrifuged for 10 min at 13000 *g*. The lower phase, containing phospholipids, was washed with an equal volume of a methanol–water (1 : 1) mixture and centrifuged to separate the phases. The solvents were removed in a rotary evaporator. The remainder was suspended in 100 μl of chloroform and kept at –20°C. Phospholipids were separated by one-dimensional TLC on Kieselgel 60 F_{254} plates (10 × 10 cm, Merck, Germany) in a chloroform–methanol–water–ammonia (60 : 34 : 3 : 1) system. The plates were dried and placed into a closed glass vessel containing several iodine crystals. Lipids developed as yellow-brown spots, which were carefully outlined with a pencil. Upon decolorization, the marked portions of Kieselgel were transferred with a spatula to bottles containing 5 ml of a scintillation mixture and the radioactivity was measured in a SL-30 liquid spectrometer (Intertechnique, France). Phospholipids were identified by using standards from Sigma (United States).

Ubiquinones were extracted as described by Collins [14] and analyzed on a Finnigan MAT 8430 MS (Germany) mass spectrometer under standard conditions by direct injection of a sample; the injector temperature was 160°C.

Isolation of DNA and PCR amplification. Isolation of total DNA from silage and manure samples and pure cultures and PCR amplification were performed as described earlier [8, 15, 16] using the oligonucleotide primers for the functional genes of sMMO and mMMO (Table 1). Nucleotide sequences of the PCR products were determined on a CEQ 2000XL Beckman Coulter (United States) automatic sequencer using the BigDye Terminator Cycle Sequencing kit (Perkin-Elmer, United States) according to the manufacturer's recommendations.

RESULTS AND DISCUSSION

Obtaining of enrichment and pure cultures. Wastes of cattle-breeding farms, such as silage and manure, are rich in organic matter and can be viewed as a peculiar natural fermenter for aboriginal microflora. Intense microbial processes of organic matter decomposition and methane production occur with significant liberation of heat. We used the intensity of ^{14}C-methane consumption at elevated temperatures as a primary criterion of the presence of thermophilic or thermotolerant methanotrophs in silage and manure samples.

The potential rate of methane consumption in samples of silage (O-1) and manure (H-1) at 55°C was 24 and 32 nmol/(g sample day), respectively. Subsequent PCR amplification with specific primers (Table 1) revealed the presence of the *pmoA* gene and the absence of the *mmoX* gene in the total DNAs of the samples investigated. In addition, the use of the primers specific to the *mxaF* gene, encoding the large subunit of methanol dehydrogenase, yielded PCR products of the expected size. In combination, these data unambiguously suggested the presence of methanotrophic bacteria in silage and manure.

After incubation of the samples in liquid medium under a methane-containing atmosphere at 55°C for a week, surface bacterial films developed. It should be noted that the bacterial film that developed from sample O-1 was obtained on medium with KNO_3 as the nitrogen source, whereas sample H-1 yielded a bacterial film on medium with NH_4Cl. Transfer of the bacterial films to fresh media and cultivation with continuous agitation allowed stable enrichment cultures O-1 and H-1 to be obtained. These enrichments were associations of methanotrophic bacteria and heterotrophic satellites. Due to the differences in the cell sizes of methanotrophs and the heterotrophic satellites, it proved possible to decrease the content of the latter organisms by filtration through membrane filters. Further plating of the cultures onto agarized P medium supplemented with the culture liquid of the heterotrophic satellites yielded separate methanotroph colonies, from which two pure cultures, O-12 and H-11, were isolated.

Physiological properties. During growth on agarized medium under an atmosphere of methane and air,

Table 2. Comparison of the characteristics of new thermophilic methanotrophs and type strains of *Methylocaldum* species

Characteristic	Strain O-12	Strain H-11	*M. gracile*	*M. tepidum*	*M. szegedienze*
Morphology	Rods	Rods	Rods	Ovoids	Ovoids
Cell size, μm	0.6–0.8 × 1.8–2.0	0.4–0.6 × 1.4–1.6	0.4–0.5 × 1.0–1.5	1.0–1.2 × 1.0–1.5	0.6–1.0 × 1.0–1.5
ICM type	I	I	I	I	I
sMMO	–	–	–	–	–
mMMO	+	+	+	+	+
Motility	+	+	+	+	+
Azotobacter-type cysts	+	–	+	+	+
Colony color	Cream	Light cream	Grayish brown	Grayish brown to brown	Brown to light brown
Temperature range, °C	30–61	30–59	20–47	30–47	37–62
Temperature optimum, °C	55	55	42	42	55
pH range	6 – 8.5	6 – 8.5	ND	ND	ND
pH optimum	7.2	7.1	ND	ND	ND
Growth on methanol (0.1%)	–	–	–	–	–
Pathways of C_1 assimilation	RuMP, serine, RuBP	RuMP, serine, RuBP	RuMP, serine, RuBP	RuMP, serine, RuBP	RuMP, serine, RuBP
α-Ketoglutarate dehydrogenase	–	–	–	–	–
Fatty acids (% of total)					
$C_{16:0}$	63.5	65.0			
$C_{16:1}$	13.3	12.0	ND	ND	ND
C_{17cyc}	9.0	6.0			
$C_{9\text{-OMe-}16:0}$	4.6	4.9			
Cardiolipin	+	+	ND	ND	ND
Phosphatidylethanolamine	+	+	ND	ND	ND
Phosphatidylcholine	–	–	ND	ND	ND
Ubiquinone	Methylene-Q-8	Methylene-Q-8	ND	ND	ND
G+C in DNA, mol %	58.5	58.5	56–58	57.2	56.5
Isolation source	Silage	Manure	Activated sludge	Soil	Hot spring

Note: ND, not determined; "–" means that the characteristic is negative.

the cultures formed cream, round, convex colonies with an even edge, 0.2–2 mm in diameter. During growth in liquid medium, strain O-12 produced a yellow pigment diffusing into the medium. Both strains failed to grow on solid media with methanol or formaldehyde or on polycarbon compounds: glucose, acetate, citrate, malate, succinate, pyruvate, or sucrose. Weak growth was recorded in liquid medium with 0.05% formate as the carbon source. Nitrate and ammonium salts but not urea, amino acids, or yeast extract could be used as the nitrogen sources.

Strains O-12 and H-11 grew in the temperature ranges 30–61 and 30–59°C, respectively. The temperature optimal for the growth of both strains was 55°C (μ_{max} = 0.07 h^{-1}; generation time, 10 h). At 30°C, the growth of both cultures was possible only after their preadaptation for 2 days at 40°C. The growth pH range was 6–8.5 for both strains; the pH optimum was 7.2. The strains showed better growth in medium supplemented with 0.1% NaCl; no growth occurred at NaCl concentrations higher than 0.5%. Thus, strains H-11 and O-12 are neutrophilic and thermophilic obligate methanotrophs (Table 2).

Cell morphology and ultrastructure. The isolates were characterized by cell polymorphism: along with rods with rounded ends (measuring 0.6–0.8 × 1.8–2.0 μm in strain O-12 and 0.4–0.6 × 1.4–1.6 μm in strain H-11), cocci of varying diameter also occurred (Figs. 1a, 2a). Thin sections revealed the cell wall structure characteristic of gram-negative bacteria and the occurrence of developed intracytoplasmic membranes (ICM) of type I, appearing as stacks of vesicular disks (Figs. 1d, 2b). Irrespective of the growth phase, the cells were virtually completely filled with ICM. Inter-

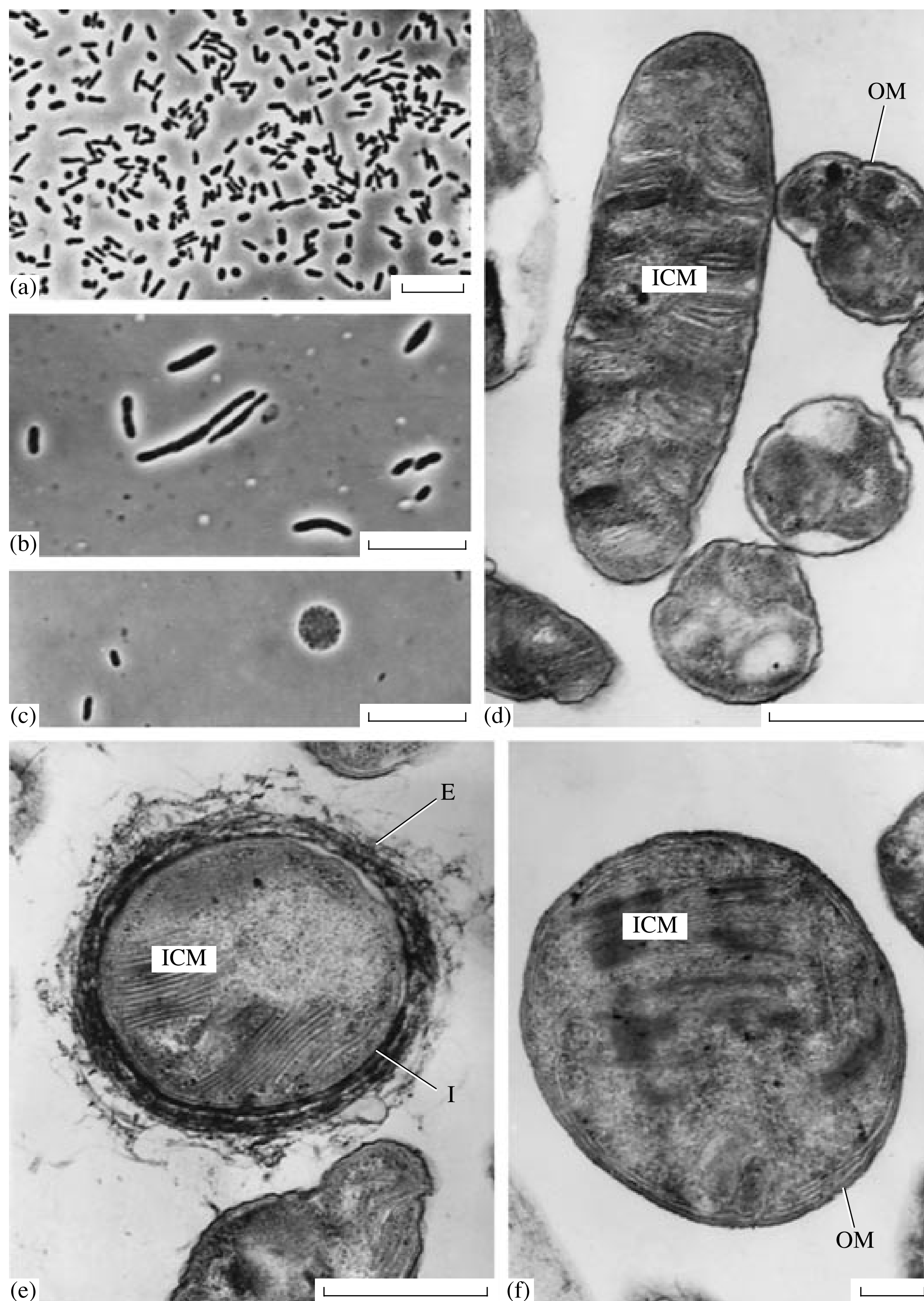

Fig. 1. Morphology and ultrastructure of strain O-12 cells: (a–c) phase-contrast micrographs; (d–f) electron micrographs of thin sections; (e) *Azotobacter*-type cysts. (a, d, e) Cells grown at 55°C; (b, c, f) cells grown at 37°C. ICM, intracytoplasmic membranes; OM, outer membrane; I, intina; E, exina. Bars represent (a–c) 10 μm and (d–f) 0.5 μm.

estingly, they lacked granules of polyhydroxybutyrate, glycogen, and polyphosphate, typical of methanotrophs. Strain O-12 formed *Azotobacter*-type cysts, whose peculiarity was the presence of a thickened multilayered cell wall (composed of exina and intina) and a developed ICM system (Fig. 1e). No resting cells were observed in strain H-11. Cell morphology depended on the growth temperature. Cells of strain O-12 grown at 37°C increased in size and acquired the shape of spindles, balls, or gigantic rods (Figs. 1b, 1c, 1f). The cyto-

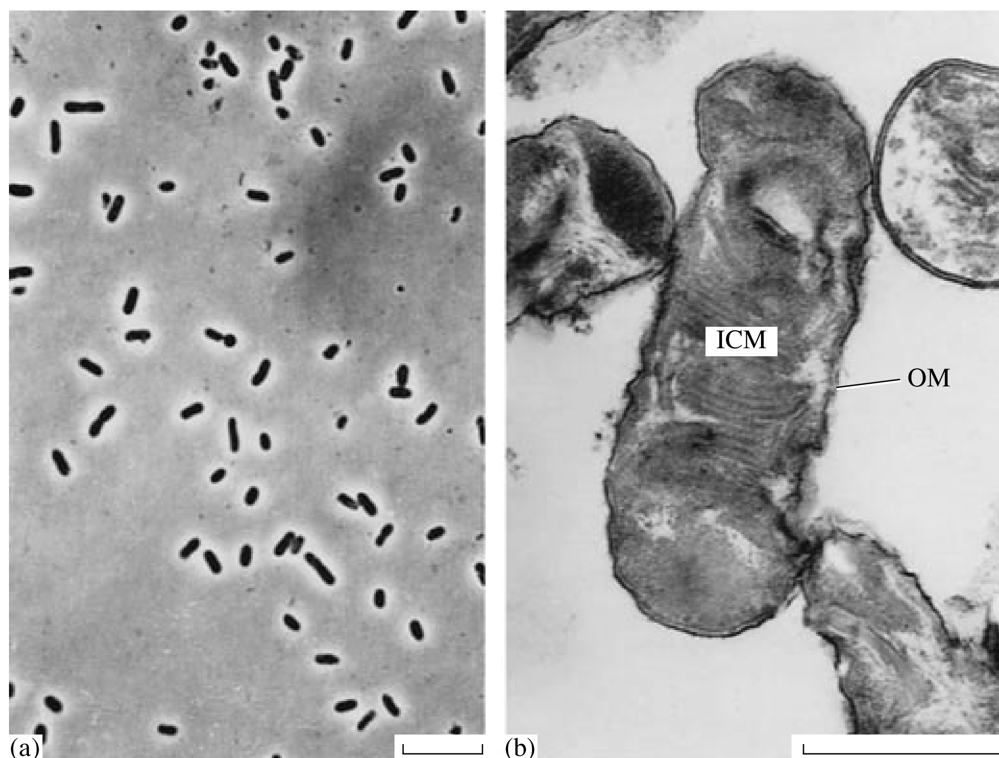

Fig. 2. Morphology and ultrastructure of strain H-11 cells: (a) phase-contrast micrograph; (b) electron micrograph of thin sections. ICM, intracytoplasmic membranes; OM, outer membrane. Bars represent (a) 10 μm and (b) 0.5 μm.

plasm of such cells was virtually completely filled with ICM (Fig. 1f).

The above morphological changes are probably related to the methane solubility in water. A decrease in the concentration of dissolved methane with increasing temperature or an increase in the bacterial population density causes the bacterial cells to increase their surface/volume ratio, i.e., to decrease their size or to acquire the shape of rods; most probably, this determines the polymorphism of thermophilic methanotrophs. However, polymorphism is not characteristic of the thermotolerant methanotrophs of the genus *Methylococcus.* The latter bacteria have additional glycoprotein structures on the outer surface of their cell walls, which, apparently, provide for higher resistance to stress factors, including fluctuations of temperature and of the concentration of solutes [17].

Phospholipid and fatty acid composition. The phospholipid pools of both cultures included phosphatidylglycerol, phosphatidylethanolamine, cardiolipin, and phosphatidylserine (Table 3). A decrease in the cultivation temperature resulted in an increase in the relative content of phosphatidylethanolamine at the expense of cardiolipin.

The fatty acids compositions of both strains grown at 55°C were dominated by hexadecanoic (63–65%) and hexadecenoic (11–13%) fatty acids. In addition, we found rather high contents of the cyclic C_{17cyc} and the $C_{9\text{-OMe-}16}$ methylated fatty acids, rarely occurring in eubacteria (Table 4). However, cells grown at 37°C contained virtually equal amounts of saturated and monounsaturated C_{16} fatty acids; the $C_{9\text{-OMe-}16}$ fatty acids were not detectable. Among the methanotrophs thus far investigated in this respect, a high content of saturated fatty acids is characteristic only of the thermotolerant methanotroph *Methylococcus capsulatus* Bath [18]. Saturated fatty acids and their methylated and cyclic derivatives apparently contribute to membrane stabilization by decreasing membrane fluidity at high temperatures. The major ubiquinone was 18-methylene-ubiquinone-8, earlier found in a number of thermophilic and thermotolerant methanotrophs [19].

Genotypic properties. The G+C content of the DNAs of both strains was 58.5 mol %. Comparative analysis of the nucleotide sequences of the 16S rRNA genes of the isolates revealed closest relatedness with *Methylocaldum* species: *M. szegediense* (99% similarity), *M. gracile* (96%), and *M. tepidum* (96%). The similarity of the nucleotide sequences of the MMO-encoding *pmoA* genes of these organisms (99, 96, and 93%, respectively) confirms the genetic relatedness of strains H-11 and O-12 with earlier described thermophilic methanotrophs.

Although the nucleotide sequences of the 16S rRNA genes and *pmoA* genes of our thermophilic methanotrophic isolates exhibit high levels of similarity with *Methylocaldum* representatives, DNA–DNA hybridiza-

Table 3. Phospholipid profile of the methanotrophic isolates

Phospholipid	% of total phospholipids			
	Strain O-12		Strain H-11	
	55°C	37°C	55°C	37°C
Phosphatidylserine	0.2	0.2	0.6	0.2
X_1	0.6	0.3	1.1	0.2
Phosphatidic acid	0.7	0.4	1.5	0.5
X_2	1.6	0.3	0.6	0.2
Phosphatidyldimethylethanolamine	6.9	3.5	6.9	5.4
Phosphatidylethanolamine	58.0	71.2	63.2	73.8
Phosphatidylglycerol	19.5	16.8	14.0	14.3
Cardiolipin	15.6	8.5	15.9	6.5

Note: X_1 and X_2, unidentified phospholipids.

Table 4. Fatty acid composition of the methanotrophic isolates grown at different temperatures

Fatty acid	% of total fatty acids			
	Strain O-12		Strain H-11	
	55°C	37°C	55°C	37°C
12:0	0.00	0.11	0.10	0
14:0	1.97	3.26	2.40	3.12
14:1ω5	0	0.20	0	0.25
15:1ω6	0	0.22	0	0.32
15:0	3.51	2.54	2.50	2.49
16:1	11.90	46.77	13.27	43.4
16:1ω5	0	0.22	0	0.24
16:0	63.67	43.22	64.99	43.57
16:0 3OH	0	0	0	0.37
17:1	0.34	0.15	0.44	0
17:1ω8	0.26	0	0.24	0
17:1ω6	0.43	0	0.44	0
17cyc	8.99	3.35	6.07	5.78
17:0	0.68	0.25	0.44	0
3h16:0	0.64	0	0.98	0
9-OMe-16:0	4.62	0	4.85	0
18:1	0.17	0	0.29	0.24
18:0	0.26	0	0.20	0.23
9-OMe-17:0	0.60	0	0.59	0
11-OMe-17:0	0.60	0	0.59	0
19:1cyc	1.37	0	1.62	0

Table 5. Activities of the enzymes of the primary and central metabolism in cell extracts of strains O-12 and H-11 grown at different temperatures (nmol/(min mg protein))

Enzyme	Cofactor	Strain O-12		Strain H-11	
		55°C	37°C	55°C	37°C
Methanol dehydrogenase	PMS	3	2	5	3
Formaldehyde dehydrogenase	PMS	5	0	10	0
	NAD^+	0	0	0	0
Formate dehydrogenase	PMS	45	211	2	104
	NAD^+	4	111	6	85
Hexulosephosphate synthase		174	176	81	97
Hydroxypyruvate reductase	NADPH	0	0	0	0
	NADH	22	2	119	25
Serine–glyoxylate aminotransferase		8	2	27	19
Ribulose-bisphosphate carboxylase		10	5	22	6
6-Phosphofructokinase (PP_i)		3	2	4	3
Fructose-1,6-bisphosphate aldolase		8	5	8	9
KDPG aldolase		22	1	6	1
Glucose-6-phosphate dehydrogenase	$NADP^+$	29	52	22	36
6-Phosphogluconate dehydrogenase	NAD	0	0	0	0
	$NADP^+$	11	16	8	10
Pyruvate dehydrogenase	NAD^+	8	10	7	10
α-Ketoglutarate dehydrogenase	NAD^+	0	0	0	0
Isocitrate dehydrogenase	NAD^+	20	22	26	28
	$NADP^+$	0	0	0	0
Glutamate dehydrogenase	NADH	0	0	0	0
	NADPH	17	13	19	20
Glutamine synthetase	Mg^{2+}, ATP	3	5	5	2
Glutamate synthase	NADPH	20	34	34	37

tion is required for the assignment of the new isolates to one of the currently recognized species. In addition, the physiological and biochemical characteristics of the validated species of *Methylocaldum* should be studied in greater detail.

Metabolic peculiarities. Cells of strains H-11 and O-12 grown on medium not supplemented with copper or on medium containing 1 μM $CuSO_4$ lacked the ability to oxidize naphthalene, which suggests the absence of sMMO. The activities of the enzymes of primary and central metabolism are presented in Table 5. It is worth noting that the activity of methanol dehydrogenase was either extremely low or could not be detected at all in the cell extracts of the isolates. In both strains, the ribulose monophosphate (RuMP) cycle of formaldehyde fixation is operative, as evidenced by the activities of its key enzyme, hexulosephosphate synthase. In addition, we revealed high activities of the serine pathway enzymes (hydroxypyruvate reductase and serine–glyoxylate aminotransferase) and a relatively high activity of the key enzyme of the Calvin cycle, ribulose-bisphosphate carboxylase (Table 5). The operation of the serine and ribulose-bisphosphate pathways along with the RuMP cycle, which is also characteristic of the thermotolerant methanotrophs of the genus *Methylocaldum*, is probably related to the thermophilic nature of the bacteria under consideration. The activity of hexulosephosphate synthase was virtually independent of the cultivation temperature. Conversely, the activities of hydroxypyruvate reductase and ribulose-bisphosphate carboxylase were considerably higher in cells grown at 55°C than in cells grown at 37°C.

Given that carbon assimilation via the serine pathway or the Calvin cycle requires more energy in the form of NADH and ATP than carbon assimilation via the RuMP cycle, it is reasonable to assume that the additional energy-consuming pathways may be realized by thermophilic methanotrophs as sinks for excessive energy; i.e., they may be involved in the regulation of the energy state of the cells under conditions of temperature fluctuations. Occurrence of the activities of 2-keto-3-deoxy-6-phosphogluconate aldolase and fruc-

tose-1,6-bisphosphate aldolase suggests that the methylotrophic isolates, like other type I and type "X" methanotrophs studied in this respect, realize two pathways of carbohydrate degradation: the Embden–Meyerhof–Parnas pathway and the Entner–Doudoroff pathway. The phosphorylation of fructose-6-phosphate in glycolysis most probably involves inorganic pyrophosphate (PP_i) instead of ATP, although the activity of the corresponding enzyme was rather low. In addition, the bacteria under study displayed rather high activities of the pentose-phosphate pathway enzymes: glucose-6-phosphate and 6-phosphogluconate dehydrogenases (Table 5). Both strains possess an NAD^+-specific isocitrate dehydrogenase, and in both strains the TCA cycle is disconnected at the level of α-ketoglutarate. NH_4^+ assimilation occurs through reductive amination of α-ketoglutarate and via the glutamate cycle. These metabolic peculiarities are typical for type "X" methanotrophic bacteria [20].

Thus, based on a number of characteristics, our isolates H-11 and O-12 should be assigned to the type "X" methanotrophic bacteria, which exhibit flexible structural and functional adaptability to changes in environmental conditions, first of all to temperature fluctuations. Investigation of the mechanisms underlying the thermophilic nature of these bacteria would be of considerable interest.

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