JOURNAL OF BACTERIOLOGY, Feb. 1972, p. 707-713

Vol. 109, No. 2
Printed in U.S.A.

Methanobacterium thermoautotrophicus sp. n., an Anaerobic, Autotrophic, Extreme Thermophile

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Received for publication 15 October 1971

The isolation of a new methanogenic bacterium, *Methanobacterium thermoautotrophicus* sp. n., is described. Successful isolation required a medium containing inorganic salts, an atmosphere consisting of an 80:20 mixture of hydrogen-carbon dioxide, and incubation temperatures of 65 to 70 C. Isolates of *M. thermoautotrophicus* were gram-positive, nonmotile, irregularly curved rods which frequently formed long filaments. The organism was found to be an autotroph and a strict anaerobe, and to have a *p*H optimum of 7.2 to 7.6. The optimal temperature for growth was 65 to 70 C, the maximum being 75 C and the minimum about 40 C. The generation time at the optimum was about 5 hr. The deoxyribonucleic acid of *M. thermoautotrophicus* had a guanine plus cytosine (GC) content of 52 moles per cent, whereas *Methanobacterium* sp. strain M.O.H. had a GC content of 38%. When heated, intact ribosomes of *Methanobacterium* sp. strain M.O.H. were stable up to 55 C and had a T_m of 73 C. In contrast, ribosomes of *M. thermoautotrophicus* were stable up to 75 C and had a T_m of 82 C. Upon complete thermal denaturation, ribosomes of strain M.O.H. underwent a 59% hyperchromic shift, whereas those of the thermophile showed only a 20% increase in hyperchromicity. Methane formation in cell-free extracts of *M. thermoautotrophicus* was temperature-dependent and required hydrogen and carbon dioxide; methyl cobalamin served as a methyl donor, and addition of coenzyme M stimulated methanogenesis.

Although thermophilic organisms have been well characterized for several decades, investigations concerning extremely thermophilic bacteria in natural ecological niches have only recently been initiated (1, 2, 6, 16). These studies have been limited to aerobic gram-negative species, primarily *Thermus aquaticus*. The existence of thermophilic methane bacteria has been reported (8, 10; G. G. T. P. Schnellen, Dissertation, Technological Univ., Delft, The Netherlands, 1947) but only in mixed cultures. The present communication deals with the isolation and characterization of an extremely thermophilic, methanogenic bacterium isolated from sewage sludge. This nonmotile, strict anaerobe is gram-positive, displays autotrophic nutritional requirements, and morphologically resembles the hydrogen-utilizing, methane-producing mesophile *Methanobacterium* sp. strain M.O.H. isolated from cultures of *Methanobacillus omelianskii*. This newly described organism is named *Methanobacterium thermoautotrophicus* sp. n.

MATERIALS AND METHODS

Inocula. Fresh, fermenting sludge obtained from the sewage treatment plant of the city of Urbana, Ill., was employed for enrichment cultures. Cultures of *Methanobacterium* sp. strain M.O.H. were kindly supplied by M. P. Bryant.

Growth media. Growth media were prepared and sterilized under a strictly anaerobic carbon dioxide or hydrogen and carbon dioxide (80:20) atmosphere by a modification of the Hungate technique (7) as described by Bryant and Robinson (4). Maintenance medium was prepared by mixing 83.5 parts of distilled water with the following components as described previously (4): 5 parts of mineral solution 2 [which contained the following in grams/liter: KH_2PO_4, $(NH_4)_2SO_4$, 6.0; NaCl, 12.0; $MgSO_4 \cdot 7H_2O$, 2.4; $CaCl_2 \cdot 2H_2O$, 1.6], 2.5 parts of mineral solution 1 (6.0 g of K_2HPO_4/liter), 5 parts of 8% Na_2CO_3, and 2 parts of cysteine-sulfide reducing agent. In addition 1 part each of vitamin solution and trace mineral solution (15) were added. The final *p*H of the sterile medium was 7.2. Unless stated otherwise, maintenance medium was employed for growth of *M. thermoautotrophicus*.

Standard medium was prepared by mixing 83.5

parts of distilled water with the following components: 5 parts of mineral solution 2, 2.5 parts of mineral solution 1, 5 parts of 8% Na_2CO_3, and 2 parts of 7.5% $Na_2S \cdot 9H_2O$.

The growth medium described by Bryant et al. (3) was employed for cultivation of *Methanobacterium* sp. strain M.O.H.

Culture techniques. The technique described by Hungate (7) as modified by Bryant and Robinson (4) was used in the isolation, maintenance, and subculture of organisms. Test tubes were not shaken and were incubated in an upright position. This was found to be a convenient and sensitive method which enabled us to determine methane without collecting the gas over water or taking samples from tubes with strong negative pressure due to complete hydrogen oxidation. The apparatus and procedure employed for mass culturing of bacteria were those described by Bryant et al. (3), with high-temperature incubation. Growth was measured turbidimetrically in matched test tubes (18 by 150 mm) at 660 nm with a Bausch & Lomb Spectronic 20 colorimeter.

Microscopy. A Carl Zeiss Universal phase microscope was employed for all microscopic observations. Wet mounts of cultures were made on glass slides or slides coated with 2% washed agar.

DNA base composition. Bacteria were disrupted by passage through a French pressure cell at 1,800 psi. Deoxyribonucleic acid (DNA) was isolated and purified from the cell homogenate by the method of Marmur (12). Base compositions were calculated according to the method of Schildkraut et al. (14) from the buoyant density of DNA in CsCl as determined in a Beckman model E ultracentrifuge. *Micrococcus lysodeikticus* DNA (kindly provided by C. L. Hershberger) served as a standard.

Ribosome thermal stability. Ribosomes were isolated and purified according to the method of Zeikus et al. (17). Thermal denaturation of intact ribosomes, suspended in 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 7.8) which contained 0.01 M $MgCl_2$ and 0.06 M KCl, was followed in a Gilford (model 2000) spectrophotometer equipped with a heated cell compartment and a temperature readout assembly. All samples had initial absorbances at 260 nm of 0.40 to 0.50.

Gas chromatography. Methane was detected with a Packard gas chromatograph equipped with a silica gel column connected to a hydrogen flame detector. Methane was quantitated as described by Bryant et al. (3) and is reported as total micromoles of methane produced.

Preparation of cell-free extracts. Cells grown at 65 to 70 C were harvested at the mid-logarithmic phase of growth and placed under an atmosphere of hydrogen. Cell extracts were prepared by subjecting 20 g (wet weight) of cells suspended in 20 ml of hydrogen-saturated 0.01 M TES (*N*-Tris-hydroxymethyl, methyl-2-aminoethane sulfonic acid) buffer, *p*H 7.1, to passage through a French press at 15,000 psi. The cell homogenate was centrifuged at 30,000 $\times$ *g* for 25 min at 2 C. The cell-free extract (supernatant fraction) was decanted and placed under a hydrogen atmosphere. The protein content of the extracts was determined by the method of Lowry et al. (9).

Chemicals. Methyl cobalamin was synthesized as described by Wolin et al. (15). Coenzyme M was purified from *Methanobacterium* sp. strain M.O.H. according to the method of McBride and Wolfe (11).

RESULTS

Enrichment, isolation, and cultivation. To enrich for thermophilic methane bacteria, a sample of sewage sludge was added to 10 ml of reduced maintenance medium which contained 5.0% rumen fluid, 0.1% Trypticase, 0.1% yeast extract, and 1.5% sodium formate. The test tube was gassed with an 80:20 mixture of hydrogen and carbon dioxide. Each tube was tightly sealed with a solid black rubber stopper and allowed to incubate without shaking at 65 to 70 C. Within 24 hr, the tubes had developed a negative pressure and copious amounts of methane were present. Microscopic examination of the medium revealed a dense, heterogeneous population of bacteria composed of cocci, rods of varying size and curvature, and numerous spores.

Pure cultures were obtained by repeated transfer and cultivation in maintenance medium followed by agar dilution with the use of maintenance medium solidified with 3% agar. Deep colonies in roll tubes were tannish-white, roughly round, diffuse, and somewhat filamentous. Isolated colonies were picked and used to inoculate liquid cultures.

Stock cultures of *M. thermoautotrophicus* were prepared by inoculating test tubes containing maintenance medium and a hydrogen-carbon dioxide (80:20) atmosphere followed by incubation for 24 hr at 65 to 70 C. The stocks can be stored at 25 or 4 C and remain viable for weeks. By removal of the vitamin solution, trace mineral solution, and cysteine from the reducing agent, cultures having an optical density above 0.6 can be obtained in 1 week on a totally inorganic salts medium (standard medium). Tubes were flushed daily with an 80:20 mixture of hydrogen and carbon dioxide for 1 min at a flow rate of 300 cc per min.

Morphology. Cells of *M. thermoautotrophicus* grown at 65 to 70 C (Fig. 1) proliferated as long, irregularly curved rods which displayed a great deal of flexibility. This organism was nonmotile and gram-positive. Morphologically, it resembled *Methanobacterium* sp. strain M.O.H. with the exception of possessing longer filaments.

DNA base composition. The base composition of *Methanobacterium* sp. strain M.O.H. was 38% GC (moles per cent guanine plus cy-

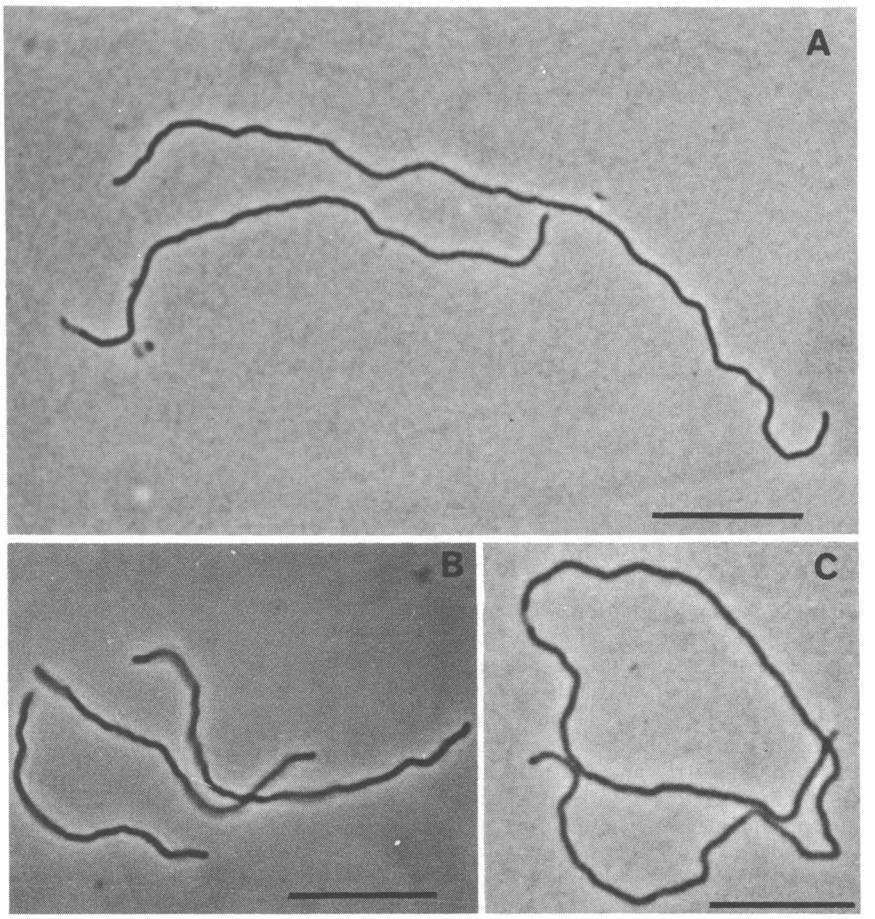

FIG. 1. *Phase-contrast photomicrographs of Methanobacterium thermoautotrophicus grown at 65 to 70 C. Bar represents 10 μm.*

tosine), and that of *M. thermoautotrophicus* was 52% GC.

Ribosome thermal stability. The stability of intact ribosomes from *Methanobacterium* sp. strain M.O.H. and from the thermophilic organism was compared by following the increase in absorbance at 260 nm upon raising the temperature. It can be seen in Fig. 2 that the ribosomes of strain M.O.H. were stable up to 55 C and displayed a T_m (temperature at which 50% of the hyperchromic effect is observed) of 73 C. On the other hand, ribosomes of the thermophile did not show any rise in absorbance until 75 C and demonstrated a T_m of 82 C. Upon complete denaturation (above 90 C), ribosomes of *Methanobacterium* sp. strain M.O.H. underwent a 59% hyperchromic shift, whereas those of *M. thermoautotrophicus* only underwent a 20% increase in hyperchromicity.

Physiological and nutritional characteristics. The relationship between growth (increase in optical density), methane production, and temperature is shown in Fig. 3. In these experiments, test tubes containing 10 ml of maintenance medium were inoculated with 0.5 ml of a stock culture and were analyzed for

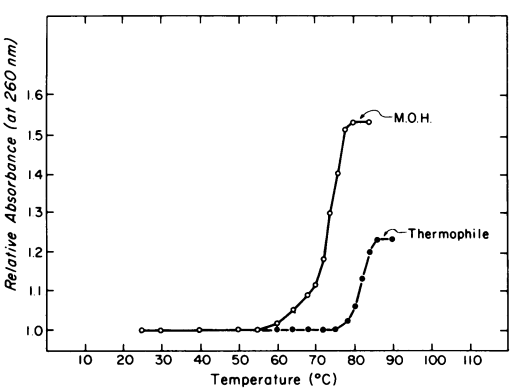

FIG. 2. *Ribosome thermal denaturation profiles.*

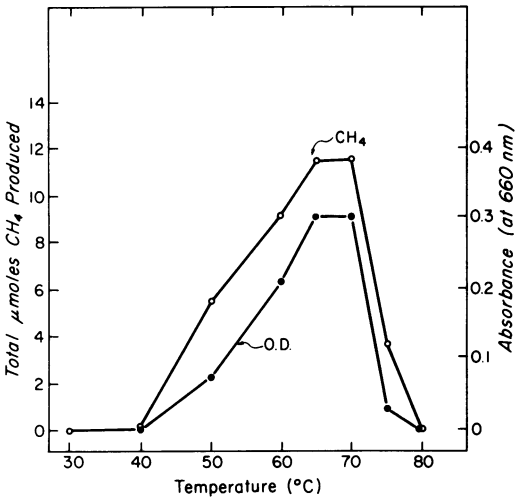

FIG. 3. *Optimal temperature for growth and CH_4 production.*

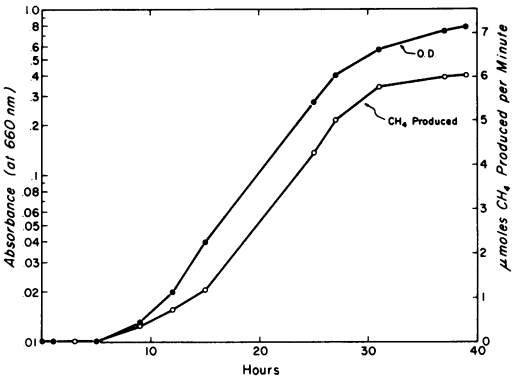

FIG. 4. *Growth and methane formation in a 12-liter fermentor at 65 C.*

TABLE 1. *Effect of various substrates on methane formation when added to standard medium*

Substrate added[a]	Atmosphere	Methane produced[b]
None	H_2-CO_2 (80:20)	7.6
Ethanol	CO_2	0.0
Methanol	CO_2	0.0
Sodium formate	CO_2	0.0
Sodium acetate	CO_2	0.0
Sodium pyruvate	CO_2	0.0
Sodium acetate	H_2-CO_2 (80:20)	7.4
Sodium pyruvate	H_2-CO_2 (80:20)	6.7

[a] Final concentration of each added substrate was 1.5%.

[b] Total methane produced in 24 hr (micromoles). Tubes closed, not flushed.

methane after 24 hr of incubation. Methane was quantitated by withdrawing samples from the gas space (14.5 ml) of the tubes and is reported as total micromoles of methane produced per tube. Optical density was measured after 72 hr, during which time the atmosphere was flushed twice daily with an 80:20 mixture of hydrogen and carbon dioxide. It can be seen that absorbance and methane production paralleled each other. No growth or methane was produced below 40 C; likewise, nothing occurred above 75 C. The optimal temperature for both growth and methane production for this organism is between 65 and 70 C.

Figure 4 demonstrates growth in a 12-liter fermentor at 65 C. In contrast to the low absorbances obtained in test tube cultures, high optical densities could be obtained in a fermentor with rapid stirring and constant gas flow (440 cc/min) of an 80:20 mixture of H_2 and CO_2. Again, as was the case in the previous experiment, methane production and growth paralleled each other. The generation time at 65 C was about 5 hr. Furthermore, by the addition of 200 ml of cysteine-sulfide reducing agent every 8 hr, higher optical densities (above 1.0) and shorter doubling times (about 3 hr) were obtained. At incubation temperatures above 60 C, coupled with constant gas flow and rapid agitation, sulfides are readily removed from the culture media (M. P. Bryant, *personal communication*).

In the experiments presented in Table 1, test tube cultures were inoculated at 65 C with 0.5 ml of a stock culture grown on standard media, and the methane formed in the closed, unflushed tubes was analyzed after 24 hr of incubation. When substrates which are utilized by other methane bacteria, such as ethanol, methanol, formate, pyruvate, or acetate, were substituted for hydrogen, methane production and growth ceased. If acetate or pyruvate was added to standard media in the presence of hydrogen and carbon dioxide, no stimulation of methane production or growth occurred. In sum, of all the substrates that we have tested, this organism can utilize only hydrogen for reducing power and only carbon dioxide as carbon source. In addition, cysteine was the only organic compound found to be stimulatory for growth, although the organism proliferated in its absence. Results of additional experiments which employed large-scale growth provided additional evidence of chemolithotrophy. For example, when a 14-liter fermentor containing 12 liters of standard medium (no organic compounds) was inoculated with a 10-ml inoculum (which had an absorbance of 0.6 and was obtained from a fermentor culture also grown on standard media) and was allowed to proliferate at 65 C, 23 g (wet

weight) of cells were harvested after 60 hr. This is the first report of sustained mass culture of a methane bacterium in a completely inorganic medium where CO_2 is the only carbon source. The ratio of 1 ml of inoculum to 1,200 ml of medium was repeated in subculture and serves to emphasize the chemolithotrophic nature of this organism as well as its ability to grow in mass culture from small inocula. However, when a 14-liter fermentor containing 12 liters of standard media supplemented with cysteine (1.5 g/liter) was inoculated with a 10-ml inoculum (which had an absorbance of 0.55 and was obtained from a fermentor culture grown in the same manner) and incubated at 65 C, 32 g (wet weight) of cells were harvested after 60 hr. It is known that cysteine lowers the oxidation-reduction potential of the medium significantly more than when sodium sulfide alone is employed as the reducing agent (M. P. Bryant, *personal communication*).

The *p*H optimum for growth of *M. thermoautotrophicus* was about 7.2 to 7.6. No growth occurred when the initial *p*H was below 6.0 or above 8.8.

Methane formation in cell-free extracts. Cell-free extracts of *M. thermoautotrophicus* were examined for methanogenic activity. In general, cells could be washed in reduced buffer without loss of activity, and better cell disruption was obtained by passage through a French press than by ultrasonic treatment. Highest activity was demonstrated in 0.05 M TES buffer at neutral *p*H values; all activity was destroyed in Tris-hydrochloride buffer, and decreased levels were observed in potassium phosphate buffer.

Figure 5 illustrates the methane-producing ability of cell-free extracts at two different temperatures. The reaction was carried out in a modified Warburg flask according to the method of Wolin et al. (15). The data presented represent total micromoles of methane produced per flask. The 1.1-ml reaction mixtures contained 56 mg of protein (crude cell extract), 15 μmoles of adenosine triphosphate, 15 μmoles of $MgCl_2$, and 400 μmoles of TES buffer, *p*H 7.0. The atmosphere consisted of an 80:20 mixture of hydrogen and carbon dioxide except in the control where argon was employed. Methane formation in cell-free extracts is a temperature-dependent reaction. More methane was produced at 65 C than at 40 C, and no methane was produced when argon was substituted for hydrogen and carbon dioxide at either 40 or 65 C.

The effect of various substrates on methane production in cell-free extracts incubated at 65 C is demonstrated in Fig. 6. The reaction mixtures contained the same components and respective concentrations as in the previous experiment (Fig. 5) except as noted. When the reaction vessel atmosphere contained hydrogen alone, no methane was formed. This was also the case if the atmosphere consisted of only carbon dioxide. When 22 μmoles of methyl cobalamin was added with a hydrogen atmosphere, methyl cobalamin served as a methyl donor, and methane was formed. However, not as much methane was produced as in a hydrogen-carbon dioxide atmosphere. If the reaction mixture contained, in addition to hydrogen and methyl cobalamin, 0.5 μmole of coenzyme M (11), then a fivefold increase in methane production occurred when compared

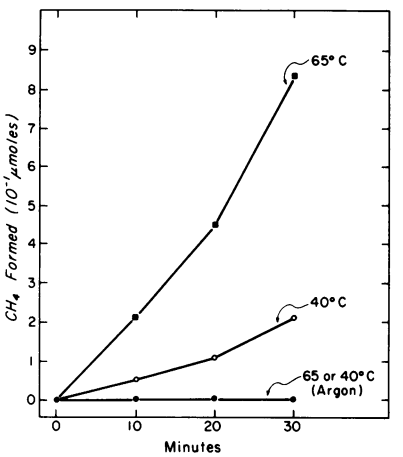

FIG. 5. *Methane production in cell-free extracts.*

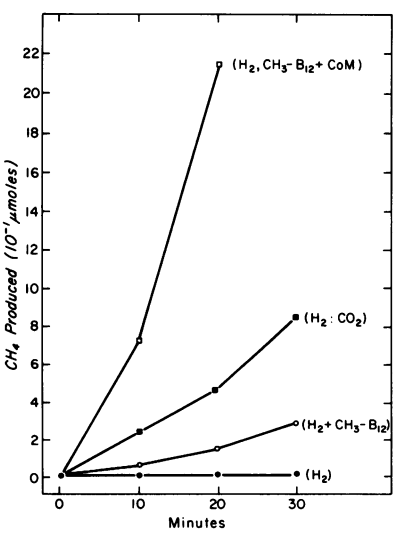

FIG. 6. *Effect of various substrates on methane formation in cell-free extracts.*

with the value obtained for hydrogen and carbon dioxide alone.

DISCUSSION

From these data, it is clear that *M. thermoautotrophicus* is a unique organism. The ability of this bacterium to proliferate at temperatures above 70 C clearly distinguishes it as an extreme thermophile. The fact that extremely thermophilic anaerobes have not been reported in the past may reflect a lack of strictly anaerobic conditions coupled with enrichment temperatures below 65 C. These problems are overcome by use of the Hungate technique modified for high temperatures. By use of this procedure, it may be possible to provide additional evidence that microbial diversity is not necessarily limited by temperature extremes.

The present study demonstrates the extraordinary biosynthetic capabilities of *M. thermoautotrophicus*, in that it can be grown in a medium containing ammonia as nitrogen source, sulfide as sulfur source, hydrogen-carbon dioxide as energy and carbon source, and inorganic salts. The organism's ability to utilize only hydrogen for reducing power and carbon dioxide as sole carbon source clearly establishes this bacterium as a hydrogen-oxidizing autotroph. In addition, doubling times under these conditions were less than 5 hr. These short generation times, relative to methane bacteria and not *Escherichia coli*, distinguish this organism as one of the fastest growing methane bacteria known.

The ribosomes of this thermophile may be unique in respect to the amount of denaturation, i.e., hyperchromic increase, that occurs upon heating. *Methanobacterium* sp. strain M.O.H. undergoes a 59% hyperchromic increase when melted; values reported for both mesophilic and thermophilic ribosomes demonstrate as high or slightly higher hyperchromic increases (5). However, the ribosomes of *M. thermoautotrophicus* only undergo a 20% increase in hyperchromicity. This indicates a difference in ribosome structure, possibly in the ribosomal proteins, ribonucleic acid (RNA), or bonding between ribosomal RNA and protein. Whether this is indeed a distinguishing characteristic of this organism or may also hold true for other extremely thermophilic anaerobes will become apparent only with further study.

The heat stabilities of ribosomes may play an important role in governing the maximal growth temperature of bacteria. A previous study by Pace and Campbell (13) demonstrated a correlation between the T_m of bacterial ribosomes and the maximal growth temperature from which they were derived. Our data are consistent with those of Pace and Campbell and may provide additional insight into this concept. The maximal temperature for growth of the thermophilic methane bacterium is 75 C; the temperature at which its ribosomes begin to denature is also 75 C. This correlation between the temperature at which the ribosomes begin to denature and the maximal growth temperature holds true for all extreme thermophiles examined. For example, the critical temperature is 79 C for *T. aquaticus* (17). This "critical temperature" concept may serve as an index of thermophily when used to compare extreme thermophiles.

In the past, the difficulty of procuring cell mass and active cell extracts has been an obstacle in the elucidation of the biochemical mechanism of methane formation. Data obtained from experiments with cell-free extracts of *M. thermoautotrophicus* demonstrate that methane formation is a temperature-dependent reaction requiring hydrogen and carbon dioxide, that methyl cobalamin can serve as a methyl donor, and that coenzyme M appears to be involved in methane formation in this new methane bacterium. *M. thermoautotrophicus* may prove to be the "organism of choice" for mass culturing of methane bacteria, as the appearance of contaminants in large fermentors is not a problem when inorganic media and high temperatures are used.

Although unique, *M. thermoautotrophicus* bears a resemblance to other methane bacteria. This organism is morphologically similar to *Methanobacterium* sp. strain M.O.H. and *M. formicicum* except for possessing longer filaments. Likewise, *Methanobacterium* sp. strain M.O.H. also displays simple nutritional requirements resembling those of an autotroph, but growth under these conditions is extremely poor, and vitamins, acetate, and cysteine are highly stimulatory (M. P. Bryant et al., Anaerobic Biological Treatment, Advan. Chem. Ser., *in press*). However, the GC contents of *M. thermoautotrophicus* (52%) and of *Methanobacterium* sp. strain M.O.H. (37.8%) differ significantly. The GC contents of other methane bacteria have not been reported.

On the basis of cellular morphology and methanogenesis, the organism is placed in the genus *Methanobacterium*, Kluyver and van Niel (1936). The name *Methanobacterium thermoautotrophicus* sp. n. is proposed, the species name being derived from the organism's ability to proliferate autotrophically at

high temperatures.

Methanobacterium thermoautotrophicus sp. n.

Morphology. Rods and filaments, 0.35 to 0.6 μm in diameter. Rods 3 to 7 μm in length, filaments of variable length from 10 μm to greater than 120 μm. Rods occurring singly or in chains. Gram-positive. Flagella and spores absent.

DNA base composition. 52% GC.

Colony characteristics: Tannish-white, roughly round, diffuse, and somewhat filamentous.

Temperature relationships. Optimum, 65 to 70 C; maximum, 75 C; minimum, 40 C.

Nutrition. Autotrophic, requires no growth factors or organic compounds. Carbon dioxide serves as sole carbon source.

Relation to oxygen. Very strict anaerobe.

Relation to pH. Optimum, 7.2 to 7.6. No growth below *p*H 6.0 or above 8.8.

Source. Sewage sludge.

ACKNOWLEDGMENTS

We thank Craig D. Taylor for preparing samples of coenzyme M, and L. L. Campbell for first directing our attention to the thermophilic methane bacteria. The assistance of C. L. Hershberger and C. C. Green in determining the buoyant density of DNA is gratefully acknowledged.

This work was supported by Public Health Service grant GM-49202 from the National Institute of General Medical Sciences.

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