APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1984, p. 807–812
0099-2240/84/100807-06$02.00/0

Vol. 48, No. 4

Variants of the Obligate Methanotroph Isolate 761M Capable of Growth on Glucose in the Absence of Methane

SHU-JIE ZHAO[1] AND R. S. HANSON[2]*

Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu, People's Republic of China,[1] and Gray Freshwater Biological Institute, University of Minnesota, Navaree, Minnesota 55392[2]

Received 25 May 1984/Accepted 24 July 1984

Isolate 761M is an unusual type I methanotroph that possesses a complete tricarboxylic acid cycle. Variants of this methanotroph that were capable of growth with methanol (isolate 761AR) or glucose (isolate 761H) have been isolated. Cultures of isolate 761H grown with glucose and casein hydrolysate as the sole carbon and energy sources retained the ability to grow on methane, contained methane monooxygenase and 3-hexulose phosphate synthase, and possessed intracytoplasmic membranes similar to those found in thin sections of isolate 761M grown on methane. Methane monooxygenase was also present in cultures of isolate 761AR grown on methanol and casein hydrolysate.

Isolate 761M is a type I methanotroph because it assimilates carbon via the hexulose monophosphate pathway and contains intracytoplasmic membranes characteristic of type I methanotrophs (2, 6). It was isolated from a rice paddy in South China by enrichment with methane as the sole source of carbon and energy (18). This bacterium is unusual because it possesses a complete tricarboxylic acid cycle, whereas other type I methanotrophs lack alpha-ketoglutarate dehydrogenase (17; S.-J. Zhao and R. S. Hanson, submitted for publication). It is also limited in its ability to synthesize cellular materials from formaldehyde and can utilize non-C_1 carbon sources for the synthesis of cellular materials (17). Supplementary carbon sources increase the rate of growth and cell yields. Methane was required as an energy source for growth (17). Therefore, it is an obligate methanotroph.

The lack of alpha-ketoglutarate dehydrogenase was considered by Anthony (2) to be the only basis for obligate methylotrophy that was generally applicable for the type I methylotrophs. Mutants of the facultative type II methylotroph *Pseudomonas* sp. AM1 lacking this enzyme are obligate methylotrophs (16). Pyruvate dehydrogenase activities were found to be low or undetectable in some obligate type II methylotrophs (14). This enzyme was present in extracts of isolate 761M (Zhao and Hanson, submitted). There is no obvious reason for the obligate requirement for C_1 compounds as energy sources by this bacterium.

A facultative methanotroph, *Methylobacterium organophilum* XX, was successfully cultivated on methane when originally isolated (12). In the past few years, we and others have had difficulty obtaining growth of *M. organophilum* XX on methane (1). Therefore, the occurrence of facultative methanotrophs has been questioned (8). In this paper, we describe two variants of isolate 761M. One, isolate 761AR, is capable of growth with methanol as an energy source, and another, isolate 761H, can utilize non-C_1 carbon and energy sources. Some properties of isolate 761AR have been described previously (17). The growth yields and growth rates of this bacterium, like those of isolate 761M, were increased by the addition of casein hydrolysate and glucose, singly or together. However, growth of isolate 761AR did not occur in the absence of methane or methanol. This strain was found to be more resistant to methanol and ethanol than isolate 761M was (17).

Washed cells of isolate 761AR grown on methane or methanol with casein hydrolysate oxidize methane and higher alkanes and form epoxides when incubated with alkenes (17). The methane-oxidizing activity of cells grown on methanol for several transfers is approximately half that of methane-grown cells. However, it is apparent that methane monooxygenase is synthesized in the absence of methane.

MATERIALS AND METHODS

Bacteria and culture conditions. Isolate 761M has been described elsewhere (Zhao and Hanson, submitted). Isolate 761AR, an alcohol-resistant isolate capable of growth on 0.5% methanol as an energy source, and isolate 761H, a variant that is capable of growth with glucose as an energy source, are described here. The mineral salts medium containing potassium nitrate (1 g · liter^{-1}) as a nitrogen source (17) was suppplemented with 0.04 M piperazine-*N*,*N*-bis(2-ethanesulfonic acid) (PIPES buffer), pH 6.8, when glucose was used as an energy source in liquid media. PIPES buffer was not used in solid media. The concentration of the phosphate salts was also increased to 0.52 g of KH_2PO_4 and 1.28 g of $Na_2PO_4 \cdot 7H_2O$ · liter^{-1} when methanol and glucose were used as energy sources in liquid and solid media. Growth in the presence of these compounds, without additional buffers, caused the pH of the culture medium to decrease below 5.8, and growth was inhibited. Casein hydrolysate (2.0 g · liter^{-1}) was added to the medium as indicated. When glucose was added to the medium, the concentration was 8.0 g · liter^{-1} unless another concentration was indicated.

Solid media were prepared by adding 15 g of Difco purified agar (Difco Laboratories, Detroit, Mich.) · liter^{-1}. It was necessary to inoculate cultures with approximately 15 mg (dry weight) of isolate 761AR and isolate H · liter^{-1} to obtain reproducible growth with methanol or glucose as an energy source. When cultures were inoculated with 10% (vol/vol) of a preculture, the pH of the medium decreased, and the cells lysed.

Isolate 761AR was grown for 5 days at 30°C on slants of mineral salts containing casein hydrolysate and methanol (0.3%, vol/vol) and was kept at 4°C for storage. The cultures remained viable for 2 months.

* Corresponding author.

Isolate 761H was maintained on agar slants containing the mineral salts medium, casein hydrolysate, and glucose. After 7 to 10 days of growth, the slants were stored at 4°C. Cultures were transferred every 2 months. Cultures were also maintained in a liquid medium and transferred (50% [vol/vol] inoculum) at 4-day intervals. Cultures were routinely streaked onto nutrient agar to test for contamination.

Oxidation of substrates by resting cells. The cells were harvested from the medium indicated by centrifugation at 17,000 × *g* for 15 min during the exponential growth phase. The cells were washed with 0.05 M phosphate buffer containing 5×10^{-3} M $MgCl_2$, pH 7.0, and were suspended in the same buffer at approximately 8 mg (dry weight) · ml^{-1}. The rate of oxidation of substrates was measured in a Rank oxygen electrode (Rank Brothers, Boisham, United Kingdom) at 22°C as described by Patt et al. (12). Phosphate buffer (0.05 M, pH 7.0) was saturated with 1 atm (101.3 kPa) of each gaseous substrate tested. After the rate of endogeneous respiration was determined, 0.1 ml of substrate solution was added to 0.9 ml of cell suspension in the electrode chamber. The dry weight of cells was measured by previously described methods (17).

Enzymatic assays. Crude extracts were prepared as descried previously (17). 3-Hexulose-6-phosphate synthase was assayed by the procedure of Ferenci et al. (7) with the following modifications. The concentrations of ribulose-5-phosphate and formaldehyde used in the reaction mixtures were 5 and 0.55 mM, respectively. The incubation temperature was 35°C

DNA isolation, digestion of DNA with restriction endonucleases, and Southern hybridization. Isolate 761M was grown on the mineral salts-casein hydrolysate medium under an atmosphere of methane and air (1:3). The mineral salts-glucose-casein hydrolysate medium was used for growth of isolate 761H, and the mineral salts-casein hydrolysate medium supplemented with 0.3% (vol/vol) methanol was used for growth of isolate 761AR. Cells from 500 ml of culture were harvested by centrifugation at 17,000 × *g* and suspended in 100 ml of 0.5 N NaCl. After 30 min of incubation at 37°C, EDTA was added to give a final concentration of 0.01 M. The suspension was centrifuged at 12,000 × *g* for 15 min, and the pellet was suspended in 10 ml of TE buffer (0.04 M Tris, 0.15 M NaCl, 0.01 M EDTA, pH 8.0) for each gram of cells (wet weight). An equal volume of lysozyme solution (6 mg · ml^{-1} in TE buffer) was added with gentle mixing, and the suspension was incubated at 37°C for 30 min. After 15 min of cooling on ice, 10% (wt/vol) sodium dodecyl sulfate was added to give a final concentration of 1% (wt/vol). The suspension was incubated at 65°C for 30 min. After centrifugation at 8,000 × *g* for 10 min, the DNA solution was extracted twice with a phenol-chloroform-isopentyl alcohol mixture (100:96:4) and twice with ethyl ether to remove protein. The aqueous phase was saved. The phenol used for deproteinization was equilibrated twice with 1 M Tris, three times with 1 M Tris buffer (pH 8.0), and twice with 0.1 M Tris buffer. Sodium acetate (0.3 M) was added to the aqueous layers after solvent extraction, and DNA was precipitated by adding 0.55 volumes of isopropanol and chilling the solution on ice for 15 min. The DNA fibers were collected by winding on a glass rod, redissolved in TE buffer, and treated with pancreatic RNase to hydrolyze RNA (12). The DNA solution was extracted again with solvents as described above, precipitated with isopropanol, and dissolved in TE buffer. DNA (50 μg · ml^{-1}) was added to the buffers recommended for restriction endonuclease treatment (Bethesda Research Laboratories, Gaithersburg, Md., bulletin 047). The solution was heated to 65°C for 10 min and cooled to 37°C. Digestions with restriction endonucleases were carried out as recommended in the Bethesda Research Laboratories technical bulletins provided with the enzymes. Restriction fragments were separated by electrophesis for 20 h in 0.7% (wt/vol) agarose gels (12.5 by 18 cm) with a constant current of 20 mA (4).

Southern blots were prepared by the method of Southern (15). DNA was radioactively labeled with [α-^{32}P]dCTP (13) as recommended in Bethesda Research Laboratories bulletin 047. Hybridization reactions were carried out at 65°C in the presence of 50% (wt/vol) formamide as described by Southern (15).

Materials. The restriction enzymes used for nick translation and Ultra-PURE agarose used in this study were obtained from Bethesda Research Laboratories. [5′-(α-^{32}P]dCTP was purchased from Amersham Corp. (Arlington Heights, Ill.). The sources of other reagents are described elsewhere (Zhao and Hanson, submitted).

RESULTS

Isolate 761AR possesses alpha-ketoglutarate dehydrogenase and hexulose-6-phosphate synthase. Isolate 761AR possessed alpha-ketoglutarate dehydrogenase at approximately the same specific activity as the wild-type strain (3.4 and 3.0 U · mg of protein^{-1}, respectively). Units are expressed as nanomoles per milligram of protein. Crude extracts of cells grown on methanol also contained 3-hexulose-6-phosphate synthase when grown on methanol and casein hydrolysate (see Table 2).

Isolation of a variant of isolate 761M capable of growth on glucose and casein hydrolysate. When isolate 761M was spread on the minimal medium with casein hydrolysate and glucose (6 to 8 g · liter^{-1}), occasional colonies appeared in 10 to 20 days on plates incubated at 30°C in the absence of methane or methanol. Approximately 1 cell in 10^8 formed colonies. These colonies were picked and purified by repeatedly streaking them onto plates of the same medium. One isolate, isolate 761H, was chosen for further study. When inoculated into the liquid medium containing glucose, casein hydrolysate, and PIPES buffer (2 ml in 13-mm culture tubes) and incubated without shaking, growth occurred without methane or methanol. The generation time was approximately 24 h. The pH of the medium decreased unless PIPES buffer was added. It was found that large inocula (25% [vol/vol]) were required to obtain growth reproducibly after transfer of liquid cultures. Growth occurred in liquid cultures when methane or glucose was added as an energy source. Growth of isolate 761H occurred in liquid media containing casein hydrolysate and PIPES buffer under a methane-air (1:3) atmosphere. The observed increase in dry weight was 0.89 g · liter^{-1}. When glucose (5.41 g · liter^{-1}) was used as an energy source in the absence of methane, the observed increase in dry weight was 0.6 g · liter^{-1}. Under these conditions, 0.75 g of glucose · liter^{-1} was consumed. Growth was not observed in the absence of methane or glucose. The best growth was obtained when flasks were filled with approximately 10% of their volume of liquid medium and the cultures were incubated at 30°C without shaking. The shape and size of cells were the same as those of isolate 761M, and the cultures had the same light pink color as cultures of the parent strain grown on methane. Colonies and liquid cultures, like the parent strain, did not grow in liquid or solid medium containing nutrient broth, casein hydrolysate, or peptone in the absence of supplemental energy sources. Colonies grew to 0.5 to 1.0 mm in

diameter in 9 days when glucose was added to the minimal casein hydrolysate medium containing PIPES buffer and to 1 to 2 mm in diameter in 7 days on the minimal casein hydrolysate medium with methane as the energy source.

D-Fructose, D-galactose, D-ribose, D-xylose, sucrose, maltose, lactose, and cellobiose did not support growth of isolate 761H at concentrations of 8 g · liter^{-1} after 13 days of incubation when added to the minimal casein hydrolysate medium containing PIPES buffer. Growth of isolate 761H was totally inhibited by 2-deoxyglucose and alpha-methylglucoside when glucose was used as an energy source.

To determine whether cells grown on glucose and casein hydrolysate retained the ability to grow and form colonies on methane, a culture of isolate 761H that had been grown for more than 10 transfers in the minimal casein hydrolysate-glucose medium was transferred to the same medium. The culture was incubated without shaking. Samples were removed at intervals and diluted, and dilutions were plated onto the minimal agar medium (minimal salts with and without casein hydrolysate). The plates were incubated with a methane-air (1:3) atmosphere. Colonies were counted after 8 days of incubation at 30°C. The results of the experiment (Fig. 1) show that the number of cells capable of growing on methane as the sole carbon and energy source or on methane with casein hydrolysate increases during growth of this strain on glucose. All colonies that grew on minimal casein hydrolysate agar with methane also grew on a minimal glucose-casein hydrolysate agar without methane after transfer with sterile toothpicks. Single-colony transfers of isolate 761H that had grown on agar with glucose and casein hydrolysate also grew on agar with methane and casein hydrolysate. The ability of isolate 761H to grow on methane has been retained during growth on glucose and casein hydrolysate for over 6 months.

DNA-DNA homology studies. DNA was prepared from cells of isolate 761M grown on minimal casein hydrolysate with methane, isolate 761AR grown on minimal casein hydrolysate plus 0.3% methanol, and isolate 761H grown on minimal casein hydrolysate plus 0.6% glucose. Each DNA preparation was treated with the restriction endonucleases *Pst*I and *Bgl*II, and the DNA fragments were separated by electrophoresis in an agarose gel (0.7% [wt/vol] agar). The DNA restriction fragments produced from DNA preparations from all three organisms were very similar (Fig. 2). The DNA fragments in the agarose gels were transferred to sheets of nitrocellulose and were hybridized with radioactive DNA prepared by nick translation of DNA from isolate 761M. It is apparent that the radioactive probe hybridized with DNA from all three strains of isolate 761, and there were no significant differences in the hybridization between restriction fragments produced from the DNA of the different strains. The radioactive probe did not hybridize with any restriction fragments of DNA from organism SB1, *M. organophilum* XX, or *Methylococcus capsulatus* (Bath) (data not shown).

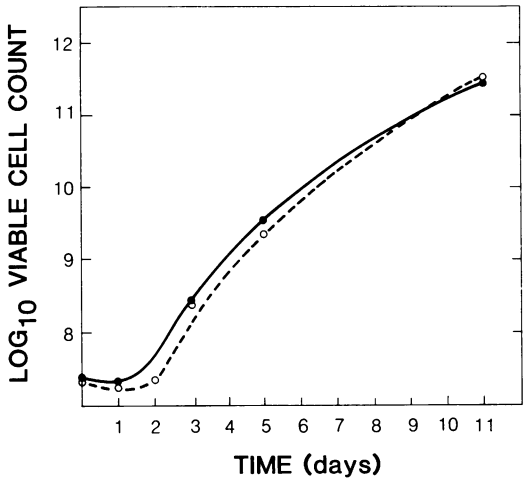

FIG. 1. Increase in the population of cells capable of utilizing methane as an energy source during growth of isolate 761H on glucose and casein hydrolysate. A culture of isolate 761H was inoculated into the liquid glucose-casein hydrolysate-PIPES medium. The culture was incubated at 30°C without shaking. Samples were removed, diluted, and spread onto mineral salts-PIPES agar (○) or mineral salts-PIPES agar with casein hydrolysate (●). All plates were incubated with a methane-air atmosphere (1:3). Colonies were counted after 2 weeks of incubation. Colonies were not detected when plates were incubated without methane.

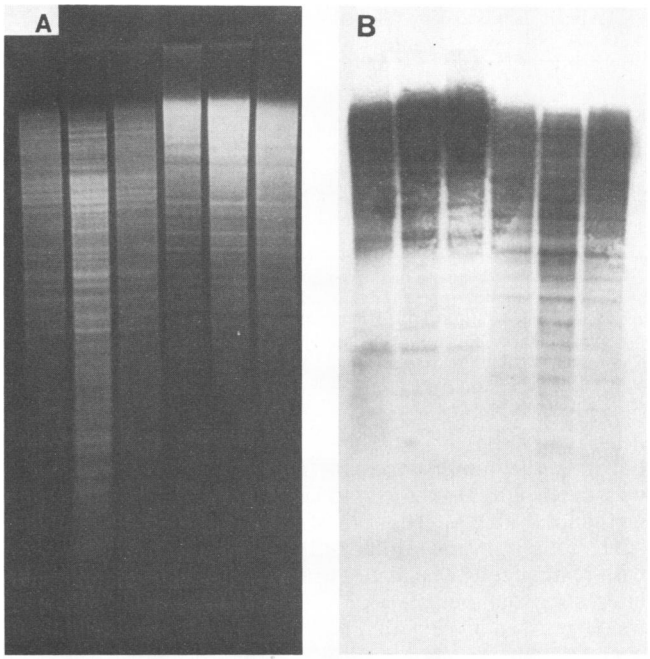

FIG. 2. Restriction enzyme fragments and homology between DNA from isolate 761M and variants 761AR and 761H. DNAs isolated from isolate 761M grown with methane and casein hydrolysate, isolate 761AR grown with methane and casein hydrolysate, and isolate 761H grown with glucose and casein hydrolysate were treated with restriction endonucleases *Pst*I and *Bgl*II. The fragments were separated by electrophoresis in 0.7% (wt/vol) agarose gels. (A) Restriction fragments of DNA from isolate 761M and its variants. Lanes show DNAs from (left to right) *Pst*I-digested 761M, 761AR, and 761H and *Bgl*II-digested 761M, 761AR, and 761H. (B) Radioautograph of filters prepared by Southern blotting fragments from agarose gel (A), after hybridization with radioactively labeled DNA from isolate 761M.

The results of these experiments further indicate that isolate 761H is a facultative methylotroph. The culture of isolate 761H used for the preparation of the DNA used in this experiment had been cultivated in the absence of methane and methanol for over 100 generations and was free of contaminants that grew on nutrient agar.

Oxidation of substrates by isolate 761H. The rate of oxidation of glucose by isolate 761H grown for over 100 genera-

TABLE 1. Oxidation of methane, methanol, and glucose by *isolates* 761M and 761H[a]

Substrate added	Oxygen consumed (nmol · min^{-1} · mg [dry wt]$^{-1}$)	
	Isolate 761M	Isolate 761H
Methane	23	15
Methanol	33	37
Glucose	1.1	1.8

[a] Isolate 761M was grown on the minimal casein hydrolysate medium with a methane-air (1:3) atmosphere for 72 h at 30°C with shaking. Isolate 761H was grown for 30 generations on the minimal casin hydrolysate-glucose medium (without methane) before inoculating the culture from which the cells were harvested. This culture was incubated for 100 h at 30°C without shaking. Substrates were added to the mineral salts-casein hydrolysate media described in the text.

tions on the minimal casein hydrolysate-glucose medium was much less than the rate of oxidation of methane and methanol (Table 1). The rate of glucose oxidation by isolate 761H cells was not significantly higher than that observed with resting cells of isolate 761M grown with methane. It is interesting that the ability of isolate 761H to oxidize methane was expressed by cells that were not exposed to methane for over 100 generations.

To obtain clear evidence that isolate 761H was a facultative methylotroph, a culture was transferred over 100 times (50% [vol/vol] inoculum) with glucose and casein hydrolysate as the carbon and energy sources. This preculture was used to inoculate the same medium (10% [vol/vol] inoculum). The rate of oxidation of methane and methanol was measured during growth of the culture (Fig. 3). It was reasoned that if methane and methanol oxidation were constitutive and the culture was not a mixed population, the rate of increase in their oxidation should approximately equal the rate of increase in cell mass. If the culture was a mixed population, the rate of oxidation of these substrates should remain constant or decrease. An obligate methylotroph would not grow in the absence of methane, methanol, or methylamine. Methane and methanol would not be produced under aerobic conditions, and isolate 761H would not grow with methylamine and casein hydrolysate.

Isolate 761H does not grow well when shaken until the cell density is above 3.0 g · liter^{-1}. When the cells reached this density, growth was not inhibited when they were placed on a shaker. Methane oxidizing activity continued to increase after the cultures were shaken (Fig. 3).

Hexulose phosphate synthase activity in crude extracts of isolate 761H. A culture of isolate 761H that had been grown in the minimal casein hydrolysate-glucose-PIPES medium for over 100 transfers at 3-day intervals was used to inoculate the same medium. Cells were harvested during exponential growth, and crude extracts were assayed for 3-hexulose-6-phosphate synthase. Extracts were also prepared from cells of isolate 761H grown with methane and casein hydrolysate rather than with glucose. The results (Table 2) show that cells grown for long periods of time with glucose in the absence of methane still contain 3-hexulose-6-phosphate synthase at a specific activity over half of that found in the wild-type strain grown on methane. The assay used did not detect enzymatic activity in extracts of *Escherichia coli* or organism SB1, a type II methanotroph.

Fine structure of isolates 761AR and 761H cells grown with methanol and glucose. After growth for 100 transfers in media with glucose and casein hydrolysate as the sole sources of carbon and energy, cells of isolate 761H contained intracytoplasmic membranes resembling those of isolate 761M grown on methane (17) and other type I methanotrophs (Fig. 4A). Similar intracytoplasmic membranes were observed in isolate 761AR grown with methanol and casein hydrolysate for over 40 generations (Fig. 4B).

DISCUSSION

An alcohol-resistant strain of isolate 761M, designated isolate 761AR, has been isolated. This strain, like the parent strain, requires peptone or casein hydrolysate for rapid growth. Glucose also stimulates growth of isolate 761AR in the presence of methanol. Radioactive glucose was assimilated into all macromolecular fractions by both strains, and exogenous amino acids were also assimilated into macromolecules (17). Isolate 761AR, unlike the parent strain, can utilize methanol as the sole energy source when it is provided at concentrations above 0.1% (vol/vol). Several methylotrophs can grow on methanol when it is provided at low concentrations (2). Higher concentrations are bactericidal to isolate 761M and some other methylotrophs (10). Isolate 761AR has developed resistance to alcohols (methanol and ethanol). Resistance to and growth on methanol cannot be explained by higher rates of methanol oxidation by the alcohol-resistant strain. We could not detect differences in the rates of oxidation of methanol by the two strains. It is possible that formaldehyde accumulates when the cultures of isolate 761M are provided with methanol and that isolate 761AR has increased levels of formaldehyde dehydrogenase. This hypothesis has not been tested. There is more than one possible route for formaldehyde oxidation in type I methylotrophs (2).

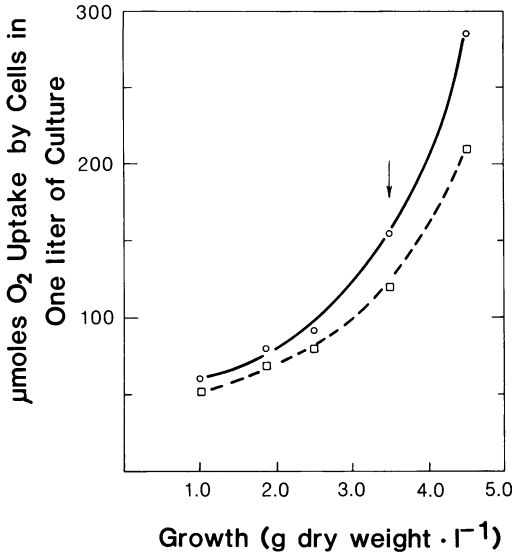

FIG. 3. Methane oxidizing activity of isolate 761H cells during growth on glucose and casein hydrolysate. Cells of isolate 761H grown in mineral salts-glucose-casein hydrolysate-PIPES medium for over 100 transfers were used to inoculate 12-ml flasks containing the same medium. The inoculum size was 10% (vol/vol). The cultures were incubated at 30°C without shaking until the dry weight of the cells reached 3.5 g · liter^{-1}. At that time (arrow), the culture was placed on a shaker. Absorbance at 650 nm was measured each day. Ten milliliters of a culture was centrifuged (10,000 × *g*, 10 min) at intervals, and the cells were suspended in 1 ml of 0.1 M potassium phosphate buffer plus 0.02 M $MgSO_4$. The rates of oxidation of methane (●) and methanol (○) and the dry weights of cells were measured as described in the text.

The ability to oxidize methane and to utilize it as an energy source is stably maintained through several transfers of isolate 761AR on media containing methanol as an energy source. The ability to oxidize methane is expressed during growth on methanol (17). These characteristics of the alcohol-resistant strain indicate that it is well suited for genetic studies of methane monooxygenase. It should be possible to isolate mutants capable of growth on methanol but not on methane.

Isolate 761H is capable of growth on heterotrophic media in the absence of C_1 compounds. This is the first report of a facultative type I methylotroph that contains 3-hexulose-6 phosphate synthase and lacks hydroxypyruvate reductase, an enzyme of the serine pathway found in type II methanotrophs. Unlike other type I methylotrophs (2, 5), isolate 761M and its variants possess a complete tricarboxylic acid cycle. The inability to oxidize acetate and pyruvate has been used to explain the obligate requirements for C_1 compounds for growth of type I methanotrophs (2). However, isolate 761M oxidized [2-^{14}C]acetate to [^{14}C]carbon dioxide (17). Impermeability to exogenous carbon sources and the formation of toxic metabolites have also been proposed as explanations for the obligate requirement for C_1 carbon and energy sources (3, 9). Radioactive glucose is assimilated into all macromolecular fractions, and exogenous amino acids are also assimilated into macromolecules (17; Zhao and Hanson, submitted). Therefore, impermeability to these substrates cannot explain the obligate requirement of isolate 761M for methane as an energy source. Glucose causes the formation of acidic metabolites that inhibit growth of all variants in the absence of additional buffers in the mineral salts media. It is not known at this time whether isolate 761H is more resistant to these or other metabolites of glucose or produces lower concentrations of potentially toxic metabolites. Further studies of the differences in metabolism should be useful in explaining the obligate requirement of isolate 761M for methane as an energy source.

It is also unclear why the range of substrates utilized as energy sources by isolate 761H is so limited. This strain, like the wild-type strain and isolate 761AR, will not grow on peptone or casein hydrolysate in the absence of an additional energy source and does not utilize sugars other than glucose as energy sources.

Isolate 761H, unlike the facultative methanotroph *M. organophilum* XX (17), possesses methane monooxygenase and intracytoplasmic membranes when grown on complex media and retains the ability to grow on methane after many transfers on media without C_1 compounds.

TABLE 2. Hexulose phosphate synthase activities in crude extracts of isolates 761M and 761H[a]

Bacterium	Growth substrates	Sp act of 3-hexulose-6-phosphate (nmol · min^{-1} · mg of protein^{-1})
Isolate 761M	Methane, casein hydrolysate	255
Isolate 761H	Methane, casein hydrolysate	288
Isolate 761H	Glucose, casein hydrolysate	148
Isolate 761AR	Methanol, casein hydrolysate	250
E. coli	Glucose, casein hydrolysate	0
Organism SB1	Methane	0

[a] Cultures were grown and enzymatic activities were assayed as described in the text. The cells were harvested during the mid-exponential growth phase.

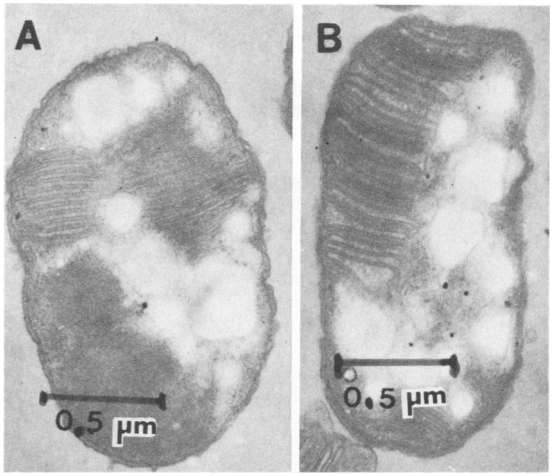

FIG. 4. Intracytoplasmic membranes of isolates 761AR and 761H. A culture of isolate 761H (A) was grown with glucose and casein hydrolysate for over 100 transfers (50% [vol/vol] inoculum) and harvested after 4 days of growth at 30°C without shaking. A culture of isolate 761AR (B) was grown with methanol and casein hydrolysate. The procedures used for harvesting of the cells, fixation, sectioning, and electron microscopy are described elsewhere (Zhao and Hanson, submitted). Magnification, ×32,600.

The restriction fragments produced from glucose-grown isolate 761H DNA that hybridize with a radioactive DNA from isolate 761M grown on methane were not detectably different from those produced from methane grown on isolate 761M DNA or methane grown on isolate 761AR DNA. In addition, the strains possess several characteristics in common, including their inability to grow and form visible colonies on L agar, Penassay agar, minimal salts agar with peptone or casein hydrolysate, Antibiotic Assay Agar no. 3, and nutrient agar.

ACKNOWLEDGMENTS

This research was supported by grant DOE-ACO2-8ZER12029 from the U.S. Department of Energy, by the University of Minnesota Graduate School, and by the Freshwater Foundation. Shu-jie Zhao is a Visiting Scholar from China at the Gray Freshwater Biological Institute.

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