Arch Microbiol (1987) 147:109–116

Archives of Microbiology

Polysaccharide reserve material in the acetotrophic methanogen, *Methanosarcina thermophila* strain TM-1: accumulation and mobilization

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Abstract. The ability of *Methanosarcina thermophila* strain TM-1 to store a reserve polysaccharide was studied using both biochemical methods and thin-section electron microscopy. When grown under conditions of excess carbon and energy (either methanol or acetate) and limiting nitrogen, *M. thermophila* accumulated a polysaccharide which could be hydrolyzed to glucose by the enzyme amyloglucosidase. This polysaccharide reached levels of 20 mg polysaccharide per g protein in nitrogen-limited cells, while cells limited for carbon, as well as cells in the exponential phase of growth, did not accumulate significant amounts of this polysaccharide. Thin-section electron micrographs of *M. thermophila* showed glycogen-like inclusion granules in nitrogen-limited cells but not in carbon-limited or exponential-phase cells. These granules were stained by a polysaccharide-specific staining procedure, the PATO stain. The polysaccharide was purified from cell extracts, the iodine-polysaccharide complex gave a maximum absorption at between 500 and 510 nm. The polysaccharide was mobilized within 21 h by cells starved for a carbon/energy source. N-Limited (polysaccharide-containing) acetate-grown cells could shift to methanogenesis from methanol more quickly than did C-limited acetate-grown cells lacking polysaccharide, and ATP levels remained higher in N-limited cells. The results are consistant with the hypothesis that this polysaccharide can provide carbon and energy for metabolic shifts but other storage compounds, such as polyphosphate, may also play a similar role.

Key words: Methanogenesis – Glycogen (or polysaccharide) – Acetotroph – N-Limitation – ATP

There have been limited studies on reserve materials in methanogens. Zeikus (1977) pointed out that in *Methanosarcina barkeri* strain MS, numerous electron dense granules of "unknown composition" were seen in thin-section electron micrographs, and suggested that they may be storage material. Scherer and Bochem (1983) described two types of electron-dense granules in thin-section electron micrographs of various *Methanosarcina* strains. One type which they identified as polyphosphate, and the other type which they referred to as "granula", but did not determine their exact chemical nature. In *Methanosarcina barkeri* morphotype 2, Zhilina (1976) called these granules glycogen, but did not demonstrate their composition. More recently, a glycogen-like polysaccharide was isolated from *Methanolobus tindarius* and four *Methanococcus* species (König et al. 1985).

The possibility that *Methanosarcina thermophila* strain TM-1 (Zinder and Mah 1979; Sowers et al. 1984; Zinder et al. 1985) stored a granular reserve material, possibly glycogen (or polyglucose), came to our attention upon examination of thin-section electron micrographs. Even though members of the genus *Methanosarcina* are the most catabolically versatile of the methanogenic bacteria (Hippe et al. 1979; Mah et al. 1978; Smith and Mah 1978; Smith et al. 1980), they are limited to growth and methanogenesis on one-carbon compounds (H_2/CO_2, methanol, methylamines) and acetate. Growth and methanogenesis from more complex compounds, such as glucose, have never been demonstrated. Therefore, the ability of *M. thermophila* to store and utilize a polysaccharide was of interest, since it would expand the known range of metabolic processes in this methanogenic bacterium. There are examples of bacteria with limited metabolic capacities, such as the aerobic, obligate methylotroph, *Methylococcus* (Linton and Cripps 1978), and the phototroph, *Chlorobium thiosulfatophilum* (Sirevag 1975), as well as the obligate chemolithotroph, *Thiobacillus neapolitanus* (Beudeker et al. 1981) which can use their reserve polysaccharide as an energy source. Here, we demonstrate the growth conditions under which *M. thermophila* strain TM-1 stored a glycogen-like polysaccharide. We also show that *M. thermophila* could mobilize its reserve polysaccharide within 21 h in the absence of an external carbon/energy source. In addition, we present experiments which suggest that, when mobilized, this reserve polysaccharide provided energy to the cells.

Materials and methods

Growth medium. The basal medium contained (g/l): K_2HPO_4, 0.34; $MgCl_2 \cdot 6\,H_2O$, 0.1; resazurin, 0.001, trace metals solution (Zeikus 1977), plus 0.02 g/l $NiCl_2 \cdot 6\,H_2O$, 10 ml/l. The pH of the medium was adjusted to 6.7 with 1.2 N HCl. The medium was boiled and then cooled under N_2, and dispensed inside an anaerobic glove box (Coy Laboratory Products, Ann Arbor, MI) into serum bottles in either 50 ml or 500 ml amounts. After autoclaving, the following sterile, anaerobic solutions were added (final concentration, g/l): $Na_2S \cdot 9\,H_2O$, 0.4; $NaHCO_3$, 0.5; $CaCl_2 \cdot 2\,H_2O$, 0.1; p-aminobenzoic acid, 5.0×10^{-5};

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NH_4Cl, 0.025–1.0; 70% N_2/30% CO_2 headspace. Cells limited for carbon received either 10 mM methanol or 12–16 mM sodium acetate and 20 mM NH_4Cl. Cells grown with excess carbon were provided with up to 72 mM methanol (in 24 mM amounts at a time) or 120 mM sodium acetate and 0.5–1.0 mM NH_4Cl (Murray and Zinder 1985). Culture vials received a 2% (v/v) inoculum and were incubated at 50°C.

Analysis of gases. Methane was determined using Gow-Mac 550 thermal conductivity gas chromatograph (Gow-Mac Instrument Co., Inc., Bound Brook, NJ), as described by Zinder et al. (1984).

Polysaccharide assay. Cells grown in 500 ml of medium in 1 l bottles were harvested aerobically by centrifugation at 17,200 × *g* for 20 min at 4°C. The cell pellet was washed twice with 10 mM potassium phosphate buffer (pH 6.7) with a final wash in distilled water. The cells were resuspended in 2 ml distilled water in a 16 mm screw-capped test tube, along with approximately 1 ml of 0.45–0.5 mm diameter glass beads (VWR Scientific, Inc., Rochester, NY). Cells were broken by vortexing with ghe glass beads at top speed for several minutes using a Fisher Scientific Vortex Genie (Fisher Scientific Co., Rochester, NY). The tube was returned to an ice bucket after every 2 min of vortexing to keep the suspension cold. Cell breakage was observed by phase microscopy, and when approximately 90% breakage was achieved, the cell extract was centrifuged for 4 min in a Fisher micro-centrifuge model 235 A (Fisher Scientific Co., Rochester, NY). The broken cell supernatant was assayed for protein using the Coomassie brilliant blue method (reagents from Bio-Rad Laboratories, Richmond, VA) (Bradford 1976).

The supernatant, and as a control, the resuspended broken cell pellet, were assayed for the presence of glycogen-like polysaccharide as follows. The alpha-linked glucose in a 200 µl sample was hydrolyzed to glucose units using 0.03 mg of the enzyme amyloglucosidase (Sigma Chemical Co., St. Louis, MO) per ml reaction mixture. KH_2PO_4 (final concentration, 0.2 M) was used to buffer the reaction mixture at pH 4.5. This reaction mixture was incubated at 55°C for 5 min, and then cooled on ice for 1 min. The glucose formed was measured using the Sigma glucose oxidase/peroxidase (PGO) method (Sigma Chemical Co., St. Louis, MO). As a control, oyster glycogen (final concentration 1 mg/ml) was assayed for glucose content each time we assayed cell extracts. This polysaccharide assay could detect levels of oyster glycogen as low as 0.005 mg/ml.

Electron microscopy. All electron microscopy was done by thin-sectioning of cells. Cells were prepared following a modified Ryter-Kellenberger fixation procedure (Kellenberger et al. 1958). Cells, in 50 ml quantities, were prefixed in 3% (final concentration) glutaraldehyde for at least 30 min, and was washed twice and resuspended in 2 ml 0.03 M PIPES buffer (pH 7.0) (Sigma Chemical Co.) and 0.01 M $CaCl_2 \cdot 6\ H_2O$, and 2 ml of 2% OsO_4 was added as a fixative. The fixed cells were embedded in 1.5% agar and post-fixed for 2 h in 0.5% uranyl acetate in water. After post-fixation, the preparations were dehydrated and embedded in Spurr low-viscosity epoxy resin (Electron Microscopy Sciences, Ft. Washington, PA). Thin-sections were cut with a Dupont diamond knife on a LKB Bromma 2080 ultratome V. These sections were placed on 300-mesh, copper grids and post-stained by floating the grids on a drop of saturated uranyl acetate (in 70% ethanol), followed by submersion in a 0.3% lead citrate solution (Venable and Coggeshall 1965).

A polysaccharide-specific post-staining procedure, called the PATO stain (periodic acid-thiosemicarbazide-OsO_4), as outlined by Hardie et al. (1983), with the deletion of the ruthenium red fixation, was used to detect intracellular polysaccharide.

All preparations were examined with a Phillips 300 electron microscope operated at 80 kV. Images were recorded on Kodak type-4489 electron microscope film.

Polysaccharide purification. One volume (10 ml) of ice cold 10% (w/v) trichloroacetic acid (TCA) was added to 10 ml of a broken cell extract (prepared from 6 l of methanol-grown cells starved for NH_4^+ at 1 mM) and incubated for 15 min on ice. The mixture was centrifuged at 12,000 × *g* for 10 min and the pellet was discarded. The polysaccharide in the supernatant was precipitated with 2 volumes (40 ml) ice cold 95% ethanol on ice for 2 h, reprecipitated and collected as outlined by Brana et al. (1982).

Analysis of purified polysaccharide. The dried, crystalline polysaccharide (16 mg) was suspended in 2 ml warm distilled water and this solution was analyzed. A 1/20 dilution of the polysaccharide solution was hydrolyzed with the enzyme amyloglucosidase as outlined above. However, acetic acid/acetate (final concentration, 10 mM) was used in place of KH_2PO_4 to buffer the system, to avoid interference with the high performance liquid chromatographic analysis for glucose. The hydrolyzed end product was assayed for glucose by the PGO method as outlined above. In addition, the hydrolysate was analyzed by high performance liquid chromatography (HPLC) using a Bio-Rad (Richmond, CA) Ion Exclusion HPX-87H column.

The absorption spectrum of the iodine-polysaccharide complex was measured using a Gilford spectrophotometer 240 (Gilford Instrument Laboratories, Inc., Oberlin, OH). The complex was prepared using 125 µl of the polysaccharide solution and 125 µl of a solution of 0.2% (w/v) I_2 and 2% (w/v) KI (Lindner et al. 1979). The optical density was measured against an I_2/KI blank at wavelengths between 350 and 700 nm.

Determination of radioactivity. The radioactivity in ^{14}C-labeled methane was measured using the gas chromatographic-proportional counter technique of Nelson and Zeikus (1974).

Polysaccharide mobilization experiment. Methanol-grown cells limited for NH_4^+ were grown anaerobically in 500 ml quantities in 1 l bottles sealed with rubber stoppers. After NH_4^+ limitation of growth, as indicated by the cessation of methanogenesis, the cells were harvested in the anaerobic glove box by allowing the *Methanosarcina* clumps to settle out of the medium, and harvesting the remaining cells with a clinical centrifuge (Clay-Adams Safety Head, VWR Scientific Co., Rochester, NY). The cell pellets were washed 4 times in basal medium (see above), and were divided approximately in half. One half of the cells were broken and assayed for initial (time 0) polysaccharide content using the enzyme amyloglucosidase as described above, and were also assayed for protein content. The cell extracts contained

approximately 1.0–1.5 mg protein/ml. The remaining cells were added to culture vials containing 50 ml of mineral medium with 20 mM NH_4^+, but lacking a carbon/energy course, and were incubated at 50° C. After incubation for the indicated times, the cells were harvested aerobically at 4° C by centrifugation and were broken and assayed for polysaccharide and protein, as above.

Metabolic shift experiments. M. thermophila cells were grown in 500 ml bottles, either in the presence of excess acetate (120 mM) and limiting NH_4^+ (0.5 mM), or in the presence of limiting acetate (16 mM) and excess NH_4^+ (20 mM). The cells in each vial were collected after 2 days after CH_4 production ceased, and were harvested and washed anaerobically as outlined above. Each washed cell pellet was resuspended in 45 ml of mineral medium with 20 mM NH_4^+, but lacking a carbon/energy source. Fifteen milliliters of suspended cells were added to three separate 35 ml culture vials. One vial received no carbon/energy source. The second vial received 10 mM sodium acetate and the third received 10 mM methanol and 1 μCi $^{14}CH_3OH$ (sp. activ., 0.01 μCi/μmol). Prior to incubation, 1 ml of cells was removed for protein determination. The vials was incubated at 50° C. CH_4 and $^{14}CH_4$ were monitored over time, as described.

Assays for intracellular ATP. Acetate-grown cells limited either for NH_4^+ or acetate were grown and anaerobically harvested and washed, as above. The washed cells were resuspended in 30 ml of mineral medium with no carbon/energy source, and, in 15 ml amounts, were placed into two 35 ml serum vials. One vial received 10 mM methanol, the other received no carbon/energy source. At various times during incubation of the cells, two 1 ml samples of cells were anaerobically removed by syringe from each vial. The cells in one sample were collected, washed, broken and the protein content in the cell extract was measured. Intracellular ATP was extracted from the second 1 ml sample of cells by immediately injecting the sample into 1 ml ice cold 12% (v/v) $HClO_4$ and incubating for 2 h (Blaut and Gottschalk 1984). This procedure has been shown to give good reproducible extraction of ATP from *Methanosarcina* cells (Blaut and Gottschalk 1984; Sprott et al. 1982). Using these techniques, we have found that intracellular ATP levels measured in duplicate samples were within 5% of each other. Extracts were carefully neutralized using 0.72 M KOH with 0.16 M $KHCO_3$ (Sprott et al. 1982). ATP in the extracts was measured using the Firefly luciferin-luciferase method (reagents from Analytical Luminescence Laboratory, Inc., San Diego, CA). The peak height of light emitted by the reaction was measured using a photometer (Turner TD-20e Luminometer), and these values were compared to an ATP standard curve. The ATP standard was diluted into 1 ml sterile culture medium, 1 ml 12% (v/v) $HClO_4$, and neutralized with 0.72 M KOH with 0.16 M $KHCO_3$ to reproduce the experimental assay conditions for intracellular ATP.

Chemicals and radiochemicals. All chemicals used were at least reagent grade. $^{14}CH_3OH$ (56.9 mCi/mmol) was obtained from Amersham and stored at 4° C as an anaerobic, filter sterilized stock solution.

Results

Polysaccharide assay. Using a chemically defined growth medium (Murray and Zinder 1985), we were able to examine the effects of nutrient limitation on accumulation of alpha-linked polysaccharide (as assayed as glucose liberated from cell extracts by amyloglucosidase) in *Methanosarcina thermophila.* Cells limited for nitrogen accumulated an alpha-linked polysaccharide at levels of up to 25 mg/g cell protein (Table 1), while cells limited for carbon/energy (either methanol or acetate), or cells undergoing exponential growth did not accumulate significant amounts of this polysaccharide.

Table 1. Reserve polysaccharide content (determined as glucose liberated after amyloglucosidase treatment) of *Methanosarcina thermophila* under various growth conditions

Growth conditions		Growth phase (of harvested cells)	mg Glucose (hydrolyzed)/g protein
Carbon source	NH_4^+		
Limiting acetate	Excess	Stationary	0–3
Limiting methanol	Excess	Stationary	0–2
Excess acetate	Limiting	Stationary	10–20
Excess methanol	Limiting	Stationary	10–25
Excess acetate	Excess	Exponential	0
Excess methanol	Excess	Exponential	0
Excess methanol and excess acetate	Limiting	In methanol phase	10

Two important controls were run on cells containing the polysaccharide. The outer sacculus of *M. thermophila* consists of a condroitin-like heteropolysaccharide which contains 0.5% glucose by dry weight (Kreisl and Kandler 1986), and it is possible that contaminating cell wall material could be the source of the glucose liberated in our assay. However, no detectable glucose was released by amyloglucosidase from a pellet consisting of cell wall fragments produced after cell breakage. Secondly, glucose was not detected in cell extracts unless amyloglucosidase was added, indicating that no free glucose was present in the cells.

Electron microscopy. Thin-section electron micrographs of *M. thermophila* cells grown under various conditions were examined for glycogen-like inclusion granules. As seen in Fig. 1, thin-section electron micrographs of methanol grown cells limited for nitrogen (A), showed the inclusion granules typical of glycogen reserve material, as seen in other bacteria (Cheng et al. 1977; Kamio et al. 1981). However, cells limited for methanol (B) or cells in the exponential phase of growth (C) had significantly fewer granules. Results similar to these were seen in thin-section electron micrographs of acetate grown *M. thermophila* (electron micrographs not shown).

To demonstrate that these inclusion granules were a polysaccharide material, and not merely polyphosphate or calcium deposits as seen in other *Methanosarcina* (Scherer and Bochem 1983), a polysaccharide-specific staining procedure, the PATO stain (Hardie et al. 1983), was performed on thin-sections of *M. thermophila.* As seen in Fig. 1D, nitrogen-limited acetate-grown cells contained granules which were stained by this method. Thin sections of acetate-limited cells subjected to the PATO stain did not contain stained granules. As a further control, no polysaccharide granules were seen after the initial treatments, without the OsO_4 staining. In addition, no granules were detected in thin sections of nitrogen-limited methanol-grown cells when periodic acid or thiosemicarbazide were deleted (electron micrographs not shown).

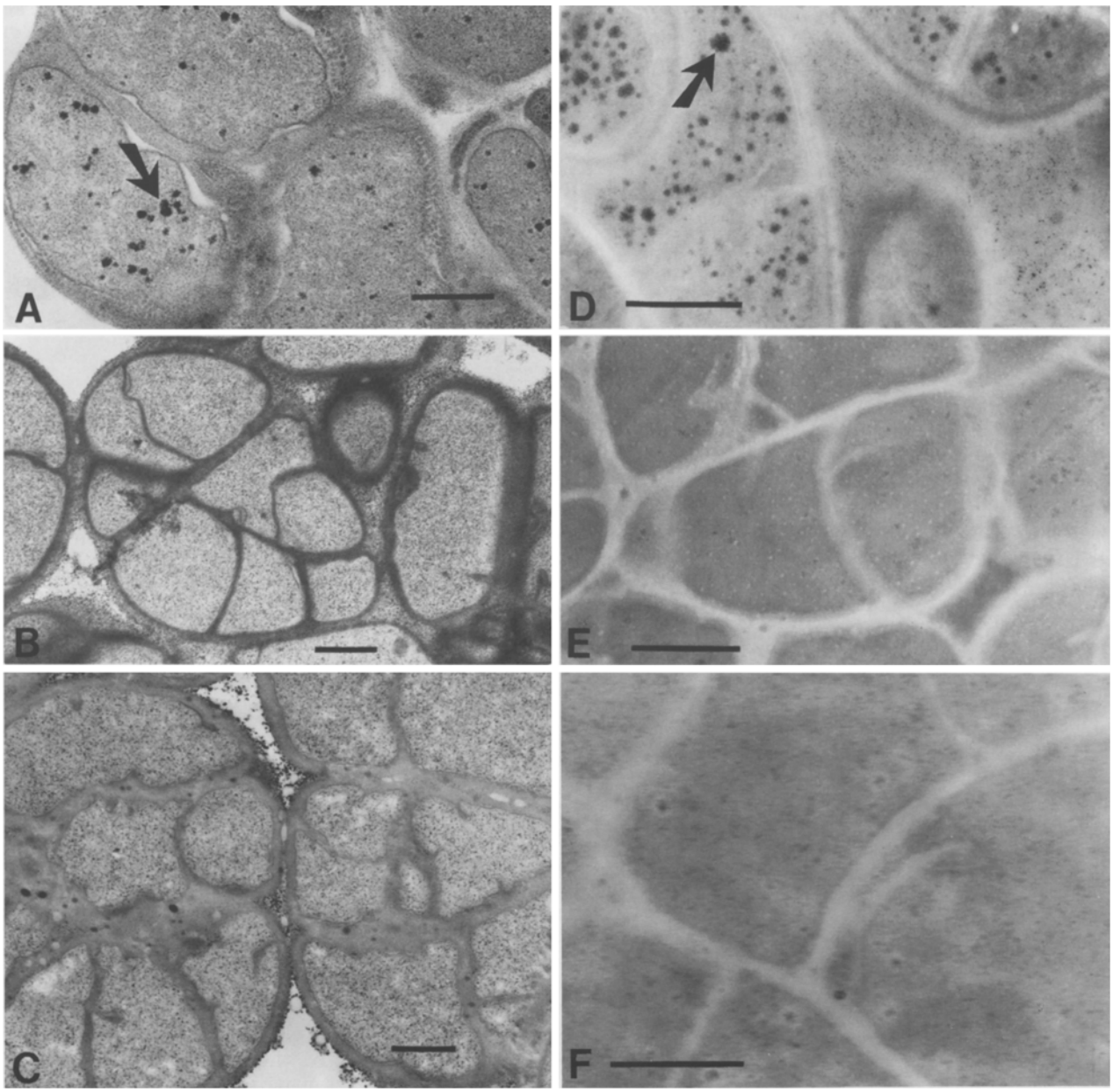

Fig. 1 A–C. Thin-section micrographs of *Methanosarcina thermophila* strain TM-1. Arrow indicates typical glycogen-like polysaccharide inclusion granules. **A** Cells grown with methanol and limiting nitrogen; **B** cells limited for methanol; **C** cells in exponential phase of growth; **D–F** Thin-section electron micrographs of *M. thermophila* strain TM-1 subjected to the polysaccharide specific PATO staining procedure (6). Arrow indicates specifically stained polysaccharide inclusion granules. **D** cells grown with excess acetate and limiting nitrogen; **E** cells grown with limiting acetate; **F** control thin-section of cells grown with excess methanol and limiting nitrogen lacking the OsO_4 stain. Bar, 0.5 μm

Polysaccharide characterization. To verify the ability of *M. thermophila* to store an intracellular polysaccharide, this polysaccharide was purified from extracts of methanol grown cells limited for nitrogen. The cells were broken and the large macromolecules precipitated from the broken cell extract with 10% TCA. The polysaccharide remaining in the TCA supernatant was precipitated by repeated treatments with cold 95% ethanol (Brana et al. 1982).

The dried polysaccharide was suspended in warm distilled water and partially characterized. Glucose was the only hydrolysis product, after treatment with amyloglucosidase, detected by high performance liquid chromatography, while maltose was detected after treatment with alpha-amylase. As seen in Fig. 2, the absorption spectrum of the iodine-*M. thermophila* polysaccharide complex gave a maximum absorption value of between 500 and 510 nm. This compared to a maximum absorption of 520 nm obtained for an iodine-oyster glycogen complex.

Mobilization of polysaccharide. Since *M. thermophila* accumulated an alpha-linked polyglycose when grown in a medium with excess carbon/energy and limiting NH_4^+, we

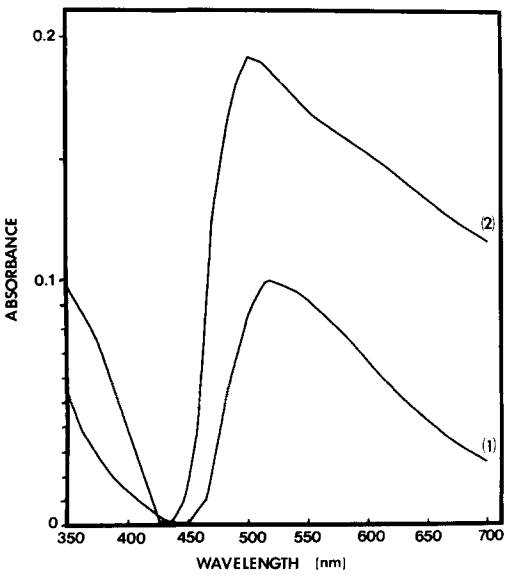

Fig. 2. Absorption spectra of the iodine-polysaccharide complex of oyster glycogen *1*, and *M. thermophila* polysaccharide *2*

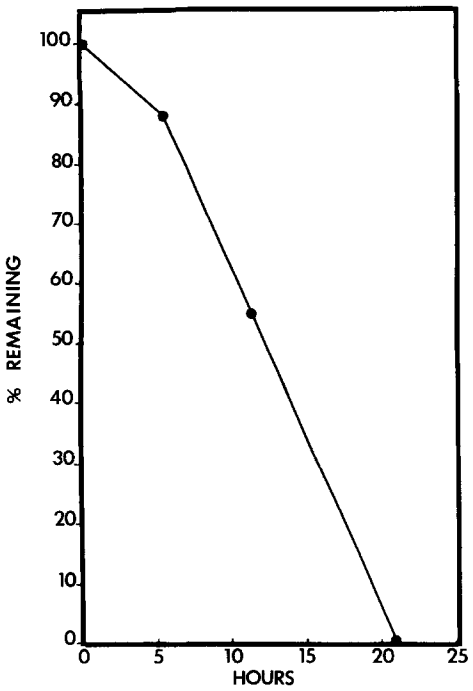

Fig. 3. Mobilization of reserve polysaccharide by N-limited methanol-grown *Methanosarcina thermophila* strain TM-1 incubated at 50° C in the absence of a carbon/energy source

examined whether these cells could mobilize this polysaccharide during starvation. After NH_4^+ limitation of growth on methanol, the initial polysaccharide content of a portion of the cells was determined. The remaining washed cells were placed in medium lacking only a carbon/energy source and samples were removed over time for determination of polysaccharide and protein. The initial concentration of polysaccharide in the cells, which ranged from 20 – 22 mg/g protein, was compared with the polysaccharide content after starvation. As seen in Fig. 3, the percentage of polysaccharide remaining in the starved cells decreased with time, until 21 h, when no detectable polysaccharide remained.

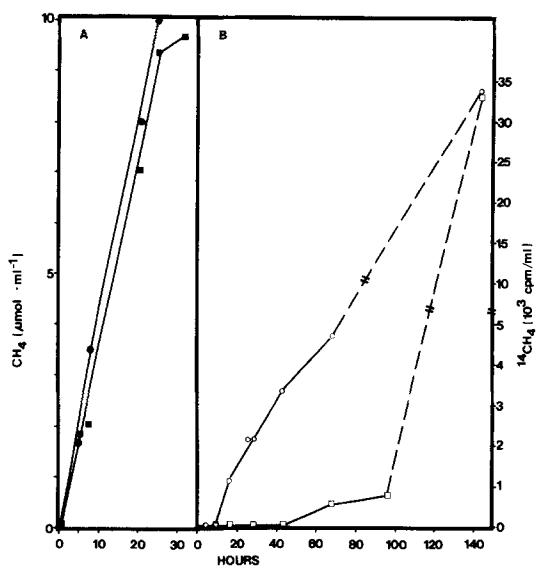

Fig. 4. A Methanogenesis from acetate by washed acetate-grown *Methanosarcina thermophila* strain TM-1. Symbols: N-limited cells containing polysaccharide, ●; C-limited cells lacking polysaccharide, ■; **B** Methanogenesis from $^{14}CH_3OH$ by washed acetate-grown *M. thermophila*. From the specific activity of $^{14}CH_3OH$ added, the amounts of CH_4 produced at 144 h were determined. N-Limited cells containing polysaccharide produced 3 mM CH_4, while C-limited cells lacking polysaccharide produced 2.9 mM CH_4. Symbols: N-limited cells containing polysaccharide, ○, C-limited cells lacking polysaccharide, □

Metabolic shift experiments. Zinder and Elias (1985) have shown that when acetate-grown *M. thermophila* cells were harvested in the exponential phase of growth, washed, and presented with methanol as the sole methanogenic substrate, the cells metabolized the methanol at only about 1% of the rate of methanogenesis from acetate and the rate didn't increase after 10 h of incubation. However, if acetate was added to these cells, along with the methanol, the rate of methanogenesis from methanol increased with time over 10 h. Presumably, the acetate served as an energy source to allow for the synthesis of proteins necessary to metabolize methanol. Therefore, if energy was required for acetate-grown cells to shift to methanol utilization, it might be possible for polysaccharide-containing cells to degrade their reserve polysaccharide to obtain energy for this shift. To examine the possibility, acetate-grown cells were harvested after either NH_4^+ limitation of growth (and polysaccharide accumulation) or acetate limitation of growth (when cells would not contain polysaccharide) (Murray and Zinder 1985). The washed cells were concentrated and provided with either acetate, methanol, or no carbon source. The initial cellular protein levels in these preparations were similar (0.2 – 0.23 mg/ml).

Both acetate-grown cells containing polyglucose (N-limited) and those lacking this reserve material (C-limited) were able to metabolize acetate and produced CH_4 rapidly and at nearly equal rates (see Fig. 4A). Control vials with no carbon/energy source showed no CH_4 production. Cells containing polysaccharide showed detectable methanogenesis from ^{14}C-labeled methanol (Fig. 4B) within 16 h, while cells lacking polysaccharide did not begin to metabolize methanol until after 50 h. In this experiment, radiolabeled

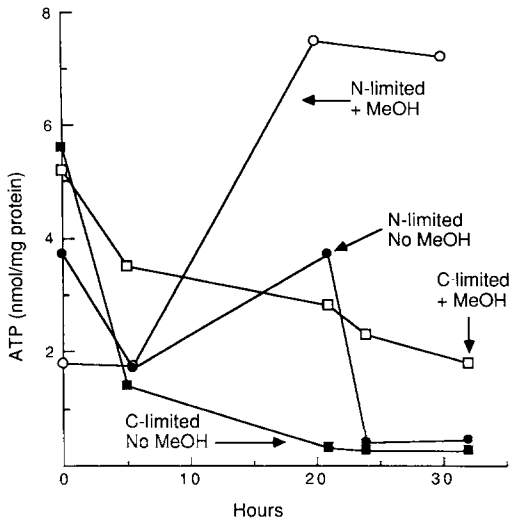

Fig. 5. Intracellular ATP levels in washed acetate-grown *Methanosarcina thermophila* strain TM-1. Symbols: N-limited cells containing polysaccharide incubated in the presence of 10 mM methanol, ○; N-limited cells incubated with no carbon/energy source, ●; C-limited cells lacking polysaccharide incubated in the presence of 10 mM methanol, □; C-limited cells incubated with no carbon/energy source, ■

methanol was used because the method for detection of $^{14}CH_4$ is more sensitive than that for unlabeled CH_4.

ATP determinations. To further examine whether polysaccharide mobilization correlated with the ability of *M. thermophila* cells to make a metabolic shift to methanol utilization, we analyzed ATP levels in these cells (Fig. 5). C-Limited acetate-grown cells incubated in medium without methanol showed a rapid decline in ATP levels, while N-limited (polysaccharide-containing) cells incubated without methanol showed somewhat higher ATP levels until 24 h, by which time the polysaccharide was depleted (Fig. 3). ATP levels in C-limited cells incubated in medium containing methanol decreased during the incubation, but not as completely as those in medium lacking methanol, presumably because of ATP generated from the small amount of methanol metabolized by the cells. N-Limited (polysaccharide-containing) cells showed an increase in ATP levels by 21 h by which time they had already begun to metabolize significant amounts of methanol (0.2 μmol/ml CH_4 produced), and ATP levels remained high at 32 h. The protein content of all these cell preparations remained constant (at approximately 0.2 mg/ml) during the course of this experiment.

Discussion

Evidence is now accruing for the presence of reserve materials in methanogens. Polyphosphate inclusion bodies were demonstrated in several species of *Methanosarcina* by Scherer and Bochem (1983) and in *Methanolobus* by König and Stetter (1982). In addition, cyclic-2,3-di-phospho-D-glycerate (cyclic DPG) has been shown to accumulate in members of the Methanobacteriales (Seely and Fahrney 1983, 1984; Tolman et al. 1986), and may serve as a reserve for carbohydrate synthesis (Evans et al. 1986). König et al. (1985) have recently demonstrated the presence of glycogen in a *Methanolobus* and several species of *Methanococcus*, and that the glycogen was consumed under conditions of energy starvation. Likewise, we have shown that *Methanosarcina thermophila* strain TM-1, an acetotrophic methanogen, has the ability to accumulate an intracellular reserve polysaccharide, which it can mobilize during carbon/energy starvation.

A wide variety of eubacteria can store polyglucose (or glycogen) (Preiss 1984). Typically, in bacteria, reserve polysaccharide is accumulated in large amounts (up to 50% of cell dry weight) when cells are starved for an essential nutrient, such as nitrogen, in the presence of an excess of a carbon/energy source (Preiss 1984). This was also the case in *M. thermophila*, although polysaccharide levels were considerably lower (up to 20 mg/g protein).

As seen with purified glycogen-like material isolated from other bacteria (Cheng et al. 1977; König et al. 1982), the purified *M. thermophila* polysaccharide was opalescent in aqueous solution, developed a reddish-brown color with iodine, and was hydrolyzed by amyloglucosidase to yield only glucose. The maximum absorption of the iodine-*M. thermophila* polysaccharide complex was between 500 and 510 nm, similar to oyster glycogen, and to iodine-polyglucose complexes from other strictly anaerobic bacteria, such as *Ruminococcus albus*, 500 nm (Cheng et al. 1977) and *Selenomonas ruminantium*, 520 nm (Kamio et al. 1981). The spectrum of the iodine-polysaccharide complex can suggest the degree of branching of the polysaccharide, with a shorter wavelength for the absorption maximum correlating with more branching (Beudeker et al. 1981). By this criterion, *M. thermophila* polysaccharide seems to be slightly more branched than glycogen from *S. ruminantium* which has an average chain length of approximately 20–25 (Kamio et al. 1981).

The inability of acetate-grown *M. thermophila* to readily shift to methanogenesis from methanol (Zinder and Elias 1985) provided us with a potential means to test the ability of *M. thermophila* to mobilize its reserve polysaccharide as a useful energy source. N-Limited polysaccharide-containing acetate-grown *M. thermophila* could make the shift to methanol utilization much more readily than C-limited acetate-grown cells without endogenous polysaccharide. This result suggested that by mobilizing its reserve polysaccharide, acetate-grown *M. thermophila* might obtain the energy which could be necessary for the synthesis of proteins required for methanol utilization. Similarly, in *Thiobacillus* A2, Gottschal et al. (1981) demonstrated that acetate-grown cells containing poly-β-hydroxybutyrate could shift to the oxidation of thiosulfate more rapidly than cells lacking this reserve material, and hypothesized that the poly-β-hydroxybutyrate was providing ATP for protein synthesis.

An increased level of intracellular ATP/mg protein was seen, corresponding with the onset of methanogenesis from methanol by polysaccharide-containing *M. thermophila*. ATP levels in acetate-grown cells lacking polysaccharide decreased steadily over time. The levels of ATP seen in *M. thermophila* were similar to levels measured in other methanogenic bacteria (Blaut and Gottschalk 1984; Gunnarsson and Ronnow 1982). Blaut and Gottschalk (1984) showed that the ATP levels in *Methanosarcina barkeri* growing on methanol were approximately 10–20 nmol/mg protein, while Gunnarsson and Ronnow (1982) demonstrated that when *Methanobacterium thermoautotrophicum* was limited for ammonium, the intracellular ATP levels dropped

from between 5 to 7 nmol/mg protein to below 1 nmol ATP/mg protein.

These data suggest that polysaccharide was degraded, potentially yielding ATP, which the cells could use to their advantage in shifting to methanogenesis from methanol. It is also possible that *M. thermophila* could utilize this reserve polysaccharide as a cell wall precursor, since the cell wall of the *Methanosarcinae* consists of a heteropolysaccharide (Kreisl and Kandler 1986). In addition, it is likely that the conservation of carbon-carbon bonds by these methanogens is a high priority, and that the polysaccharide is degraded to important cellular carbon intermediates. Furthermore, other storage compounds such as polyphosphate can exist in *M. thermophila*, and may play important roles in endogenous metabolism. An assessment of the function of glycogen-like polysaccharide in *M. thermophila* was severely hampered by the fact that this methanogen only stores about 1% of its cell dry weight as this polysaccharide.

Our demonstration, and that of König et al. (1985), of the ability of methanogenic archaebacteria to produce and use reserve polysaccharide raises interesting questions about the pathway of utilization, since methanogens are not generally considerated to be able to use substrates as complex as carbohydrates. König et al. (1985) found that glycogen levels decreased after 24 h of starving *Methanolobus* cells for methanol and that there was production of CH_4 in amounts roughly stoichiometrically equal to the amount of glucose units degraded. We have found (unpublished data) that a small amount of acetate was produced by starving polysaccharide-containing *M. thermophila* cells, but we have not been able to associate this acetate production directly with polysaccharide degradation. The suggestion that methanogens have a glycolysis-like pathway for generation of energy from polysaccharide is reasonable if one considers that methanogens carry out gluconeogenesis (Fuchs et al. 1983) to synthesize cell wall polysaccharide and other carbohydrate-containing structural components.

Acknowledgements. This research was funded, in part, by USDA/Hatch funds and the Cornell University Graduate Fellowship Office. The authors thank Jon Kemp for excellent technical assistance and Dr. William Ghiorse for advice concerning the electron microscopy presented here. In addition, we thank Dr. Jane Gibson for help in writing this manuscript and Mrs. Jackie Armstrong for excellent typing.

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Received May 26, 1986/Accepted December 2, 1986