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EXPERIMENTAL ARTICLES

Physiological, Biochemical, and Cytological Characteristics of a Haloalkalitolerant Methanotroph Grown on Methanol

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Abstract—The halotolerant alkaliphilic methanotroph *Methylomicrobium buryatense* 5B is capable of growth at high methanol concentrations (up to 1.75 M). At optimal values of pH and salinity (pH 9.5 and 0.75% NaCl), the maximum growth rate on 0.25 M methanol (0.2 h^{-1}) was twice as high as on methane (0.1 h^{-1}). The maximum growth rate increased with increasing medium salinity and pH. The growth of the bacterium on methanol was accompanied by a reduction in the degree of development of intracytoplasmic membranes, the appearance of glycogen granules in cells, and the accumulation of formaldehyde, formate, and an extracellular glycoprotein at concentrations of 1.2 mM, 8 mM, and 2.63 g/l, respectively. The glycoprotein was found to contain 23% protein and 77% carbohydrates, the latter being dominated by glucose, mannose, and aminosugars. The major amino acids were glutamate, aspartate, glycine, valine, and isoleucine. The glycoprotein content rose to 5 g/l when the concentration of potassium nitrate in the medium was augmented tenfold. The activities of sucrose-6-phosphate synthase, glycogen synthase, and NADH dehydrogenase in methanol-grown cells were higher than in methane-grown cells. The data obtained suggest that the high methanol tolerance of *M. buryatense* 5B is due to the utilization of formaldehyde for the synthesis of sucrose, glycogen, and the glycoprotein and to the oxidation of excess reducing equivalents through the respiratory chain.

Key words: haloalkalitolerant methanotrophs, growth on methanol, adaptation and detoxification mechanisms.

Methanotrophic bacteria are able to use, as sources of carbon and energy, not only methane but also methanol, which is toxic to most bacteria at a concentration of 0.1% or higher. The ability to grow on 0.2% methanol is even used as a specific phenotypic signature of methanotrophs [1–3]. Some methanotrophs can grow at considerably higher methanol concentrations. For instance, *Methylococcus capsulatus* is able to grow in batch culture at a methanol concentration of 0.2% or in a continuous culture at a methanol concentration of 1%. It should, however, be noted that the residual concentrations of methanol in chemostat cultures are relatively low [1]. After the long-term adaptation of the methanotrophic bacteria *Methylocystis parvus* OBBP and *Methylosinus trichosporium* OB3b with the serine pathway of C_1-assimilation to high methanol concentrations, they acquired the ability to grow in the presence of 4% methanol [4, 5]. Although the mechanisms responsible for the methanol tolerance of methanotrophs are not clearly understood, it is obvious that their ability to grow on methanol is limited by the accumulation of formaldehyde, which is a central intermediate of methylotrophic metabolism. When the oxidation and assimilation of C_1 compounds are unbalanced, the accumulated formaldehyde can inhibit some enzymes and the entire systems of transcription and translation [6].

Taking into account our recent finding that some haloalkaliphilic and haloalkalitolerant methanotrophs grown on methane or methanol can excrete into the medium not only formaldehyde but also formate [7–9], it can be suggested that such methanotrophs possess some mechanisms involved in the oxidation and assimilation of C_1 compounds, including those responsible for the removal of toxic intermediates of C_1 metabolism.

The aim of the present work was to comparatively study the physiological, biochemical, and cytological characteristics of *Methylomicrobium buryatense* 5B grown on methane and methanol in order to gain insight into the mechanisms responsible for the ability of this haloalkalitolerant methanotroph to grow at high methanol concentrations.

MATERIALS AND METHODS

Bacterium and growth conditions. The halotolerant alkaliphilic methanotrophic bacterium used in this study was isolated from mud samples taken from soda Lake Gorbunka (Chita region, Russia). The bacterium was preliminarily identified as *Methylomicrobium buryatense* 5B [7]. The bacterium was cultivated at

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26°C in 750-ml flasks with 100 ml of mineral P medium containing various amounts of NaCl. The pH of the medium was adjusted to required values (9.5 or 7.5) by adding 50 mM sodium carbonate buffer (pH 9.5) or 50 mM sodium phosphate buffer (pH 7.5) [8]. The cultivation medium was inoculated with 10 ml of a culture grown under optimal conditions and then either flushed with methane in an amount of 0.5 l or supplemented with 0.2 to 9 vol % methanol. Cultivation was performed at 26°C on a rotary shaker (160 rpm). Growth was monitored by measuring culture turbidity at 600 nm using a Specol 221 spectrophotometer (Germany).

The effect of respiratory-chain inhibitors on growth. 2-Heptyl-4-hydroxyquinoline *N*-oxide (HQNO) and rotenone (inhibitors of sodium- and proton-translocating NADH dehydrogenases, respectively) were dissolved in 0.1 ml of CH_3OH and added to the growth medium at concentrations of 50 and 200 μM. The extent of growth inhibition was calculated with respect to the specific growth rate of culture in the absence of the inhibitors.

Enzyme assays. Cells grown to the mid-exponential growth phase were collected by centrifugation at 10000 *g* for 30 min, washed twice with 50 mM Tris–HCl buffer (pH 7.5) containing 0.1 M NaCl, resuspended in the same buffer, and disrupted by sonication (3 × 1 min) using an MSE disintegrator (150 W; 20 kHz). The cell homogenate was centrifuged at 30000 *g* for 15 min, and the supernatant was assayed for the activity of methanol dehydrogenase, formaldehyde dehydrogenase, formate dehydrogenase, NADH dehydrogenase, 3-hexulose-6-phosphate synthase, sucrose-6-phosphate synthase, and glycogen synthase [7, 9].

The isolation of extracellular glycoprotein. Cells from a certain volume of the *M. buryatense* 5B culture were removed by centrifugation at 30 000 *g* for 30 min, and the supernatant was passed through a 0.45-μm-pore-size Millipore membrane filter. Extracellular glycoprotein (EGP) was precipitated from the filtrate with two volumes of acetone. The precipitate was washed with acetone, dehydrated under a vacuum, and dissolved in distilled water. The EGP solution was dialyzed against distilled water for 24 h and lyophilized. The EGP yield was determined gravimetrically.

Analytical procedures. The total content of carbohydrates in the EGP was determined by reaction with phenol and sulfuric acid [10]. The protein concentration was evaluated by a modification of the Lowry method [11]. The monosugar and amino acid composition of the EGP was determined after hydrolyzing it in 1 M trifluoroacetic acid for 6 h at 120°C. The hydrolysate was analyzed using Biotronic carbohydrate and amino acid analyzers (Germany). The concentration of formaldehyde and formate in the culture liquid was evaluated by the methods described by Nash [12] and Lang and Lang [13].

The ^{1}H-NMR analysis of glycogen. Cells (1 g) from the late exponential growth phase were collected by centrifugation and resuspended in 2 ml of D_2O. Analysis was performed using a WP-80SY NMR spectrometer (Switzerland) as described earlier [14].

Electron microscopy. Cells for electron microscopic examinations were collected from the lag, early-, mid-, and late-exponential growth phases, when the optical density of the culture was 0.15, 1.3, 2.7, and 3.8 units, respectively. Cells were fixed in the presence of ruthenium red, cut into thin sections, contrasted, and examined in an electron microscope as described elsewhere [14].

RESULTS

M. buryatense 5B cells that were preliminarily grown at optimal pH and salinity values (pH 9.5 and 0.75% NaCl) and then transferred to a medium containing various amounts of methanol grew with a lag phase, whose duration increased with increasing methanol concentration (Fig. 1). The maximum growth rate at methanol concentrations between 0.25 and 0.75 M was 0.157–0.159 h^{-1}, i.e., higher than during growth on methane (μ = 0.1 h^{-1}). The growth rate of methanol-adapted *M. buryatense* 5B cells pregrown on 0.25 M methanol reached 0.2 h^{-1}. This culture was able to grow at methanol concentrations up to 1.75 M.

When *M. buryatense* 5B was grown at a nearly neutral pH (7.5), the maximum growth rate (0.12 h^{-1}) was observed in the presence of 0.125 M methanol, whereas higher CH_3OH concentrations considerably inhibited growth (Fig. 2). When NaCl was not added (in this case, the concentration of Na^+ ion in the medium was about 0.1 M), the growth rate on almost all the methanol concentrations used was lower than in the presence of 0.75 or 3% NaCl. In the latter case, the most intense growth with μ_{max} = 0.17 h^{-1} was observed at a methanol concentration of 0.75 M. The upper halotolerance limit of growth on methanol (6% NaCl) was higher than that of growth on methane (4% NaCl).

The *M. buryatense* 5B culture grown on 0.25 M methanol accumulated formaldehyde and formate at concentrations of 1.2 and 8 mM, respectively. When methane-grown cells were transferred to the medium with methanol, the accumulation of formaldehyde occurred only in the early period of cell adaptation to methanol. Formaldehyde decline in the medium was accompanied by the accumulation of formate and the initiation of bacterial growth. After the exhaustion of formaldehyde in the medium, the culture grew actively (Fig. 3). In methane-grown cultures (μ_{max} = 0.1 h^{-1}), formaldehyde could not be detected in the medium, and the concentration of formate did not exceed 2 mM.

The growth of *M. buryatense* 5B on methanol was accompanied by the accumulation of a viscous glycoprotein in the medium. The carbohydrate moiety of the EGP contained predominantly glucose, mannose, and amino sugars, as well as minor amounts of galactose, xylose, ribose, and rhamnose (Table 1). The protein

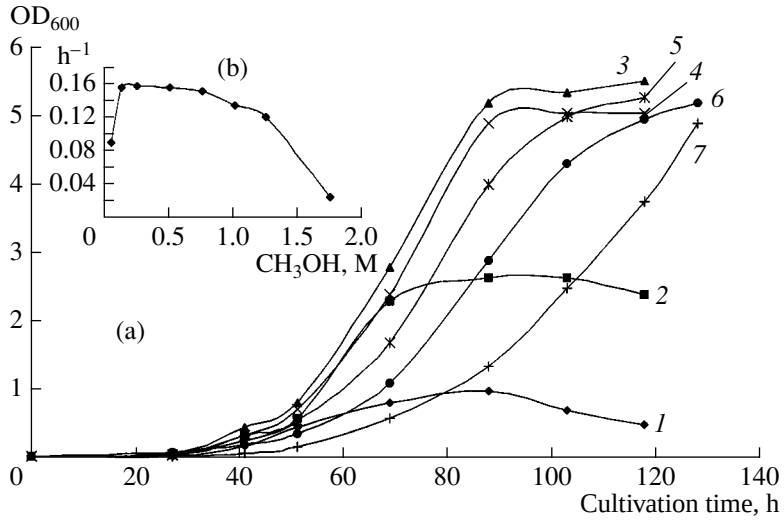

Fig. 1. Growth of *M. buryatense* 5B at pH 9.5 in the medium containing 0.75% NaCl and methanol at various concentrations (M): *1*, 0.05; *2*, 0.13; *3*, 0.25; *4*, 0.5; *5*, 0.75; *6*, 1.0; and *7*, 1.25. The inset shows the dependence of the specific growth rate on the methanol concentration in the medium.

moiety of the EGP was dominated by glutamate, aspartate, glycine, valine, and isoleucine. The glycoprotein content increased from 2.63 to almost 5 g/l (3.4 g/g cells) when the concentration of potassium nitrate in the medium was augmented tenfold (Table 2). However, the biomass yield did not change. Figure 4 shows the ultrastructure of methanol-grown *M. buryatense* 5B cells from different growth phases, when the growth medium was inoculated with logarithmic-phase methane-grown cells (the ultrastructure of such cells is shown in Fig. 5). Methanol-grown cells from the lag phase (OD_{600} = 0.16) were found to contain intracytoplasmic membranes (ICMs) of type I (Fig. 4a), which filled most of the cytoplasm, and some amount of polysaccharide inclusions, analogous to those of methane-grown cells. Methanol-grown cells from the early exponential phase (OD_{600} = 1.3) contained ICMs and polysaccharide inclusions (Fig. 4b) in proportions typical of methane-grown cells from the late exponential phase. In the course of further growth (mid- and late-exponential cells from cultures with OD_{600} = 2.7 and 3.8, respectively), the cytoplasm was completely filled with polysaccharide granules, whereas the ICMs became reduced (Figs. 4c and 4d). The fact that the

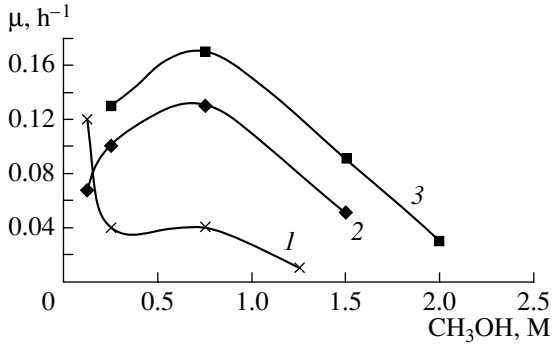

Fig. 2. The specific growth rates of *M. buryatense* 5B at different concentrations of methanol in the medium containing (*1*) 0.75% NaCl (pH 7.5), (*2*) 0% NaCl (pH 9.5), and (*3*) 3% NaCl (pH 9.5).

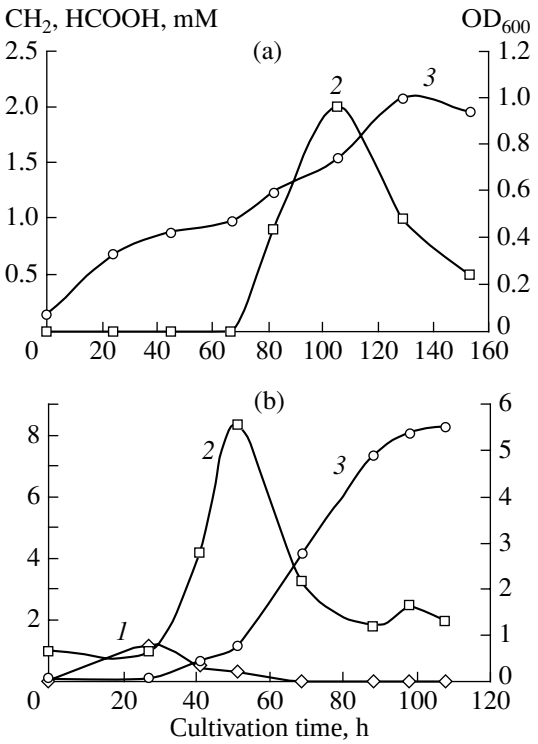

Fig. 3. Accumulation of (*1*) formaldehyde and (*2*) formate during (*3*) the growth of *M. buryatense* 5B on (a) methane and (b) 0.25 M methanol at pH 9.5 in the presence of 0.75% NaCl.

granules had a polysaccharide nature was evident from their intense staining with ruthenium red. The ^{1}H-NMR spectra of methanol-grown cells were similar to those of plant glycogens (Fig. 6). Estimations showed that glycogen may comprise up to 35% of the mass of methanol-grown cells. Unlike the polysaccharide granules of methanol-grown cells, those of methane-grown cells showed up as faint objects under the electron microscope (Figs. 4 and 5). Presumably, this was due to the lower permeability of the cell wall of methane-grown bacteria to ruthenium red.

The activities of glycogen synthase, sucrose-6-phosphate synthase, and NADH dehydrogenase in methanol-grown cells were higher than in methane-grown cells (Table 3). The activities of the dehydrogenases of methanol, formaldehyde, formate, and NADH in these two types of cells were almost the same. The cell-free extracts of *M. buryatense* 5B actively oxidized formaldehyde at alkaline pH values in the presence of NH_4^+, i.e., under conditions favorable to methanol dehydrogenase.

Rotenone at a concentration of 200 μM decreased the growth rate of cells on methanol by 14% and did not influence their growth on methane. At the same time, HQNO inhibited the growth of both methane- and methanol-grown cells by 17 and 16%, respectively.

DISCUSSION

The haloalkalitolerant methanotroph *M. buryatense* 5B is able to grow within a wide range of pH values (7.0–10.5) and salinities (0–4% NaCl), showing the most active growth at pH 9.5 and 0.75% NaCl. This methanotroph is tolerant to relatively high concentrations of methanol in the medium. Its growth on methanol at an optimum concentration of 0.25 M is faster than with methane as the growth substrate. The methanol resistance of the strain is higher at alkaline than at neutral pH values. The halotolerance of *M. buryatense* 5B is at a maximum (6% NaCl) when this bacterium grows on methanol.

Methanol-grown *M. buryatense* 5B cells excrete formaldehyde and formate in greater amounts than methane-grown cells, indicating that the formation and assimilation of these intermediates in the methanol-grown cells are unbalanced. It is known that the reaction of methane oxygenation by soluble and particulate methane monooxygenases (sMMO and pMMO) requires NADH and reduced cytochromes, respectively. In turn, NADH and reduced cytochromes are produced during the oxidation of formate by NAD-dependent and PMS-dependent dehydrogenases. In the presence of methanol as the growth substrate, NADH and reduced cytochromes are scarce, which favors the accumulation of formaldehyde and formate in the cells and medium.

In the course of the adaptation of *M. buryatense* 5B to methanol, formaldehyde is gradually exhausted in

Table 1. The carbohydrate and amino acid composition of the extracellular glycoprotein of *M. buryatense* 5B

Compound	Content, wt % of dry cells
Carbohydrates	
Glucose	35.1
Xylose	2.78
Galactose	4.18
Mannose	11.21
*X*1	Traces
Ribose	2
Rhamnose	0.69
*i*2	4.5
Cellobiose	0.66
Aminosugars	15.77
Total carbohydrates	77
Amino acids	
Lysine	0.83
Histidine	0.37
Arginine	0.77
Aspartate	2.72
Threonine	1
Serine	1
Glutamate	3.5
Proline	0.03
Glycine	3.07
Alanine	0.9
Valine	2.5
Methionine	0.34
Isoleucine	1.97
Leucine	0.63
Tyrosine	1.3
Phenylalanine	1.7
Total amino acids	23

Table 2. The content of extracellular glycoprotein in the culture liquid of *M. buryatense* 5B grown on methane or methanol in the presence of different concentrations of KNO_3

Growth substrate	KNO_3 concentration, g/l	Cell biomass, g/l	Glycoprotein, g/l
Methane	1	0.41	0.8
Methanol, 0.25 M	1	1.69	2.63
	3	1.48	3.57
	10	1.44	4.96

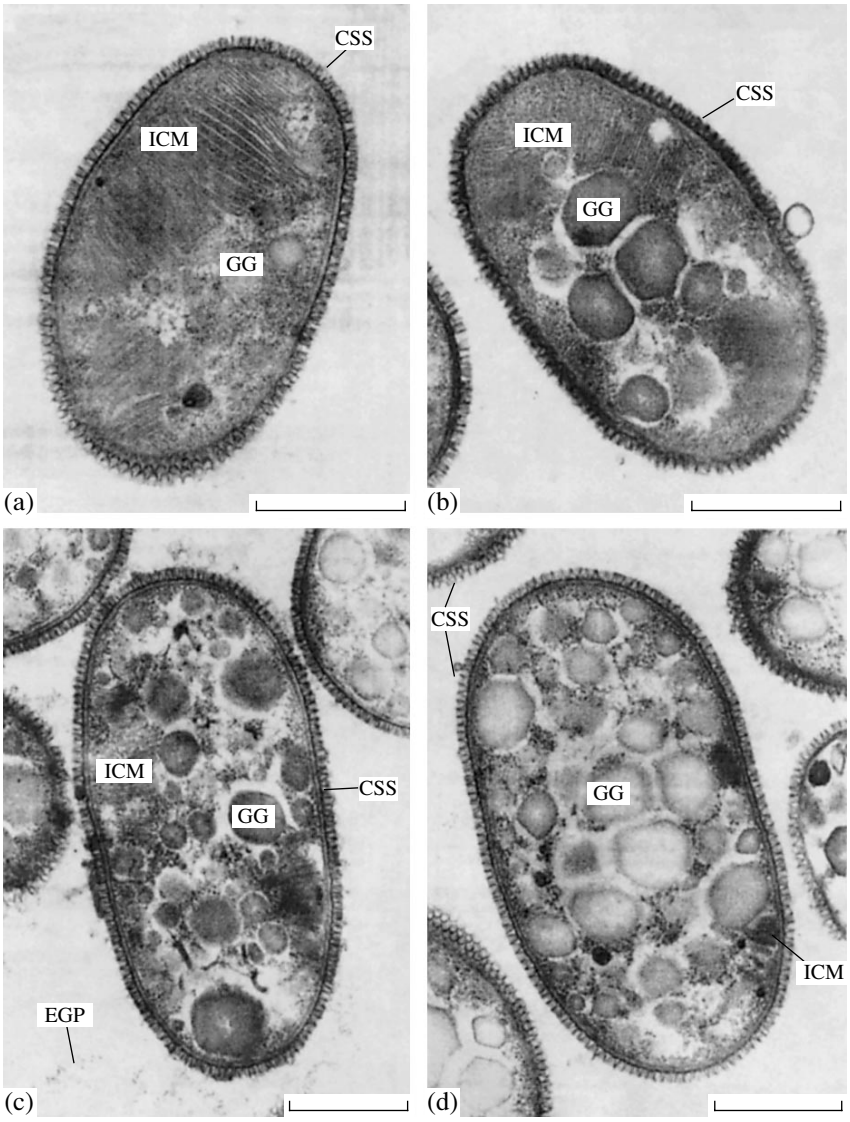

Fig. 4. The ultrastructure of *M. buryatense* 5B cells grown at pH 9.5 in the medium containing 0.25 M methanol and 0.75% NaCl to a culture density (OD_{600}) of (a) 0.15, (b) 1.3, (c) 2.7, and (d) 3.8. CSS, cup-shaped structures; ICM, intracytoplasmic membranes; GG, glycogen granules; and EGP, extracellular glycoprotein. The scale bars represent 0.5 μm.

the medium, presumably due to the synthesis of intra- and extracellular polysaccharides. This bacterium assimilates formaldehyde through the ribulose monophosphate (RuMP) pathway, in which 3-hexulose-6-phosphate synthase (HPS) catalyzes the condensation of formaldehyde and ribulose-5-phosphate with the formation of hexulose-6-phosphate. The latter isomerizes into fructose-6-phosphate. Therefore, phosphohexoses (precursors of glycogen, sucrose, and polysaccharides) are formed already at the early stages of methanol assimilation. The increased activities of sucrose-6-phosphate synthase and glycogen synthase in methanol-grown cells suggest that the synthesis of polysaccharides serves to sink formaldehyde and thus to mitigate its toxic effect on the cells. On the other hand, the activity of HPS in both methanol- and methane-grown cells is the same, suggesting that this enzyme is regulated by the concentration of the reaction substrates. It should be noted in this regard that the K_M of the HPS of *Methylococcus capsulatus* with respect to formaldehyde (0.47 mM) [6] is comparable with the concentration of formaldehyde accumulated by methanol-grown *M. buryatense* 5B cells in the cultivation medium (1 mM). If the kinetic properties of the HPSs of these two bacteria are close, this concentration of formaldehyde should correspond to the maximum rate of the primary C_1-assimilation.

The consumption of formate from the medium during the period of the active growth of the methanotroph suggests that formate is oxidized by formate dehydrogenase to CO_2. This process may be associated with the functioning of the respiratory chain and, therefore, with

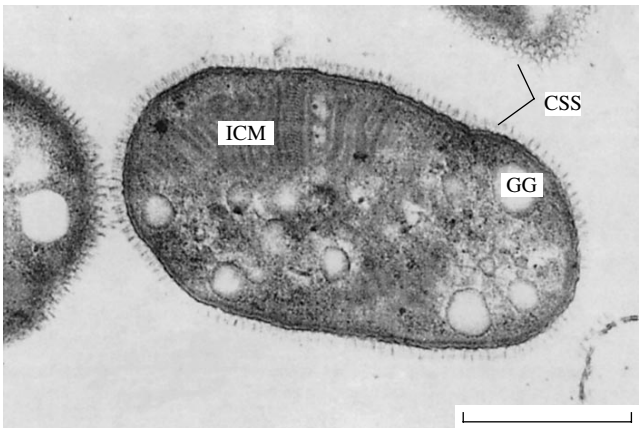

Fig. 5. The ultrastructure of *M. buryatense* 5B cells grown on methane at pH 9.5 in the presence of 0.75% NaCl to the late logarithmic growth phase. Designations and the scale bar are as in the legend to Fig. 4.

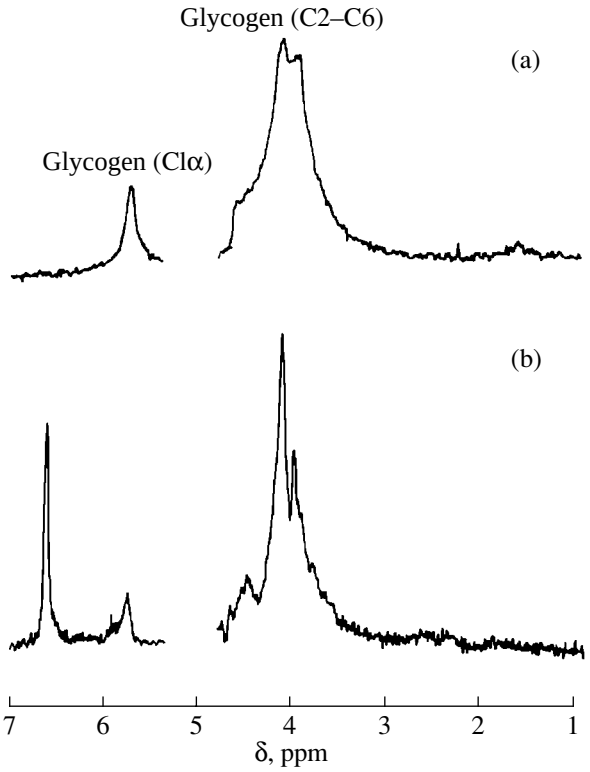

Fig. 6. The ^{1}H-NMR spectra of (a) plant glycogens and (b) *M. buryatense* 5B cells grown at pH 9.5 in the medium containing 0.25 M methanol and 0.75% NaCl.

the formation of ATP. Taking into account that the latter is necessary for the synthesis of polysaccharides, it can be suggested that the respiration of cells increases in the process of their adaptation to methanol.

The NADH dehydrogenase of *M. buryatense* 5B seems to be also involved in the adaptation of this bacterium to methanol, as judged from the increased activity of this enzyme in methanol-grown cells and the enhanced sensitivity of their respiration to rotenone. The enhanced methanol tolerance of *M. buryatense* 5B cells at alkaline pH values and high medium salinities suggests that the reactions maintaining intracellular ionic homeostasis are associated with the oxidation of NADH. The origin of these reactions in halotolerant alkaliphilic methanotrophs needs to be studied.

Marine methanotrophs of the genus *Methylomicrobium* have also been found to be resistant to methanol [7, 15]. Some of the isolates of this genus synthesize glycogen, whereas the halotolerant and halophilic isolates additionally synthesize sucrose [9, 14]. The haloalkaliphilic methanotroph *M. alcaliphilum* 20Z utilizes NADH and ATP to maintain the sodium gradient and the flagellum rotation [16]. The activity of NADH dehydrogenase peaked at neutral and alkaline pH values, indicating that this enzyme may occur in different isoforms and that it is involved in the maintenance of intracellular pH [17].

Table 3. Some enzymatic activities in *M. buryatense* 5B cells grown on methane or methanol

Enzyme	Coenzyme	Growth substrate	
		methane	methanol, 0.25 M
Methanol dehydrogenase with methanol	PMS	296	274
	PMS	192	157
Methanol dehydrogenase with formaldehyde	PMS	7	10
Formate dehydrogenase	PMS	41	35
	NAD^+	3	12
3-Hexulose-6-phosphate synthase		224	201
Sucrose-6-phosphate synthase		39	96
Glycogen synthase		4	7
NADH dehydrogenase		426	605

Note: Enzymatic activities were expressed in nmol/(min mg protein). PMS is phenazine methosulfate.

Neutrophilic nonhalophilic methanotrophs with the serine pathway of C_1 metabolism may also adapt to high methanol concentrations. For instance, after prolonged adaptation to methanol, *Ms. trichosporium* OB3b and *Mcs. parvus* OBBP acquired the ability to grow on 4% methanol [4, 5]. The former bacterium grown on methanol synthesized polyhydroxybutyrate, whereas the latter accumulated a heteropolysaccharide in amounts of up to 62% of the cell biomass. Unlike methylotrophs with the RuMP pathway, bacteria with the serine pathway utilize not only ATP but also NADH for the synthesis of storage polymers [18–20]. Such a synthesis in type II methanotrophs is believed to serve to detoxify formaldehyde and to sink excess energy.

To conclude, haloalkaliphilic and haloalkalitolerant methanotroph, which are able to grow within wide ranges of pH, salinity, and methanol concentrations, may present not only theoretical but also practical interest as producers of valuable biopolymers.

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