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EXPERIMENTAL ARTICLES

Synthesis of Osmoprotectants by Halophilic and Alkaliphilic Methanotrophs

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Abstract—The ^{1}H-NMR analysis of methanol extracts of halophilic and halotolerant alkaliphilic methanotrophs isolated from the soda lakes of Southern Transbaikal and Tuva showed that bacterial cells grown at an optimum salinity accumulated mainly sucrose and 5-oxo-1-proline, whereas cells adapted to 0.5–1.0 M NaCl additionally synthesized ectoine. A more detailed study showed that nitrogen deficiency in the growth medium of *Methylobacter alcaliphilus* 20Z decreased the synthesis of nitrogen-containing osmoprotectants, ectoine and 5-oxo-1-proline. *M. alcaliphilus* 20Z cells exhibited activities of UDP-glucose pyrophosphorylase and sucrose-phosphate synthase involved in sucrose synthesis. Glutamine synthetase in vitro did not require NH_4^+ ions, which implies that this enzyme is involved in 5-oxo-1-proline synthesis. Cells grown at high salinity exhibited elevated levels of aspartate kinase, aspartate-semialdehyde dehydrogenase, and ectoine synthase. This suggests that ectoine is synthesized via aspartate and aspartate-semialdehyde, i.e., via the route earlier established for extremely halophilic bacteria.

Key words: osmoprotectants, ectoine, sucrose, 5-oxo-1-proline, methanotrophs, halophiles, alkaliphiles.

The adaptation of microorganisms to high concentrations of NaCl in a medium is associated with the synthesis and accumulation in their cells of special organic and inorganic solutes (osmoprotectants or osmolytes) in concentrations sufficient to balance the increased external osmotic pressure [1–3]. The osmoprotectants of halophilic and halotolerant aerobic bacteria are, as a rule, low-molecular-weight organic compounds compatible with the bacterial metabolism. The accumulation of osmoprotectants by bacterial cells in high amounts suggests that these compounds, which are also employed as stabilizers of biomolecules and whole cells [4], can be produced on an industrial scale by microbiological methods. Of much interest in this respect are halophilic bacteria [5, 6].

Recently, a number of salt-dependent methanotrophs have been isolated from seawater [7, 8], limans, and the soda lakes of Ukraine, Tuva, and Southern Transbaikal [9–12]. The aim of the present work was to investigate the accumulation of osmoprotectants and to elucidate the pathways of their biosynthesis in some novel strains of haloalkaliphilic methanotrophs.

MATERIALS AND METHODS

Strains and cultivation conditions. The four strains of methanotrophs used in this study were isolated from the Southern Transbaikal soda lakes [11] and preliminarily assigned to the genus *Methylomicrobium*. The halophilic alkalitolerant methanotrophic strains 5G and 7G and halotolerant alkaliphilic strains 5B and 6G were grown batch-wise in mineral medium P in a methane–air (1 : 1) atmosphere in the presence of different concentrations of NaCl [12]. *Methylobacter alcaliphilus* 20Z, which was isolated from the saline alkaline Lake Shara-Nur of Tuva, was grown as described earlier [10]. The neutrophilic nonhalophilic methanotroph *Methylobacter chroococcum* 72, obtained from the Culture Collection of the Skryabin Institute of Biochemistry and Physiology of Microorganisms, was grown in mineral medium P without NaCl.

The effect of methionine D,L-sulfoximine (MSI) on the growth of *M. alcaliphilus* 20Z was studied at different salinities of the mineral growth medium. In these experiments, MSI was sterilized by filtering it through 0.2-μm-pore-size membrane filters.

Extraction and analysis of osmoprotectants. Low-molecular-weight organic compounds were extracted from cells grown at an optimum (0.75% NaCl) and maximum (4–6% NaCl) salinity of the medium. For this purpose, 100 mg of cells (wet biomass) were suspended in 2 ml of methanol and shaken for 1 h. The suspension was then centrifuged at 5000 *g* for 5 min, and the supernatant was dried in a vacuum. The residue was suspended in 0.5 ml of a 0.01 M solution of maleic acid in D_2O and analyzed in a high-resolution WP 80 SY NMR spectrometer (Bruker, Germany) with

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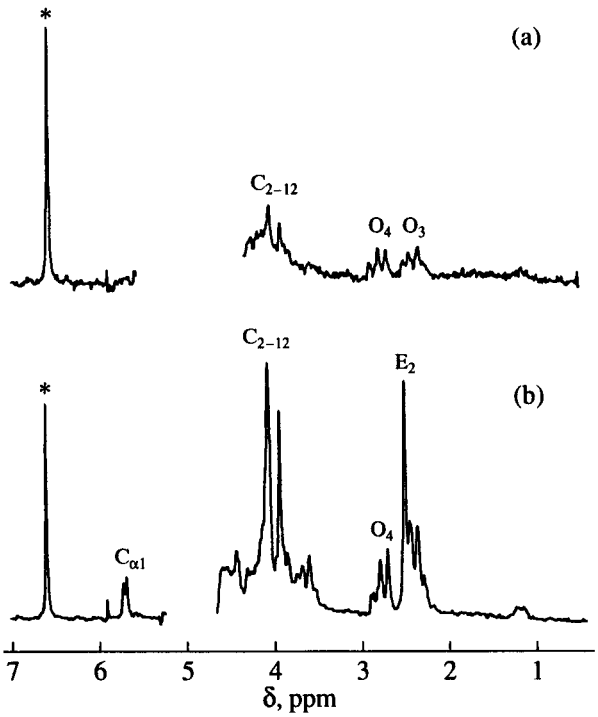

Fig. 1. ^{1}H-NMR spectra of methanol extracts of strain 7G cells grown in media with (a) 0.75% NaCl and (b) 5% NaCl. Proton signals $C_{\alpha 1}$ and C_{2-12} correspond to the carbon atoms of sucrose, signals O_3 and O_4 correspond to the -CH_2 groups of 5-oxo-1-proline, and signal E_2 corresponds to the methyl group of ectoine.

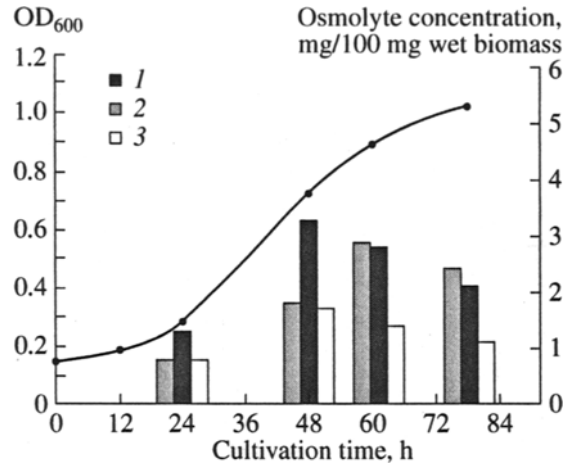

Fig. 2. Dynamics of osmolytes in *M. alcaliphilus* 20Z cells during their cultivation in a medium with 5 mM KNO_3 and 0.2% methanol: *1*, ectoine; *2*, sucrose; and *3*, 5-oxo-1-proline.

a working frequency of 80 MHz. The content of extracted metabolites in samples was estimated by comparing the intensities of their proton signals with those of maleic acid [13].

Enzyme assays. Cell extracts for enzyme assays were prepared as described earlier [14]. The activity of glutamine synthetase (EC 6.3.1.2) was determined from the ADP formation rate [15] with the exogenously added lactate dehydrogenase (LDH) and pyruvate kinase (PK). The reaction mixture (1 ml) contained (μmol) Tris–HCl buffer (pH 7.3), 10; ATP, 5; NH_4Cl, 50; $MgCl_2$, 50; dithiothreitol (DTT), 4; phosphoenolpyruvate (PEP), 5; NADH, 0.5; PK, 4 U; LDH, 1 U; and cell extract. The reaction was started by adding 80 μmol of L-glutamate Na. The activities of glutamate dehydrogenase (EC 1.4.1.2), alanine dehydrogenase (EC 1.4.1.1), and glutamate synthase (EC 2.6.1.53) were evaluated from the oxidation rate of NADH or NADPH [16]. UDP-glucose pyrophosphorylase and sucrose-phosphate synthase (EC 2.4.1.14) were assayed by measuring the UDP formation rate in the reaction mixture (2 ml) containing (μmol) Tris–HCl buffer (pH 7.5), 10; UTP, 0.5; glucose-1-phosphate, 0.5; PEP, 5; $MgCl_2$, 50; NADH, 0.25; PK, 4 U; and LDH, 1 U. The reaction was started by adding 0.5 μmol of fructose-6-phosphate. Aspartate kinase (EC 2.7.2.4) was assayed by measuring the rate of formation of ADP from ATP [17]. The activity of aspartate-semialdehyde dehydrogenase was estimated from the oxidation rate of NAD(P)H in the reaction mixture (2 ml) containing (μmol) Tris–HCl buffer (pH 7.5), 10; ATP, 0.5; $MgCl_2$, 50; NAD(P)H, 0.25; and aspartate, 10. Ectoine synthase was assayed as described by Peters *et al.* [18].

The protein concentration was measured by the method of Lowry *et al.* Nitrate was quantified by the conventional method [19].

RESULTS AND DISCUSSION

Analysis of the ^{1}H-NMR spectra of methanol extracts of halophilic and halotolerant alkaliphilic methanotrophs (Fig. 1 illustrates such spectra for strain 7G, whereas the results of the analysis of all strains are summarized in Table 1) showed that cells of four strains, 5G, 6G, 7G, and 5B, grown at optimum salinity (0.75% NaCl) contained 5-oxo-1-proline (pyrrolidone-5-carboxylic acid). Cells of the moderately halophilic strains 5G and 7G additionally accumulated sucrose (Fig. 1). It should be noted that cells of all of these strains grown at a low salinity of the medium (0.2% NaCl) did not accumulate 5-oxo-1-proline or sucrose in amounts sufficient for their detection by the ^{1}H-NMR method [13].

At an elevated salinity of the medium (3–5% NaCl), all of the strains studied exhibited the presence of the cyclic amino acid ectoine (2-methyl-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid) (Table 1). The relative content of ectoine in the halotolerant alkaliphilic strains 5B and 6G was higher than in the moderately halophilic strains 5G and 7G.

Unlike these strains isolated from the soda lakes of Southern Transbaikal, the halotolerant alkaliphilic methanotroph *M. alcaliphilus* 20Z isolated from the soda lake of Tuva synthesized ectoine not only in the

presence but also in the absence of NaCl in the medium (Table 1). This fact suggests that the enzymes involved in ectoine biosynthesis in this methanotroph are constitutive.

Investigation of the dynamics of osmoprotectants in *M. alcaliphilus* 20Z cells grown batch-wise in a medium with 1 M NaCl and 5 mM KNO_3 as the source of nitrogen showed that the cellular content of the nitrogen-containing osmoprotectants 5-oxo-1-proline and ectoine was maximum in the midexponential growth phase, whereas the sucrose content was maximum at the beginning of the growth-retardation phase (Fig. 2). Then, the total cellular content of osmoprotectants decreased. Such cellular dynamics of osmoprotectants in strain 20Z suggests that they can be used for the synthesis of other cellular constituents. In particular, the decrease in the osmoprotectant content in the stationary-phase cells may be associated with the triggering of metabolism to the synthesis of glycogen, whose granules take up most of the cytoplasm of methanotrophic cells limited in nitrogen source [13].

The residual content of NO_3^- in the cultivation medium in the growth retardation phase was 1.56 mM, i.e., one-third of its initial concentration. This suggests that nitrate reductase or the transport system for NO_3^- has a low affinity for nitrate. In this case, the low rates of NH_4^+ formation in cells may limit the synthesis of nitrogen-containing osmoprotectants.

In a medium with 30 mM KNO_3, the content of 5-oxo-1-proline and ectoine but not of sucrose in the *M. alcaliphilus* 20Z cell increased (data not presented). The residual NO_3^- content in the growth medium in the stationary phase was 16 mM. In a neutral medium with 30 mM NH_4Cl, the cellular content of osmoprotectants was still higher (Table 2). These data suggest that nitrates present in the growth medium can control the synthesis of nitrogen-containing osmoprotectants.

M. alcaliphilus 20Z failed to grow in media with ammonium salts, irrespective of the presence of nitrates, at pH 9.0 (the typical optimum pH value for the growth of this alkaliphile) but grew at pH 7.2. Similar observations were published earlier by Lees *et al.* [8], who showed that marine methanotrophs cannot grow at a salinity of 2% NaCl and pH 7.6 and explained this by the absence of transport systems for ammonium ions in these methanotrophs, whose natural habitats contain both nitrates and ammonium salts.

The ability of *M. alcaliphilus* 20Z to use ammonium salts as nitrogen sources in media with neutral pH values implies that this methanotroph possesses a system for the transport of ammonium cations into cells. We also cannot exclude the possibility that NH_4^+ ions affect the cellular homeostasis of methanotrophs or that these ions competitively inhibit methane monooxygenase (MMO). The latter possibility is most likely, since, at alkaline pH values, ammonium occurs mainly in the neutral form, NH_3, which is the MMO substrate.

M. alcaliphilus 20Z cells grown in media with ammonium contained greater amounts of osmoprotectants than cells grown in media with nitrate as the nitrogen source. This may be due to the fact that the energy expenses of cells on the NH_4^+ transport are lower than on the nitrate reduction.

The growth of *M. alcaliphilus* 20Z and the neutrophilic nonhalophilic methanotroph *M. chroococcum* 72 was suppressed by low concentrations (<0.5 mM) of MSI, an inhibitor of glutamine synthase (GS), suggesting an important role of this enzyme in the assimilation of NH_4^+ by the morphotype I methanotrophs (Table 3).

Table 1. Cellular content of osmoprotectants (nmol/mg dry biomass) in some haloalkaliphilic methanotrophic isolates

Strain	NaCl concentration, %	Ectoine	Oxo-1-proline	Sucrose
5G	0.75	0	171	88
	3	99	93	94
6G	0.75	0	22	0
	5	127	85	73
7G	0.75	0	160	29
	5	250	320	205
5B	0.75	0	27	<0
	3	282	39	30
20Z	0	33	38	0
	3	448	148	140

Table 2. Content of osmoprotectants (nmol/mg dry biomass) in *M. alcaliphilus* 20Z cells grown in a medium with 6% NaCl at different concentrations of KNO_3

Osmolyte	Concentration of mineral nitrogen		
	5 mM KNO_3	30 mM KNO_3	30 mM NH_4Cl
Ectoine	739	810	1021
Sucrose	205	175	401
Oxo-1-proline	426	465	560

Table 3. Inhibition (%) of the growth of *M. alcaliphilus* 20Z and *M. chroococcum* 72 by methionine D,L-sulfoximine (MSI)

Species	MSI concentration, mM	–NaCl	6% NaCl
M. alcaliphilus 20Z	0.05	36	3
	0.5	93	74
M. chroococcum 72	0.05	72	100
	0.5	77	100

Table 4. Effect of NaCl in the growth medium on the activity (nmol/(min mg protein)) of some enzymes involved in the biosynthesis of osmoprotectants in *M. alcaliphilus* 20Z cells

Enzyme	Cofactor	–NaCl	6% NaCl
Glutamate dehydrogenase	NADPH	4	0
	NADH	0	0
Glutamate synthase	NADPH	5	6
	NADH	0	0
$+NH_4Cl$	ATP	76	74
Glutamine synthetase			
$-NH_4Cl$	ATP	28	30
Alanine dehydrogenase	NADPH	0	0
	NADH	7	19
Aspartate kinase	ATP	10	105
UDP-glucose pyrophosphorylase/sucrose-phosphate synthase	UTP	91	89
Aspartate-semialdehyde dehydrogenase	NADPH	11	70
Glutamate–oxaloacetate aminotransferase		21	76
Glutamate–pyruvate aminotransferase		0	17
Alanine–oxaloacetate aminotransferase		15	20
Ectoine synthase		10	50

At an elevated salinity of the medium (6% NaCl), strain 20Z probably employs, in addition to the GS/GOGAT system (GOGAT = glutamate–oxoglutarate aminotransferase = glutamate synthase), some other mechanisms for the NH_4^+ assimilation, due to which the growth of this strain is but slightly inhibited by MSI.

M. alcaliphilus 20Z exhibited the activities of NADPH-dependent glutamate dehydrogenase, NAD-dependent alanine dehydrogenase, GS, and NADPH-dependent glutamate synthase (Table 4). These data suggest that strain 20Z assimilates ammonium through the reductive amination of α-ketoglutarate and pyruvate and via the glutamate cycle. NaCl present in the growth medium stimulated the synthesis of alanine dehydrogenase by several times (Table 3), although the activities of GS, GOGAT, and glutamate dehydrogenase virtually did not change, indicating the constitute synthesis of these enzymes.

The GS of *M. alcaliphilus* 20Z was active even in the absence of NH_4^+. This means that this enzyme can catalyze the ATP-dependent conversion of glutamate into 5-oxo-1-proline, similarly to the way in which it occurs in *Escherichia coli* [15]. Therefore, the GS of *M. alcaliphilus* 20Z may be responsible for the synthesis of both glutamine and 5-oxo-1-proline, the proportion between these amino acids being regulated by the intracellular levels of ammonium and glutamate.

Along with the aforementioned enzymatic activities, the *M. alcaliphilus* 20Z strain displayed the constitutive activities of UDP-glucose pyrophosphorylase and sucrose-phosphate synthase, as well as the inducible activities of aspartate kinase, aspartate-semialdehyde dehydrogenase, and ectoine synthase. These enzymes may be involved in the biosynthesis of ectoine by the pathway suggested earlier for other halophilic bacteria [18].

In marine salt-dependent methanotrophs, NH_4^+ is assimilated in a similar way [8]. However, based on the low affinity of alanine dehydrogenase for NH_4^+, Lees *et al.* questioned the involvement of this enzyme in the NH_4^+ assimilation. Our data that indicate a derepression of alanine dehydrogenase synthesis at high salinities of the medium show an important role of this enzyme in the osmoadaptation of *M. alcaliphilus* 20Z cells. The derepression can be due to a deficiency of pyruvate in cells caused by its decarboxylation into acetyl-CoA, which is involved in ectoine synthesis at the stage of acetylation of diaminobutyrate [8].

The identical sets of osmoprotectants in the methanotrophs studied suggest that their primary and central metabolisms are similar. All of the strains implement the ribulose monophosphate (RuMP) pathway of formaldehyde fixation and assimilate ammonium via the GS/GOGAT enzymatic system involving alanine and glutamate dehydrogenases. The primary products of the RuMP pathway, phosphohexoses, provide sucrose synthesis with precursors (glucose-1-phosphate and fructose-6-phosphate) in the reactions catalyzed by

UDP-glucose pyrophosphorylase and sucrose-phosphate synthase.

Sucrose has long been recognized as an osmoprotectant in some halophilic phototrophic bacteria [20]. Ectoine, which was first revealed in phototrophic haloalkaliphilic bacteria of the genus *Ectothiorhodospira*, is also a well-known bacterial osmoprotectant [19]. The data presented here suggest that the halotolerant alkaliphilic methanotroph *M. alcaliphilus* 20Z and extreme halophiles [18] synthesize ectoine in similar ways. Our ^{1}H-NMR data show that salt-dependent methanotrophs can accumulate 5-oxo-1-proline in high amounts. This compound is evidently less toxic to cells than glutamate or glutamine (obligate methanotrophs are inhibited by many organic substances, including amino acids [21–23]) and, hence, conforms to one of the criteria of osmoprotectants, i.e., metabolic nontoxicity. In this connection, it is noteworthy that the activity of GS did not increase in cells grown at high salinity but decreased in cells grown in the presence of ammonium salts.

Considerable variations in the cellular content of osmoprotectants in the methanotrophs studied can be accounted for by their ability to synthesize not only low-molecular-weight osmoprotectants but also extracellular polymeric compounds involved in the synthesis of surface S layers, which are capable of stabilizing the bacterial cell wall under osmotic stress conditions. The mechanisms regulating the partitioning of the metabolic fluxes of methanotrophs to the synthesis of glycoprotein S layers or to the accumulation of low-molecular-weight osmoprotectant remain to be studied.

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