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Mode of sodium ion action on methanogenesis and ATPase of the moderate halophilic methanogenis bacterium *Methanohalophilus halophilus*

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Cells of *Methanohalophilus halophilus* swelled when exposed to hypotonic solutions of NaCl at pH 7.0. The swelling of the cells ceased in the presence of Mg^{2+}. Methane formation by non-growing cells was strongly dependent on the NaCl concentration. Among other monovalent and divalent cations only Li^+ and Mg^{2+} could partly substitute for a specific function of sodium ions. The artificial Na^+/H^+ antiporter, monensin, exerted a strong inhibitory effect on methane formation from methylamine. The membrane-bound Mg^{2+}-stimulated ATPase of these cells was enhanced at low (40 mM) NaCl concentration while higher concentrations of this solute were inhibitory. The results obtained show that sodium ions are a prerequisite for optimal methane formation and ATPase activity in these cells. However, both of these processes required different sodium ion concentrations.

Halophilic methanogen; Sodium ion; Methanogenesis; ATPase; *Methanohalophilus halophilus*

1. INTRODUCTION

Methanogenic bacteria exhibit extreme habitat diversity, but moderate and extreme halophilic methanogenic bacteria from saline and hyper-saline ecosystems have been isolated only recently [1–5]. The moderate halophilic bacteria require up to 20% sodium chloride for their growth. Their intracellular salt concentration is considerably lower than that of the medium, and the salt often can be replaced by low molecular weight organic osmolytes [6–8].

The first moderate halophilic methylotrophic methanogenic bacteria, which grow at salinities of up to 15% NaCl, were isolated from modern stromatolytes in Shark Bay, Australia [1]. This obligate halophilic methanogenic bacterium *Methanohalophilus halophilus*, grows optimally with monomethylamine as a sole carbon and energy source in the presence of 7% sodium chloride. Sodium chloride was shown to be obligatory for growth of these bacteria [1].

The function of sodium ions in halophilic bacteria is not yet completely understood. The lowering of the NaCl concentration in the medium generally can lead to irreversible structural and functional changes in these cells [9]. Many enzymes of halophilic bacteria are denatured at low concentrations, especially in the complete absence of salt [10]. In the last few years considerable evidence has been accumulated for the importance of sodium ions as coupling factors in bacterial energetics [11,12]. It has also been firmly established that sodium ions are involved in the energy coupling between some endergonic and exergonic reactions in methanogenic bacteria [13,14]. However, information on the function of sodium ions in moderate halophilic methanogenic bacteria is completely missing.

The results presented here show that sodium ions can play an inportant role in methanogenesis from methylamine, and of membrane-bound, DCCD-sensitive ATPase in cells of the moderate halophilic methanogenic bacterium *Methanohalophilus halophilus*.

2. MATERIALS AND METHODS

Methanohalophilus halophilus strain Z-7982 was cultivated with 0.5% monomethylamine and 7% NaCl in a growth medium as described earlier [1]. Cells in the late logarithmic phase of growth were harvested by anaerobic centrifugation, washed twice and resuspended in the appropriate buffer containing 7% NaCl.

For continuous monitoring of swelling of the cells the rate of absorbance decrease at 578 nm was followed with a recording spectrophotometer (Beckman M-25).

For the measurement of ATPase activity the membrane preparation was used. The membrane fraction was prepared as follows: cells of *M. halophilus* were harvested by centrifugation and washed twice with 50 mM potassium-glycyl-glycine, 100 mM KCl, 10 mM $MgCl_2$, pH 7.5, buffer solution. A few grains of DNAse were added to the cell suspension and cell breakage was effected by sonication in a Soniprep M 150 sonifier in the pulse mode for 4 min at 3°C. Disrupted cells were centrifuged at 25,000 × *g* for 15 min at 4°C. The cell-free supernatant was centrifuged aerobically at 120,000 × *g* for 1 h at 4°C. The resulting sediment was washed twice with the buffer described above. The

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membrane fraction (10–15 mg protein per ml) in 2 ml of 50 mM glycyl-glycine buffer, pH 7.5, containing 100 mM KCl and 5 mM $MgCl_2$ was used for measuring of ATPase activity.

ATPase activity was assayed by the measurement of inorganic phosphate released from ATP [23]. The reaction mixture contained in 1 ml: 3 mM ATP, 5 mM $MgCl_2$, 100 mM glycyl- glycine, pH 8.0 and 500 μg protein. After 5 min pre-incubation, the reaction was started by the addition of ATP and incubated for 10 min at 37°C. A blank sample without Mg^{2+} was substracted from every sample. Detailed descriptions of the assay conditions and of the assay mixtures are given in the legends to the figures.

Methane formation from monomethylamine in the cell suspension was measured as described earlier [24] by gas chromatography on a Chrom 5 Chromatograph equipped with a thermal-conductive detector. Samples of the gaseous phase were taken from the cultivation flasks by gas-tight syringes (Pierce, Series A-2).

Protein was determined according to [25] using bovine serum albumin as a standard.

All chemicals were of reagent grade purity and purchased from Lachema Brno, except for monensin, dicyclohexylcarbodiimide (Serva) and ATP (Calbiochem).

3. RESULTS AND DISCUSSION

Gram-negative bacterial cells can swell or shrink depending upon the relative concentration of a solute inside and outside the cell. Therefore, the measurement of osmotic swelling is a convenient method to examine the osmotic behaviour of these cells [15,16].

Cell suspensions of *Methanohalophilus halophilus* rapidly swelled when exposed to a sub-optimal salinity. The result of a typical experiment is given in Fig. 1. The extent of swelling was found to be strongly dependent on the NaCl concentration. These results have shown that cells of *M. halophilus* behave like osmometers. This indicates that, under the conditions employed, NaCl does not penetrate freely the interior of cells. Swelling was not observed when the cells were suspended in 7% NaCl (1.2 M) which was isotonic with the growth medium. These results are in good agreement with previous findings which have shown that cells of *M. halophilus* grow optimally at a concentration of 7–9% NaCl [1]. The swelling of cells ceased by addition of magnesium ions to 40 mM final concentration. The protective effect of magnesium ions might be explained by their stabilizing effect on the membrane integrity of these cells as it has been already shown for some other microorganisms [17].

Correspondingly the methane production in *M. halophilus* under non-growing conditions was decreased in the media containing suboptimal NaCl concentrations (Fig. 2). These results support the previous finding that a correct NaCl concentration is important for optimal growth and methane formation by these cells to protect them from the loss of structural and functional properties.

Further experiments were conducted to show that sodium ions are exclusively essential for methanogenesis from methylamine in these cells. The effect of other monovalent and bivalent cations on the methane

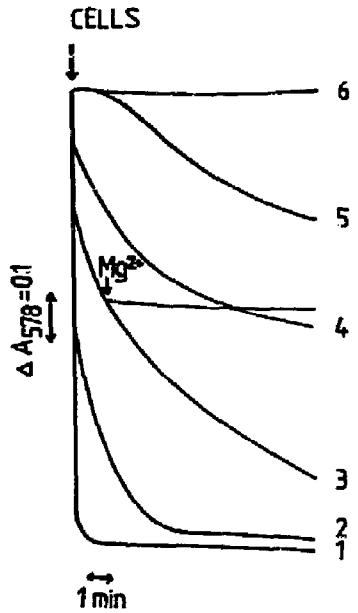

Fig. 1. Swelling of intact cells of *Methanohalophilus halophilus* exposed to different NaCl concentrations. 20 μl of cell suspension (1.2 mg protein ml^{-1}) in 50 mM Tris–HCl, 7% NaCl, pH 7.0, was added to 2 ml of NaCl solution of different concentrations. (1) 0% NaCl; (3) 2% NaCl; (4) 4% NaCl; (5) 5% NaCl; (6) 7% NaCl. $MgCl_2$ in a final concentration of 40 mM was added to the cuvette as indicated by the arrow. The changes of absorbance of the suspensions at 578 nm were recorded at room temperature on a recording spectrophotometer.

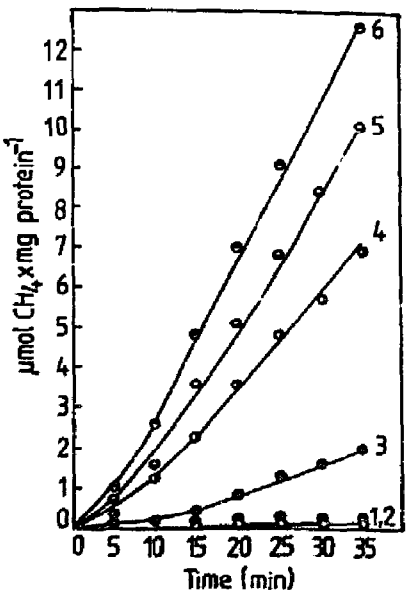

Fig. 2. Effect of different NaCl concentrations on methane formation from methylamine in cell suspension of *Methanohalophilus halophilus*. Cells (1.2 mg protein ml^{-1}) were incubated in 3 ml of 50 mM Tris-HCl, 10 mM $MgCl_2$, 30 mM methylamine, pH 7.0 (gas-phase, nitrogen) with different concentrations of NaCl. (1) 1% NaCl; (2) 2% NaCl; (3) 3% NaCl; (4) 4% NaCl; (5) 6% NaCl; (6) 7% NaCl. The suspensions in 22 ml serum bottles were shaken on a gyratory shaker at 200 rpm, 35°C and methane was measured at the times indicated.

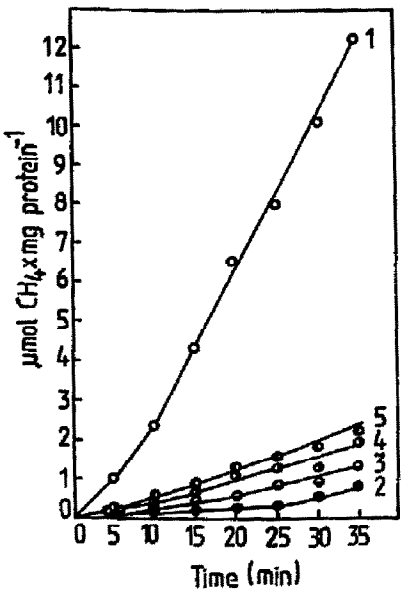

Fig. 3. Effect of monovalent (Na^+, K^+, Li^+, NH_4^+) and bivalent (Mg^{2+}) cations on methane formation from methylamine in cell suspensions of *Methanohalophilus halophilus*. Cells (1.3 mg protein ml^{-1}) were incubated in 3 ml of 50 mM Tris–HCl, 30 mM methylamine, pH 7.0, with NaCl (1), NH_4Cl (2), KCl (3), LiCl (4) and $MgCl_2$ (5) in concentrations iso-osmolar to 7% NaCl; purified nitrogen was used as the gas phase. The suspensions in 22 ml serum bottles were shaken on a gyratory shaker at 200 rpm, 35°C and methane was measured at the time indicated.

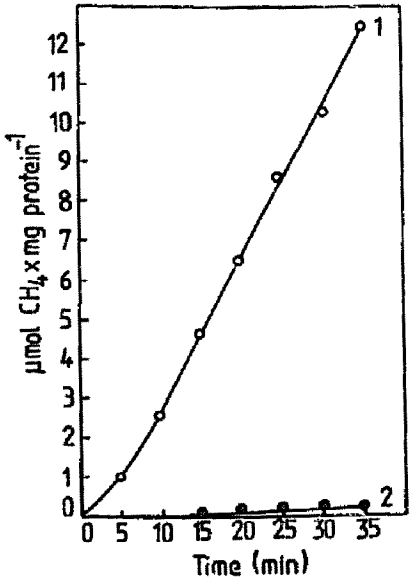

Fig. 4. The effect of monensin on methane formation from methylamine in cell suspension of *Methanohalophilus halophilus*. Cells (1.3 mg × protein ml^{-1}) were incubated in 3 ml of 50 mM Tris–HCl, 7% NaCl, 30 mM methylamine, pH 7.0, gas-phase nitrogen. (1) control; (2) 1 µM monensin, added at the beginning of the incubation. The suspensions in 22 ml serum bottles were shaken on a gyratory shaker at 200 rpm, 35°C and methane was measured at the times indicated.

formation was determined. It is apparent from Fig. 3 that neither KCl nor NH_4Cl at concentrations iso-osmotic to 7% NaCl can substitute for NaCl. On the other hand, LiCl and $MgCl_2$, at concentrations iso-osmotic to 7% NaCl, could partly substitute for the specific function of sodium ions in the methanogenesis of the cells. It is tempting to speculate that in these cells magnesium ions play an important role in the stuctural integrity of cytoplasmic membrane where some enzymes of methanogenesis are probably localized. Li^+ ions can partly substitute for Na^+ ions in some bioenergetic function of methanogenic bacteria [18]. Non-ionic osmoregulators, such as sucrose or mannitiol, in concentrations iso-osmotic to 7% NaCl, supported neither growth nor methanogenesis in *M. halophilus* (not shown). Since sodium ions play a vital role in these bacteria [1] it was important to find out whether a sodium gradient is involved in methane formation in these moderate halophilic methanogenic bacteria. The artificial Na^+/H^+ antiproter, monensin, was shown to dissipate the electrochemical sodium gradient in *Methanosarcina barkeri* and hence inhibits methanogenesis from methanol [26]. The results given in Fig. 4 show that methanogenesis from methylamine in cell suspensions of *M. halophilus* was strongly inhibited in the presence of 1 µM monensin. This finding indicates that a sodium gradient might play an important role in the energy metabolism of the halophilic methanogenic bacteria during methanogenesis from methylamine.

In some halophilic bacteria it was observed that the activity of the membrane-bound ATPase and the activity of some soluble enzymes as well, were maximal when the concentration of NaCl in the medium was high [19]. On the other hand, an inhibitory effect of high NaCl concentration of ATPase activity was observed in some other halophilic bacteria [19]. The membrane preparation of the moderate halophilic methanogen *M. halophilus* hydrolyzed ATP and this hydrolysis was strongly inhibited by DCCD. A relatively low sodium concentration (40 mM) was required for maximum activity of this ATPase. Neither KCl nor LiCl could substitute for NaCl, indicating that the ATPase activity was dependent on sodium ions only. Higher sodium ion concentrations up to 1.2 M (7%) were inhibitory (Table

Table I

Effect of Na^+ on ATPase activity (nmol P_i × mg protein^{-1} × min^{-1}) in membrane fractions of *Methanohalophilus halophilus*

Additions	Activity	Stimulation (%)	Inhibition (%)
None	30		
40 mM NaCl	55	83	
500 mM NaCl	17		44
50 µM DCCD	8		73
0.1% Triton X-100	28		

I). An enhancement of the ATPase activity in the presence of Triton X-100 was not observed. It seems to be reasonable to suppose that Na^+ ions can not properly fulfill the function of an osmotic stabilizer in the microenvironment of the membrane-bound ATPase. The existence of organic non-ionic osmoregulators was detected recently in some halophiles including some halophilic methanogenic bacteria [20–22]. Based on this observation it can be supposed that such osmoregulator(s) also exists in cells of *M. halophilus*. This could rationalise the observed inhibitory effect of higher NaCl concentrations on the membrane-bound ATPase in these cells. Further studies on this matter are in progress.

REFERENCES

[1] Zhilina, T.N. (1983) Mikrobiologija 52, 375–382.
[2] Paterek, J.R. and Smith, P.H. (1985) Appl. Environ. Microbiol. 50, 877–881.
[3] Yu, I.K. and Kawamura, F. (1987) J. Gen. Microbiol. 33, 303–310.
[4] Oren, A. (1988) Antonie van Leeuwenhoek 54, 267–277.
[5] Liu, Y., Boone, D.R. and Choy, C. (1990) Int. J. Syst. Bacteriol. 40, 111–116.
[6] Florkin, M. and Schoffeniels, E. (1969) Molecular Approaches to Ecology, Academic Press, New York, pp. 98–111.
[7] Ben-Amotz and Avron, M. (1978) in: Energetics and Structure of Halophilic Microorganisms (S.R. Caplan and M. Ginsburg eds.) Elsevier, Amsterdam, pp. 523–541.
[8] Pollard, A. and Wyn Jones, R.C. (1979) Planta 144, 291–298.
[9] Abram, D. and Gibbons, N.E. (1961) Can. J. Microbiol. 7, 741–750.
[10] Lanyi, J.K. (1974) Bacteriol. Rev. 38, 272–290.
[11] Dimroth, P. (1987) Microbiol. Rev. 51, 320–340.
[12] Skulachev, V.P. (1989) J. Bioenerg. Biomemb. 21, 635–647.
[13] Lancaster Jr., J.R. (1989) J. Bioenerg. Biomembr. 21, 717–740.
[14] Gottschalk, G. and Blaut, M. (1990) Biochim. Biophys. Acta 1018, 263–266.
[15] Avi-Dor, Y., Kuczyinski, M., Schatzberger, G. and Mager, J. (1956) J. Gen. Microbiol. 14, 76–83.
[16] Henderson, P.J.F. (1971) Annu. Rev. Microbiol. 21, 393–428.
[17] Singer, T.P. (1968) Biological Oxidations, Interscience, NY, pp. 55–112.
[18] Schönheit, P. and Beimborn, D.B. (1985) Arch. Microbiol. 142, 354–361.
[19] Meyer, B. and Imhoff, J.F. (1989) J. Gen. Microbiol. 135, 2829–2836.
[20] Galinski, E.A. and Truper, H.G. (1982) FEMS Microbiol. Lett. 13, 357–360.
[21] Imhoff, J.F. and Rodriquez-Valera, F. (1984) J. Bacteriol. 160, 478–479.
[22] Sowers, K.R., Robertson, D.E., Noll, D., Gunsalus, R.P. and Roberts, M.F. (1990) Proc. Natl. Acad. Sci. USA 87, 9083–9087.
[23] Sauer, D., Mahadevan, S. and Erfle, J.D. (1984) Biochem. J. 221, 61–69.
[24] Šmigáň, P., Friederová, A., Rusňák, P. and Greksák, M. (1984) Folia Microbiol. 29, 353–358.
[25] Lowry, O.H., Rosebourgh, A.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[26] Blaut, M., Muller, V., Fiebig, K. and Gottschalk, G. (1985) J. Bacteriol. 364, 95–101.