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Evidence for Na^+/H^+ Antiport in *Methanospirillum hungatei*

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ABSTRACT. The growth and methane formation of *Methanospirillum hungatei* were inhibited by an inhibitor of Na^+/H^+ antiport amiloride. After addition of NaCl or LiCl, when the cells had a lower intracellular pH and were deenergized, they extruded protons into the external medium. The acidification of the external medium was stimulated by protonophores and inhibited by amiloride. These findings suggest the existence of an Na^+/H^+ antiport in the cytoplasmic membrane of *M. hungatei* and its role in the energetics of methanogenic bacteria.

Methanogenic bacteria, as a special division of archaebacteria, can be categorized as one of the earliest divergences of extant life forms (Balch *et al.* 1979). Much evidence suggests that these microorganisms, with characteristics of both eubacteria and eukaryotes, represent a third superkingdom of organisms (Jones *et al.* 1987), along with eubacteria and eucytes. Methanogenic bacteria require Na^+ ions for methane formation from $H_2 + CO_2$, acetate and methanol (Perski *et al.* 1982) but not when formaldehyde or methanol plus hydrogen are used as a carbon and energy source (Blaut and Gottschalk 1985; Blaut *et al.* 1985). However, the function of Na^+ is not yet fully understood although it has been suggested that Na^+ ions might play an important role in energy transformation in methanogenic bacteria (Blaut and Gottschalk 1985; Kaesler and Schönheit 1989).

It is known that Na^+ ions can be translocated through the cytoplasmic membrane of prokaryotes in different ways (Dimroth 1987), but Na^+/H^+ exchange is the only known system for Na^+ extrusion in bacterial cells (West and Mitchell 1974; Krulwich 1986). In other words, the prokaryotic plasma membrane Na^+/H^+ antiporter might operate in either direction. An Na^+/H^+ antiporter is a membrane carrier that catalyzes the exchange of Na^+ and H^+, *i.e.* Na^+ and H^+ are translocated in opposite direction across the membrane, or Na^+ and OH^- in the same direction. Na^+/H^+ antiporters are regarded as secondary active transport systems rather than primary pumps (Krulwich 1983). In prokaryotes, some Na^+/H^+ exchange activities have been reported to be unaffected by changes in membrane potential and have been characterized as electroneutral (West and Mitchell 1974). Others require a membrane potential (outside positive), at least at some external pH values, and have been presumed to be electrogenic (Booth 1985).

A bacterial Na^+/H^+ antiport activity was first reported in *Streptococcus faecalis* (Harold and Papineau 1972) and then in *Escherichia coli* by West and Mitchell (1974). The sodium–proton antiport was first demonstrated in methanogenic bacteria *Methanobacterium thermoautotrophicum* by Schönheit and Beimborn (1985) and in *Methanosarcina barkeri* by Müller *et al.* (1987). Recently, Kaesler and Schönheit (1989) have suggested a key role for that Na^+/H^+ exchange system in coupling between sodium ion and proton energetics in methanogenic bacteria.

It was interesting to elucidate the existence of Na^+/H^+ antiport in *Methanospirillum hungatei*, as these bacteria require only very low sodium concentrations for growth. This paper reports the results which indicate the presence of an electrogenic Na^+/H^+ antiport and its similar properties in the cytoplasmic membrane of *Methanospirillum hungatei*, as it was found in *Methanobacterium thermoautotrophicum* and *Methanosarcina barkeri*.

MATERIALS AND METHODS

Methanospirillum hungatei strain GPI (DSM 1101) was kindly donated by Dr. C.D. Sprott (*National Research Council of Canada*, Ottawa).

The cells were cultivated in 300 mL NTS (*National Transfusion Service*) infusion flasks sealed with butyl-rubber stoppers in 50 mL of the anaerobic medium, prepared according to Kates *et al.* (1982). The flasks were pressurized to 150 kPa with hydrogen and carbon dioxide in the ratio 4:1 and

incubated on a gyratory shaker at 35 °C. The gases used were purified from oxygen on a column containing copper particles heated to 350 °C. Cell growth was measured by determining either suspension absorbance at 578 nm, or dry mass on a Synpor filter (0.4 μm).

Production of methane was measured in a *Carlo Erba* Fractovap 4200 gas chromatograph using a 2-m steel column packed with Sepharon AE 200 to 300 μm. Detection was done with a heat-conductive detector Model 450. Samples of the gaseous phase were collected from the cultivation flasks with gas-tight syringes Pierce Series A-2.

For measurement of pH changes in external medium, exponentially grown cells were used. These cells were collected by centrifugation, washed with an acidification buffer containing per L: 50 mmol NH_4Cl, 20 mmol 2-(N-morpholine)ethanesulfonic acid, 50 μmol Ti^{3+} citrate, pH 6.0, washed once more with a buffer composed of 2 mmol/L Tris, 140 mmol/L choline chloride, 50 μmol/L Ti^{3+} citrate, pH 7.6, and finally resuspended in the same buffer. The entire procedure was performed under strictly anaerobic conditions in the presence of purified argon as gas phase.

The Orion Research Ionanalyzer 901, equipped with semimicroelectrode Orion 910300 and Kipp-Zonen BD40 recorder for recording changes in external pH, was used. For ΔpH calibration 50 μmol/L HCl was utilized. The assay was performed in a 10 mL pH electrode vessel filled with 3 mL assay mixture (2 mmol/L Tris, 140 mmol/L choline chloride, 50 μmol/L Ti^{3+} citrate, pH 7.6), cells and other additions as indicated in the legends to figures. The assay mixture was continuously stirred (5 Hz) under a stream of purified argon (5 L/min). After 15–20 min the pH of the suspension had stabilized. Then different cations were added as indicated.

All chemicals were of analytical grade purity and purchased mainly from *Lachema* (Brno), except for amiloride (*Spolana*, Neratovice), valinomycin, Tris, monensin (*Serva*, Heidelberg) and tetrachlorosalicylanilide (*Kodak*). The gases were from *Chemika* (Bratislava).

RESULTS AND DISCUSSION

A sodium/proton antiporter is a constituent of the plasma membrane in virtually all animal cells and in at least some bacteria. It functions to extrude protons from the cytoplasm using the inwardly directed Na^+ gradient (Krulwich 1983). Na^+/H^+ antiport is inhibited by the diuretic amiloride and its derivatives which apparently compete for the Na^+ site (Benos 1982). As it appears that Na^+/H^+ antiport is tightly involved in the regulation of cytoplasmic pH and Na^+ concentration,

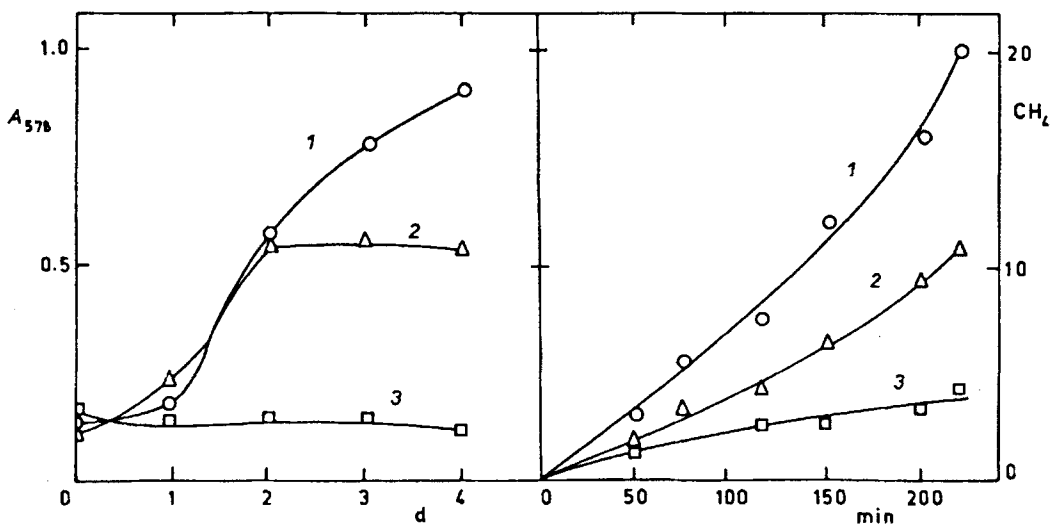

Fig. 1. Inhibitory effect of amiloride on growth (*left*) and methane formation (*right*) under growth conditions of *M. hungatei*. *Left*: *1* – control; *2* – 180 μmol/L amiloride added at 48 h of incubation; *3* – 180 μmol/L amiloride added at 0 h of incubation. *Right* (μmol CH_4 per mg dry mass): *1* – control; *2* – 200 μmol/L amiloride; *3* – 500 μmol/L amiloride. The growth medium (50 mL) in 300-mL infusion flasks was inoculated with 5 mL bacterial suspension, pressurized with H_2 + CO_2 (4:1) to 150 kPa every 24 h and incubated at 35 °C.

the control of cell volume, the initiation of cell proliferation (Grinstein and Rothstein 1986) and some mechanisms of energy transformation in cells (Schönheit and Beimborn 1985; Müller *et al.* 1987) the inhibition of the antiport by amiloride can have unfavorable consequences on cell function. As seen from Fig. 1 *left*, amiloride inhibited growth of the cells of *M. hungatei* when grown on hydrogen and carbon dioxide as energy and carbon source. The inhibitory effect of amiloride was also evident when the inhibitor was added to the cell suspension in the exponential pahse of growth. As expected, the methane formation of the cell suspension of *M. hungatei* was amiloride-sensitive under both growth (Fig. 1 *right*) and non-growth (Fig. 2) conditions. It should be noted that the inhibitory effect of amiloride on methane formation was observed only when the pH of the medium was in the alkaline range (7.1–7.7), while at pH 6.4 amiloride had no effect on the process. These results indicate the presence of an Na^+/H^+ antiport and its vital function in the cells of *M. hungatei* – particularly in an alkaline environment. This finding is in agreement with the results of Müller *et al.* (1987), showing that Na^+/H^+ antiport in *M. barkeri* is active in alkaline medium only. Similarly, some eubacteria use Na^+/H^+ exchange for the regulation of intracellular pH in the alkaline pH range (Krulwich 1983).

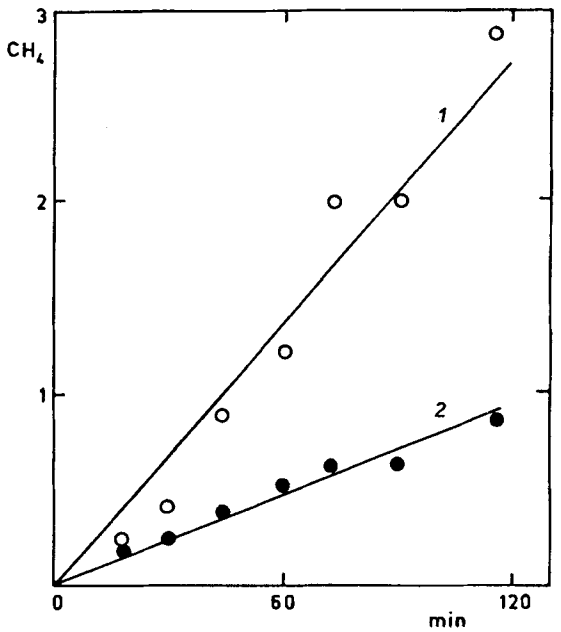

Fig. 2. Inhibitory effect of amiloride on methane formation (μmol CH_4 per mg dry mass) under non-growth conditions of *M. hungatei*; *1* – control, *2* – 380 μmol/L amiloride. The cells were incubated in 100-mL infusion flasks containing 20 mL of a medium composed of 50 mmol/L Tris-HCl, 50 mmol/L NaCl, 50 μmmol/L Ti^{3+} citrate (pH 7.7) and 1 mg dry mass of cells per mL, at 35 °C under H_2 + CO_2 (4:1) at 100 kPa pressure.

Na^+/H^+ antiport can be assayed by several approaches, but in general the finding of both Na^+-dependent H^+-translocation and H^+-dependent Na^+-translocation across a particular membrane has been taken as direct evidence for an Na^+/H^+ antiport. The former approach had been used in this work, measuring Na^+-dependent eflux of H^+ from cells into the extracellular medium. However, under steady-state conditions Na^+/H^+ exchange activity in the cells of *M. hungatei* was not found to be significant (*results not presented*). A dramatic stimulation of the antiport occurred as an acute acid load was applied by briefly pretreating the cells with a NH_4^+/NH_3-containing buffer, according to Roos and Boron (Roos and Boron 1981; Boron 1983). As seen in Fig. 3 *left* the cells extruded H^+ into the external medium after the addition of Na^+ or Li^+ to cells in which intracellular pH was reduced below its resting value by the acidifying procedure. The apparent K_M for external NaCl was found to be 14 mmol/L which is in agreement with results (10–50 mmol/L) published elsewhere (Moolenaar 1986). As demonstrated in Fig. 3 *left* the addition of K^+ to the cell suspension did not induce the proton extrusion into the extracellular medium. The results obtained imply the presence of Na^+/H^+ antiport in the cytoplasmic membrane of *M. hungatei*. The exchange process is driven by combined chemical Na^+ and H^+ gradients and does not depend on metabolic energy. Moreover, the need for internal cytoplasmic acidification of the cells shows that Na^+/H^+ antiport is in a latent state under normal physiological intracellular pH steady-state, and that the exchanger functions as an intracellular pH regulator in these cells. It cannot be excluded that the intracellular acidification is also needed for an allosteric activation of the Na^+/H^+ antiporter by protons, as originally proposed by Aronson *et al.* (1982, 1985).

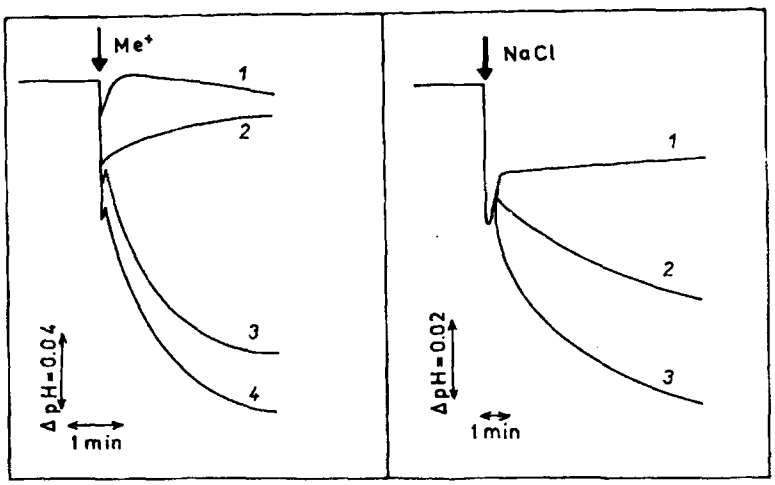

Fig. 3. Effect of monovalent cations (Me^+) on acidification of external medium of the cell suspension of *M. hungatei* (*left*) and the inhibition of Na^+-dependent acidification by amiloride (*right*). *Left*: *1* – 75 mmol/L KCl, *2* – control with 75 mmol/L NaCl added to the medium without the cells, *3* – 75 mmol/L NaCl, *4* – 75 mmol/L LiCl. *Right*: *1* – 75 mmol/L NaCl + 3.72 mmol/L amiloride, *2* – 75 mmol/L NaCl + 0.46 mmol/L amiloride, *3* – 75 mmol/L NaCl. The assay was performed with a cell concentration of 3.9 mg dry mass per mL (*left*) and 2.1 mg dry mass per mL (*right*).

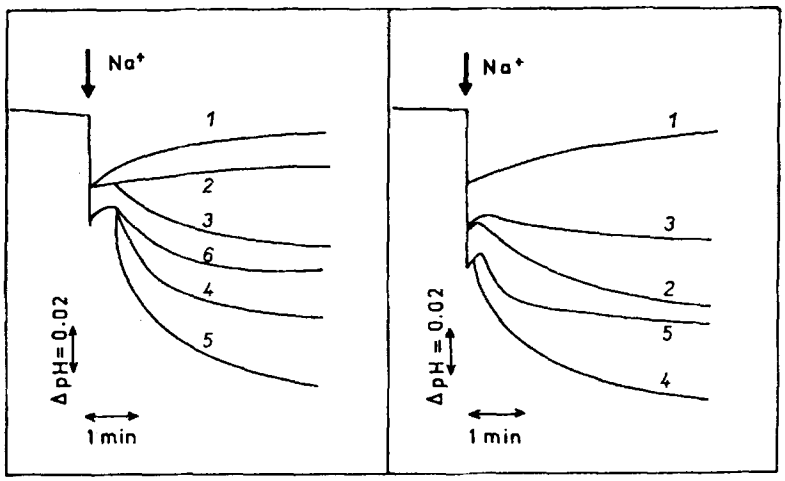

Fig. 4. Effect of tetrachlorosalicylanilide, valinomycin and thiocyanate on Na^+-dependent acidification of the cell suspension of *M. hungatei* in the presence of 75 mmol/L KCl (*left*) and in the absence of KCl (*right*); *1* – control without the cells, *2* – 6.8 μmol/L valinomycin, *3* – control with cells, *4* – 6.8 μmol/L valinomycin + 10 μmol/L TCS, *5* – 10 μmol/L TCS, *6* – 10 μmol/L TCS. The assay was performed with a cell concentration of 2.5 mg dry mass per mL. In all cases the assay was started by the addition of 75 mmol/L NaCl except in case *6* where 75 mmol/L NaSCN was added instead of NaCl.

To exclude a passive transport of protons through the cytoplasmic membrane of *M. hungatei*, the effect of amiloride on the Na^+/H^+ exchange was examined. As seen from Fig. 3 *right*, amiloride showed a potent inhibitory effect on the acidification of external medium with apparent K_i = 0.44 mmol/L. Monensin, as an artificial Na^+/H^+ antiporter, removed the effect of amiloride (*results not shown*).

When the cells had been preincubated with a protonophore (tetrachlorosalicylanilide, SF6847) the acidification of the external medium upon addition of NaCl remarkably increased though the uncouplers did not affect proton extrusion in the absence of NaCl addition. When NaSCN, which is known to dissipate membrane potential, was added instead of NaCl, the acidification of extracellular medium was much less than in the case of NaCl addition. There were no differences in these results whether or not K^+ ions were present in the medium (Fig. 4). These results indicate that at the pH used (7.6) the Na^+/H^+ antiport in the cytoplasmic membrane of *M. hungatei* is affected by the transmem-

brane electrical potential. This in turn indicates the presenee of an electrogenic exchange mechanism. To confirm the above proposal, the effect of valinomycin on the Na^+/H^+ exchange was checked. As seen from Fig. 4 *left* valinomycin in the presence of external K^+ ions (dissipation of membrane potential) inhibited the proton extrusion to the external medium whereas in the absence of K^+ ions (generation of membrane potential) the acidification was enhanced (Fig. 4 *right*). In the former case, the protonophore tetrachlorosalicylanilide reduced the effect of valinomycin, and in the latter case it exhibited a synergistic effect to valinomycin.

So far we have not determined the membrane potential nor the ΔpH, but the effects of ionophores known to dissipate or generate the membrane potential and the effect of SCN^- clearly implicate the electrogenic character of Na^+/H^+ antiport in *M. hungatei*.

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