JOURNAL OF BACTERIOLOGY, Sept. 1992, p. 5575–5583
0021-9193/92/175575-09$02.00/0

Vol. 174, No. 17

# Energy Transduction in the Methanogen *Methanococcus voltae* Is Based on a Sodium Current

MICHAEL DYBAS† AND JORDAN KONISKY*

*Department of Microbiology, University of Illinois, Urbana, Illinois 61801*

Received 1 April 1992/Accepted 22 June 1992

**We provide experimental support for the proposal that ATP production in *Methanococcus voltae*, a methanogenic member of the archaea, is based on an energetic system in which sodium ions, not protons, are the coupling ions. We show that when grown at a pH of 6.0, 7.1, or 8.2, *M. voltae* cells maintain a membrane potential of approximately −150 mV. The cells maintain a transmembrane pH gradient ($pH_{in} - pH_{out}$) of −0.1, −0.2, and −0.2, respectively, values not favorable to the inward movement of protons. The cells maintain a transmembrane sodium concentration gradient ($sodium_{out}/sodium_{in}$) of 1.2, 3.4, and 11.6, respectively. While the protonophore 3,3′,4′,5-tetrachlorosalicylanilide inhibits ATP formation in cells grown at pH 6.5, neither ATP formation nor growth is inhibited in cells grown in medium at pH 8.2. We show that when grown at pH 8.2, cells synthesize ATP in the absence of a favorably oriented proton motive force. Whether grown at pH 6.5 or pH 8.2, *M. voltae* extrudes $Na^+$ via a primary pump whose activity does not depend on a proton motive force. The addition of protons to the cells leads to a harmaline-sensitive efflux of $Na^+$ and vice versa, indicating the presence of $Na^+/H^+$ antiporter activity and, thus, a second mechanism for the translocation of $Na^+$ across the cell membrane. *M. voltae* contains a membrane component that is immunologically related to the $H^+$-translocating ATP synthase of the archaeabacterium *Sulfolobus acidocaldarius*. Since we demonstrated that ATP production can be driven by an artificially imposed membrane potential only in the presence of sodium ions, we propose that ATP production in *M. voltae* is mediated by an $Na^+$-translocating ATP synthase whose function is coupled to a sodium motive force that is generated through a primary $Na^+$ pump.**

Evidence has been obtained with whole cells of *Methanosarcina barkeri* and with whole cells, protoplasts, and crude membrane vesicles of methanogenic bacterium strain Göl that reduction of the heterodisulfide of $CH_3$-S-CoM [2-(methylthio)ethanesulfonate-methylcoenzyme M] and 7-mercaptoheptanoylthreoninephosphate leads to the generation of a proton motive force (PMF) that is coupled to the synthesis of ATP via an $H^+$-translocating ATP synthase (3, 4, 9, 10, 23, 32). In contrast, results obtained in studies directed towards elucidating the energetics of ATP formation in *Methanococcus voltae* have shown that methane formation and ATP synthesis can occur in the presence of the protonophore SF6847 and have led to the conclusion that the maintenance of a PMF is not required for ATP synthesis in this marine methanogen (7). On the basis of these findings, a novel and intriguing scheme of substrate-level phosphorylation was considered possible (27).

To study the mechanism of ATP formation in *M. voltae*, we decided to reexamine the possibility that ATP formation was mediated by a chemiosmotically based mechanism by considering a mechanism in which the coupling ion is not a proton. Since growth, amino acid transport, and ATP production in whole cells of *M. voltae* had been shown to be dependent on sodium ions (21, 22, 40), we considered it possible that the relevant ion might be sodium. Indeed, sodium-based energy transduction systems have been described for the anaerobe *Propionigenium modestum* (16), several marine bacteria, including *Vibrio parahaemolyticus*, *V. alginolyticus*, and *V. costicola* (14, 37–39), halotolerant bacterium strain BA1 (24), benthic freshwater filamentous bacterium *Vitreoscilla* sp. (18), and alkaline-grown *Bacillus* sp. strain FTU and *Escherichia coli* (1). In each of these cases, the experimental evidence supports the conclusion that energy metabolism leads to the extrusion of sodium ions through the operation of a primary sodium ion pump.

We previously described an uptake system for *M. voltae* that mediates the accumulation of exogenously added $CH_3$-S-CoM (17). We also provided evidence that upon accumulation, $CH_3$-S-CoM was not oxidized to other $C_1$ cycle intermediates but was converted directly to methane. Thus, this system allows us to examine the features of *M. voltae* ATP formation based on the terminal reactions of methanogenesis separate from other reactions of the $C_1$ cycle (15). Using $CH_3$-S-CoM–$H_2$ and $CO_2$-$H_2$ as substrate pairs, we determined the contributions of several parameters to the chemiosmotic gradients present in *M. voltae*. We conclude that ATP production in this methanogen is based on a chemiosmotic mechanism in which the coupling ion is sodium. We propose that this marine methanogen generates a membrane potential ($\Delta\psi$) through the activity of a primary sodium ion pump and that the resulting chemiosmotic gradient is coupled to an $Na^+$-translocating ATP synthase.

## MATERIALS AND METHODS

**Growth conditions and preparation of cells.** *M. voltae* was grown as previously described in the defined medium of Whitman et al. (40) supplemented with 0.1% yeast extract and 0.1% tryptone. As needed, the pH of the medium was adjusted by the addition of either HCl or KOH. In all cases, the substrate was $CO_2$-$H_2$ (20:80), and this mixture was used in all experiments in which these gases were utilized as a

* Corresponding author. Internet: Konisky@cso.uiuc.edu.
† Present address: Center for Microbial Ecology, Michigan State University, East Lansing, MI 48824-1325.

TABLE 1. Electrochemical parameters

| Parameter | Value at the following growth pH: | | | |
|---|---|---|---|---|
| | 6.0 | 7.1 | 8.2 | 8.2 (plus TCS) |
| ATP (mM) | 3.6 | 5.7 | 3.5 | 3.1 |
| Methane (nmol/min/mg) | 182 | 188 | 190 | 158 |
| $\Delta\psi$ (mV) | −146 | −153 | −143 | 0 |
| $pH_{out}$ | 6.0 | 7.1 | 8.2 | 8.2 |
| $pH_{in}$ | 5.9 | 6.9 | 8.0 | 7.8 |
| $Z\Delta pH$ (mV) | −6.0 | −12 | −12 | −24 |
| $\Delta\mu H^+/F$ (mV)[a] | −140 | −141 | −131 | 24 |
| $Na^+_{in}$ (mM) | 310 | 108 | 32 | 41 |
| $Na^+_{out}$ (mM) | 370 | 370 | 370 | 370 |
| $Na^+_{out}/Na^+_{in}$ | 1.2 | 3.4 | 11.6 | 9.1 |
| $Z\Delta pNa$ (mV) | 4.7 | 31 | 64 | 57.5 |
| $\Delta\mu Na^+/F$ (mV)[b] | −150 | −189 | −207 | −57.5 |

[a] $\Delta\mu H^+/F = \Delta\psi - Z\Delta pH$, where $\Delta pH = pH_i - pH_o$.
[b] $\Delta\mu Na^+/F = \Delta\psi - Z\Delta pNa$, where $\Delta pNa = pNa_i - pNa_o$ and $pNa = -\log[Na^+]$.

substrate. For the preparation of cell suspensions, cells were grown to an optical density at 660 nm of 0.35 to 0.4 and harvested by anaerobic centrifugation and two washes in an anaerobic buffer consisting of 400 mM sucrose, 200 mM NaCl, 100 mM HEPES (free acid) (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 10 mM KCl, 10 mM $MgCl_2$, 1 mmol of $H_2S$ per liter (20 ml of gas per liter) as a reducing agent, and 0.001% resazurin as a redox indicator. The pH was adjusted with HCl or KOH as needed. The buffer was flushed with nitrogen gas at 15 lb/in$^2$ for 1 h with stirring to remove oxygen before the addition of the reducing agent. Cells were incubated in this buffer under 100% $H_2$ for 30 min at 30°C to allow the reduction of any endogenous $C_1$ cycle intermediates. The cell suspension was adjusted to a protein concentration of 1 mg/ml in this buffer under $N_2$ for use in the experiments.

For assays with $CH_3$-S-CoM or HS-CoM (2-mercaptoethanesulfonate-coenzyme M), these compounds were added to a concentration of 50 mM. Cells were incubated under $N_2$ for 30 min to allow passive accumulation of the coenzyme M derivatives by diffusion, mediated by the methylcoenzyme M and coenzyme M transport systems, respectively (17). Cells were dispensed into 12.5-ml serum vials under $N_2$ before use.

For the determination of the parameters shown in Table 1, cells were grown to an optical density at 660 nm of 0.2 to 0.3 and harvested by anaerobic centrifugation, and the cell pellets were resuspended in a 1/20 volume of growth medium to a protein concentration of approximately 1 mg/ml. The cell suspension was dispensed in 5-ml volumes into 60-ml serum vials and then pressurized with $CO_2$-$H_2$ to 15 lb/in$^2$ before the measurements were made.

**Determination of cellular ATP levels.** ATP levels in energized cells were determined by withdrawal of 500-μl samples and extraction with a solution of 10% trichloroacetic acid–4 mM EDTA. Extracts were washed three times with 3 volumes of diethyl ether prior to the ATP level determination by the luciferin-luciferase assay as previously described (12).

For experiments in which the sodium dependence of ATP formation was determined, cell suspensions were prepared as described above, except that NaCl was omitted from the wash buffer and the number of washes was increased to three. NaCl was added as an anaerobic stock solution 10 min prior to the initiation of assays in which $CO_2$-$H_2$ served as the energy source. For experiments in which ATP formation was driven by an artificially imposed $\Delta\psi$, cells were prepared as described above, except that both NaCl and KCl were omitted from the wash buffer. NaCl was added as a stock solution 10 min prior to the initiation of assays by the addition of valinomycin to 10 μM (10 nmol/mg of cell protein).

**Determination of the phosphorylation potential.** The intracellular levels of ATP, ADP, and phosphate in perchlorate-lysed cells were determined as described by Chapman et al. (6). The phosphorylation potential was calculated from the intracellular levels of ATP, ADP, and phosphate by the following equation: $\Delta Gp = \Delta G^{\circ\prime} + RT \ln [ATP]/[ADP][P_i]$; $\Delta Gp$ is the phosphorylation potential, $\Delta G^{\circ\prime}$ is the change in the standard free energy of ATP hydrolysis (kilojoules per mole).

**Determination of $^{24}Na^+$ extrusion and intracellular levels of $Na^+$.** $^{24}Na^+$ was prepared by converting 250 μmol of $^{24}Na_2CO_3$ to $^{24}NaCl$ with 1.5 ml of 1 N HCl and drying with a heat lamp. $^{24}Na_2CO_3$ was prepared in a Triga Mark II Research Reactor located on the University of Illinois, Urbana, campus. Incubation of cells at 30°C with $^{24}Na^+$ at a concentration of 225 mM (10 μCi/ml) led to the accumulation of $^{24}Na^+$ to a constant level. In the experiments reported, equilibration was carried out for 60 min before the addition of the substrate. The amount of $^{24}Na^+$ retained in the cells was determined by removing aliquots (500 μl) and pelleting the samples through silicon oil as described for $\Delta\psi$ assays (see below). Radioactivity was determined by Cerenkov counting with a Beckman LS 5000TD liquid scintillation counter.

The intracellular levels of $Na^+$ in metabolizing cells were determined from the amount of $^{24}Na$ present in metabolically active cells that had been equilibrated at 30°C for 1 h with $^{24}NaCl$ as described above and with an intracellular volume of 1.37 μl/mg (dry weight) (22), a quantity that corresponds to 4.1 μl/mg of protein, as determined by $^{14}$C-inulin–$^3H_2O$ partitioning by the method of Rottenberg (36). Cell volume was found not to vary under the full range of growth conditions described in this paper.

**Measurement of proton extrusion from cells.** Proton extrusion from cells was measured in 0.4 M sucrose–0.1 mM HEPES (pH 7 or 8.2) reduced with 2 μmol of $H_2S$ per liter. Cells were harvested anaerobically, washed twice in this buffer, and resuspended to a protein concentration of 10 mg/ml. Cell suspensions were placed in a 25-ml scintillation vial with a cap modified to allow pH probe access and gas input via 18-gauge needles. The pH was monitored with an Ion 83 ion meter equipped with a GK 2321c combination-pH electrode (radiometer) with stirring at 100 rpm. After a stable baseline was obtained, assays were initiated by the addition of NaCl, LiCl, or KCl to a final concentration of 200 mM.

**Determination of the $\Delta\psi$ and transmembrane pH gradients.** The $\Delta\psi$ was calculated on the basis of the accumulation of $^3$H-tetraphenylphosphonium bromide ($^3$H-TPP$^+$; final concentration, 1 μM; 26 mCi/mmol; Amersham) in the presence of the counterion tetraphenyl boron (final concentration, 1 μM; $Na^+$ salt; K+K Fine Chemicals, Plainview, N.Y.). Cells were equilibrated with $^3$H-TPP$^+$ and tetraphenyl boron for 15 min prior to the initiation of assays. Aliquots (500 μl) were removed, layered over anaerobic silicon oil (DC 710; density, 1.103 g/ml; Fluka), and centrifuged in a microcentrifuge at 17,000 × *g* for 3 min. The cell suspensions remained anaerobic during this procedure. The buffer and silicon oil were removed, and the cell pellet was cut out of the Eppendorf tube with a razor blade. The pellet was

solubilized by being shaken at 100 rpm overnight in Beckman Ready-Protein scintillation cocktail before radioactivity was determined by liquid scintillation spectroscopy. Alternatively, a vacuum filtration method was used to separate the cells from the buffer. The vacuum filtration method was used for cells grown under high-pH conditions, since cells grown under these conditions could not be pelleted through silicon oil of any density tested. In the vacuum filtration procedure, aliquots (500 μl) were removed from cell suspensions and filtered over Whatman GF/F filters, and the filters were rinsed with 5 ml of label-free buffer. The entire filtration-rinse procedure took less than 15 s. The $\Delta\psi$ was calculated from the intracellular and extracellular concentrations of $^3$H-TPP$^+$ as described by Rottenberg (36) with an intracellular volume for *M. voltae* of 1.37 μl/mg (dry weight) (22) and corrected for the nonspecific binding of $^3$H-TPP$^+$ determined in parallel experiments in which samples contained 3,3',4',5-tetrachlorosalicylanilide (TCS) (10 μM; 10 nmol/mg of protein), monensin (10 μM; 10 nmol/mg), SF6847 (10 μM; 10 nmol/mg), and valinomycin (2 μM; 2 nmol/mg). The values obtained for the $\Delta\psi$ with typical samples by the pelleting or filtration method agreed within 5%.

The intracellular pH gradient was determined by measuring the steady-state accumulation of $^{14}$C-benzoic acid (16.0 mCi/mmol; NEN) or $^{14}$C-methylamine (41 mCi/mmol; NEN) in the presence of $^3H_2O$ (0.45 mCi/mmol; NEN). Cells were equilibrated with 1 μM $^{14}$C-benzoic acid or $^{14}$C-methylamine (0.2 μCi/ml) and $^3H_2O$ (1 μCi/ml) for 15 min prior to the initiation of assays. Aliquots (500 μl) were removed, and cells were separated from the buffer by centrifugation through silicon oil or filtration as described above. Radioactivity was determined as described for $\Delta\psi$ determinations. pH gradients were calculated from the $^{14}C/^3H$ concentration ratios as described by Rottenberg (36). In control experiments, methylamine was not metabolized by *M. voltae*.

**Immunoblot analysis.** Cells were grown in 500 ml of medium and harvested by centrifugation at 5,000 × *g* for 15 min at 4°C. The harvested cells were resuspended aerobically in 20 ml of buffer (50 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 10% glycerol, 1 mM phenylmethanesulfonyl fluoride), and the solution was passed through a French pressure cell at 10,000 lb/in$^2$. Cell debris was removed by centrifugation at 4°C for 10 min at 12,000 × *g* and the resulting supernatant fraction was centrifuged at 100,000 × *g* and 4°C for 1 h. The pellet (membrane fraction) was resuspended in 500 μl of the above-described buffer with a Teflon homogenizer at 1,550 rpm. After dodecyl sulfate-polyacrylamide gel electrophoresis of the extracted membrane proteins as described by Laemmli (26), the proteins were transferred to an Immobilon P membrane by use of a Bio-Rad electrotransfer apparatus in accordance with manufacturer instructions. After transfer, the Immobilon P membrane was blocked with 2.5% nonfat dry milk–3% bovine serum albumin–0.2% Tween 20–0.02 M Tris-HCl (pH 7.5)–0.5 M NaCl–10 μg of sodium azide per ml (Super Blotto) overnight at room temperature. The membrane was then incubated at room temperature in 0.02 M Tris-Cl (pH 7.5)–0.5 M NaCl–0.2% Tween 20 (TTBS) containing 10 μg of primary antibody (anti-V-type ATPase antibody raised to *Sulfolobus acidocaldarius* V-type ATPase; a gift from Mathais Lubben, European Molecular Biology Laboratory, Heidelberg, Germany) and 1% bovine serum albumin for 1 h. The incubation was followed by three washes for 10 min each with TTBS. The membrane was then incubated with biotin-labeled secondary antibody (Pierce anti-rabbit immunoglobulin G; 1 μg/ml) in TTBS for 1 h and washed three times for 15 min each with TTBS. A streptavidin-enzyme conjugate (0.75 μg/ml; Bethesda Research Laboratories) was added to the membrane in TTBS, and the membrane was incubated for 30 min and washed three times for 15 min each with TTBS. In the final step, the membrane was incubated with Bluo-gal (ferric cyanide color system; Bethesda Research Laboratories) until blue color representing cross-reactive proteins was observed.

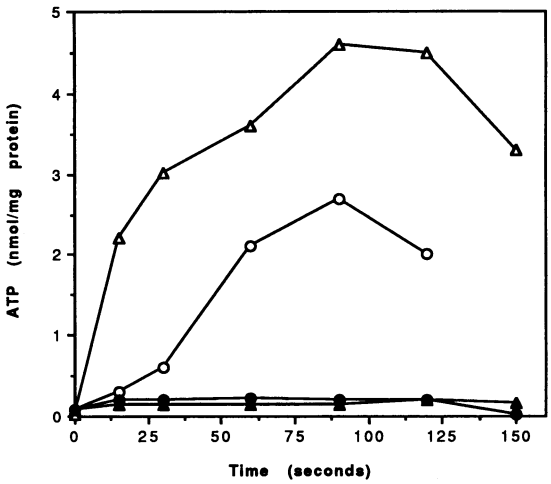


FIG. 1. ATP formation from $CO_2$-$H_2$ and $CH_3$-S-CoM-$H_2$. Cells were grown, washed, and resuspended in buffer at pH 6.5 as described in Materials and Methods. $CH_3$-S-CoM (50 mM) was added to cells under $N_2$ 30 min prior to the addition of $H_2$ to allow the passive accumulation of methylcoenzyme M. Zero time represents the time of addition of either $CO_2$-$H_2$ or $H_2$. Where indicated, TCS (0.2 μM; 0.2 nmol/mg) was added 15 min prior to the addition of either $CO_2$-$H_2$ or $H_2$. The data shown represent the average of four experiments. Symbols: △, $CH_3$-S-CoM-$H_2$; ▲, $CH_3$-S-CoM-$H_2$ plus TCS; ○, $CO_2$-$H_2$; ●, $CO_2$-$H_2$ plus TCS.

**Other analytical methods.** $CH_3$-S-CoM was synthesized from iodomethane and HS-CoM as described previously (20). Chemicals, inhibitors, and luciferin-luciferase were obtained from Sigma Chemical Co., St. Louis, Mo., unless otherwise indicated. TCS was obtained from Eastman Kodak Co. Methanogenesis rates were determined by gas chromatography. Protein was determined by a modification of the assay of Lowry et al. (28) with bovine serum albumin as a standard.

## RESULTS

**Effects of protonophores on ATP formation and growth.** The addition of $CO_2$-$H_2$ to *M. voltae* cells grown at pH 6.5 and then equilibrated in an $N_2$ atmosphere led to the rapid formation of ATP (Fig. 1). The formation of a $\Delta\psi$ of −150 mV was observed within 2 min of the addition of $CO_2$-$H_2$ (data not shown). When the cells were incubated in the presence of $CH_3$-S-CoM under $N_2$, the passive accumulation of methylcoenzyme M occurred in the cells, reaching within 30 min a level that could serve as a substrate for methanogenesis and ATP formation upon subsequent addition of $H_2$ (Fig. 1). Methanogenesis was dependent on the reduction of the methyl group supplied as $CH_3$-S-CoM, since the nonmethylated form of coenzyme M, HS-CoM, did not serve as a substrate for either methanogenesis or ATP formation (data not shown). The fact that methanogenesis did not

occur in the absence of $CH_3$-S-CoM also demonstrated that neither methanogenesis nor ATP formation was due to the presence of $CO_2$ contaminating our reagents. The reduction of $CH_3$-S-CoM by $H_2$ led to the generation of a $\Delta\psi$ of $-150$ mV within 2 min of $H_2$ addition and showed kinetics similar to those of the formation of a $\Delta\psi$ with $CO_2$-$H_2$. With either substrate pair, ATP formation was inhibited in cells treated with bromoethanesulfonate, an inhibitor of methanogenesis (20), indicating that ATP formation was coupled to methanogenesis.

We observed a $\Delta\psi$ of $-85$ mV in cells prepared under the conditions described in Materials and Methods. The $\Delta\psi$ could be collapsed by the protonophore TCS at 5 μM (5 nmol/mg) or by a combination of valinomycin, TCS, and monensin (10 μM each; 10 nmol/mg) under all conditions tested (data not shown). Thus, the $\Delta\psi$ values observed upon the addition of a substrate represented an enhancement of approximately $-70$ mV. This enhancement was required for ATP synthesis, since ATP production required the addition of the substrate $CO_2$-$H_2$ or $CH_3$-S-CoM–$H_2$.

To determine whether ATP synthesis required a PMF, we determined the effects of TCS on ATP formation in cells energized with either $CO_2$-$H_2$ or $CH_3$-S-CoM–$H_2$. ATP formation was completely inhibited by 0.2 μM (0.2 nmol/mg) TCS in cells grown and assayed at pH 6.5 (Fig. 1). Under these conditions, methanogenesis was not inhibited, and the $\Delta\psi$ was reduced by 40%. These results suggest that a component of the PMF was required to make ATP under these conditions and, therefore, that a chemiosmotic mechanism of ATP formation was being utilized.

Since it was reported that ATP formation in *M. voltae* (assayed at pH 8.0) was resistant to the protonophore SF6847 (7), we determined the effect of TCS on ATP formation in cells grown and assayed at pH 8.2. It was shown that TCS was an effective protonophore under these slightly alkaline conditions (31), and its effectiveness under our own experimental conditions was confirmed by experiments described below. In contrast to the results obtained with cells grown and assayed at pH 6.5, TCS did not inhibit ATP formation in cells grown and assayed at pH 8.2, even in the presence of up to 5 μM (5 nmol/mg) TCS, 20 times the concentration that completely inhibited ATP formation at pH 6.5 (Table 1 and see below).

Assuming that TCS functions as a protonophore under these conditions (see below), these results suggested that ATP formation in cells grown at an alkaline pH was not dependent on the presence of a PMF. This conclusion is consistent with studies in which we examined the effect of TCS on cells grown at pH 6.5 and 8.2 (Fig. 2). At pH 8.2, *M. voltae* grew in the presence of 5 μM (5 nmol/mg) TCS. In contrast, monensin, an ionophore that exchanges $Na^+$ for $H^+$ (34, 35) and, thus, dissipates an $Na^+$ gradient, completely inhibited growth when added at 5 μM (5 nmol/mg) in a medium maintained at pH 6.5 or 8.2 (data not shown). These results suggest that growth at either pH depends on a process involving sodium transport.

**Determination of parameters of energetics.** The observed uncoupler insensitivity of growth at an alkaline pH suggested that different bioenergetic motifs were functioning at acidic versus alkaline pHs in *M. voltae*. Since our conclusion was largely based on our finding that TCS was not inhibitory, it was necessary to determine that TCS was functioning as an ionophore in cells grown at pH 8.2. We therefore next defined several parameters of energetics and ascertained the effect of TCS on them (Table 1). For this purpose, cells were

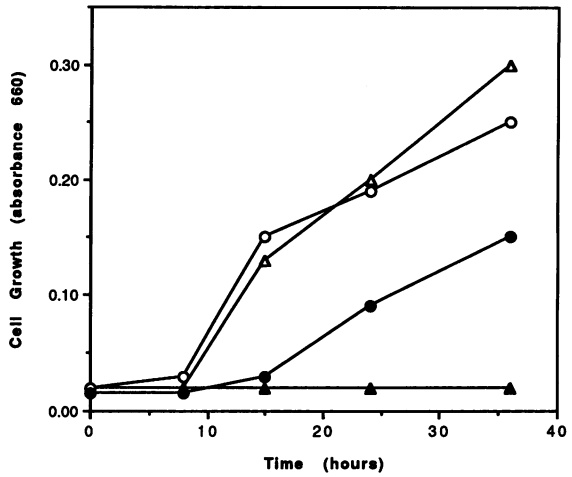


FIG. 2. Effect of TCS on cells grown at pH 6.5 or 8.2. TCS was present at 5 μM (5 nmol/mg). Symbols: △, control (pH 6.5); ▲, TCS (pH 6.5); ○, control (pH 8.2); ●, TCS (pH 8.2).

grown at a pH of 6.0, 7.1, or 8.2 and at a pH of 8.2 in the presence of TCS (5 μM; 5 nmol/mg).

The cells maintained a nearly identical $\Delta\psi$ under three of the growth conditions. In each case, the $\Delta$pH was small (acid inside), and it was apparent that *M. voltae* does not maintain a constant internal pH, as in all cases the determined internal pH was similar to the pH at which the cells were grown. Thus, under all growth conditions, the $\Delta\mu H^+/F$ was composed almost entirely of the $\Delta\psi$, and the Z$\Delta$pH detracted from rather than enhanced the $\Delta\mu H^+/F$ ($\Delta\mu H^+/F = \Delta\psi +$ Z$\Delta$pH, where Z = 60 mV). Cells grown at pH 8.2 in the presence of TCS did not maintain a $\Delta\psi$ and manifested a $\Delta$pH of 0.4 (acid inside). These results convincingly demonstrate that TCS was active under these conditions and that *M. voltae* is able to synthesize normal levels of ATP in the presence of a $\Delta\mu H^+/F$ that is in the wrong orientation to couple proton movement to ATP formation. Our conclusions fully reconcile the findings of Lancaster and collaborators, who demonstrated that ATP production in pH 8.0-grown *M. voltae* is resistant to the protonophore SF6847 (7).

When we examined the magnitude of sodium gradients in cells grown under these conditions, we observed that a sodium motive force ($\Delta\mu Na^+/F$) ($\Delta\mu Na^+/F = \Delta\psi +$ Z$\Delta$pNa, where Z = 60 mV) was maintained under all growth conditions (Table 1). While a chemical concentration gradient of sodium ions was present under all growth conditions, the magnitude of the sodium gradient ($[Na^+]_{out}/[Na^+]_{in}$) was dependent on the pH at which the cells were grown. Since the $\Delta\psi$ was nearly constant, the $\Delta\mu Na^+/F$ was maximal in cells grown at pH 8.2, at which the sodium gradient was 11.6.

The observation that cells can grow in the absence of a $\Delta\psi$ (with TCS at pH 8.2) while maintaining a $\Delta\mu H^+/F$ whose orientation will not support proton-mediated ATP synthesis suggests that the $\Delta\mu Na^+/F$ ($-57.5$ mV) maintained under these conditions drives ATP synthesis.

We next determined how the magnitude of the electrochemical gradients related to the magnitude of the phosphorylation potential. We found that cells grown at pH 7.1 or 8.2 and at pH 8.2 in the presence of TCS maintained phosphorylation potentials of $-447$ or $-450$ and $-370$ mV, respectively. These values fall within the range of phosphorylation potentials reported for other bacteria. From these results,

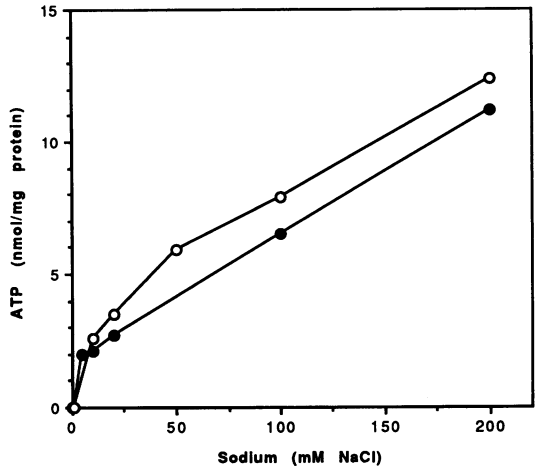


FIG. 3. Dependence of ATP production on $CO_2$-$H_2$- or $K^+$- and valinomycin-induced $\Delta\psi$. After being harvested by centrifugation, the cells were washed three times in buffer lacking both sodium and potassium and incubated under $N_2$. The experiment was initiated by the addition of either $CO_2$-$H_2$ to 15 lb/in$^2$ (○) or valinomycin to 10 μM (10 nmol/mg) (●). The amount of ATP produced was determined 2 min after energization, and the values shown represent the average of three experiments.

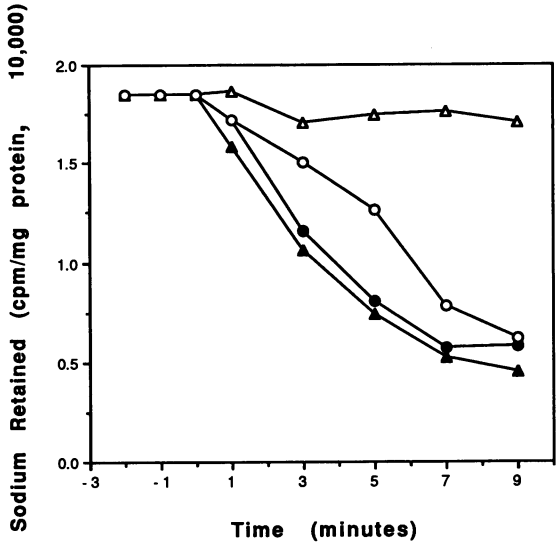


FIG. 4. $^{24}Na^+$ extrusion by cells grown and assayed at pH 6.5. The conditions and the assay were as described in Materials and Methods. The cells were energized at zero time by the addition of either $CO_2$-$H_2$ or $H_2$ (to the $CH_3$-S-CoM-containing sample) to 15 lb/in$^2$. $CH_3$-S-CoM (50 mM) was added to the cells under $N_2$ 30 min prior to the addition of $H_2$ to allow the passive accumulation of methylcoenzyme M. Where indicated, TCS (0.2 μM; 0.2 nmol/mg) was added 5 min prior to the addition of the gas substrates (zero time). Symbols: △, cells under $N_2$; ●, $CO_2$-$H_2$; ○, $CH_3$-S-CoM-$H_2$; ▲, $CO_2$-$H_2$ plus TCS.

we conclude that the ability of pH 8.2-grown cells to maintain nearly normal ATP production at a seemingly low $\Delta\mu Na^+/F$ (−57.5 mV) is not explained by the maintenance of an abnormally low phosphorylation potential and that some other explanation therefore must be operative.

**Dependence of ATP formation on a $\Delta\psi$ and sodium.** To examine further the energetic parameters that support ATP formation in *M. voltae*, we determined the minimal requirement for ATP production. For this purpose, cells suspended in a buffer solution containing no added potassium ions and various amounts of added sodium ions were treated with valinomycin to induce the electrogenic efflux of potassium ions, and the amount of ATP produced was determined 2 min later. Valinomycin-dependent ATP formation in cells grown and assayed at pH 6.5 was dependent on the presence of added sodium ions and increased over the range of 0 to 200 mM added NaCl (Fig. 3). Thus, the $\Delta\psi$ (−100 mV) generated under these conditions was not sufficient to drive ATP synthesis, indicating that the concomitantly generated $\Delta\mu H^+/F$ could not support ATP production. ATP formation was not observed in the absence of valinomycin, indicating that the addition of sodium alone could not support ATP production. The amount of ATP produced by a combination of the induced $\Delta\psi$ and sodium ions was similar to that observed in cells energized by the addition of $CO_2$-$H_2$, indicating that the amount of ATP produced by artificial means was physiologically significant. A similar pattern of results was observed in experiments in which cells were grown and assayed at pH 8.2. While the level of ATP production was only 25% of that observed for cells grown and assayed at pH 6.5, the level of ATP made in cells energerized by $CO_2$-$H_2$ was identical to that made in cells treated with valinomycin (data not shown). Consistent with our finding that the generation of a PMF was not sufficient to drive ATP formation was our failure to induce ATP production by subjecting pH 7.5-grown cells to an acid load of 2 pH units ($Z\Delta pH$ = 120 mV).

**Sodium movement during methanogenesis from $CO_2$-$H_2$ and $CH_3$-S-CoM-$H_2$.** To examine the possibility that *M. voltae* energetics might include a primary sodium pump, we monitored the level of cell-associated $^{24}Na^+$ ions after substrate addition. As shown in Fig. 4, the addition of either $CO_2$-$H_2$ or $CH_3$-S-CoM-$H_2$ to cells grown and assayed at pH 6.5 led to the loss of cell-associated sodium. Since the cells were fully equilibrated in the presence of $^{24}Na^+$ before the addition of substrate, the loss of $^{24}Na^+$ from the cells reflected true extrusion and not the exchange of intracellular $^{24}Na^+$ with extracellular nonradioactive sodium ions. While we can explain the extrusion of $^{24}Na^+$ upon the addition of $CO_2$ and $H_2$ based on the demonstration that the methyltetrahydromethanopterin:coenzyme M methyltransferase of *Methanosarcina* sp. strain Göl is a primary pump (2), the fact that we observed $^{24}Na^+$ extrusion after the addition of $CH_3$-S-CoM and hydrogen suggests the presence of an additional $Na^+$ pump that functions subsequent to the formation of $CH_3$-S-CoM.

$^{24}Na^+$ extrusion upon the addition of $CO_2$-$H_2$ was not inhibited by the addition of TCS and, thus, we conclude that $^{24}Na^+$ extrusion was mediated by a primary sodium pump and was not coupled to a PMF. To confirm that TCS was active as a protonophore under these conditions, we determined the intracellular pH in cell suspensions in a buffer at pH 6.5 and energized with $CO_2$-$H_2$. Whereas the intracellular pH of the control cells was 6.65, the addition of TCS led to an acidification of the cell cytoplasm, resulting in an intracellular pH of 6.30. We interpret these data to indicate the TCS-mediated movement of protons into the cells in response to the electrogenic extrusion of another positively charged ion, presumably $Na^+$. The addition of 300 μM harmaline, an inhibitor of the sodium/proton antiporter of *M. voltae* (see below), did not inhibit $^{24}Na^+$ extrusion in $CO_2$-$H_2$-energized cells and, thus, the activity of this antiporter

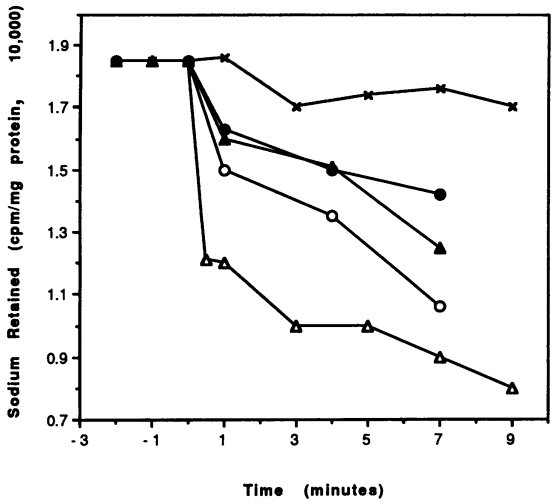


FIG. 5. $^{24}Na^+$ extrusion from cells grown and assayed at pH 8.2. The experiment was performed as described in the legend to Fig. 4. Where indicated, harmaline (300 μM; 300 nmol/mg) was added 5 min prior to the addition of $H_2$ (zero time). TCS (0.2 μM; 0.2 nmol/mg) was added 5 min prior to the addition of the gas substrates. Symbols: ×, cells under $N_2$; △, $CO_2$-$H_2$; ●, $CH_3$-S-CoM-$H_2$; ▲, $CH_3$-S-CoM-$H_2$ plus harmaline; ○, $CH_3$-S-CoM-$H_2$ plus TCS.

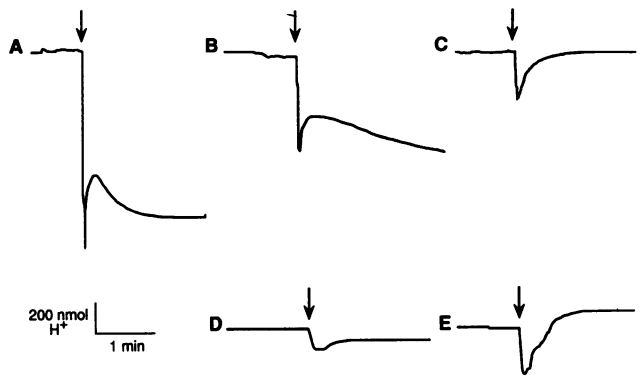


FIG. 6. Proton extrusion induced by the addition of sodium ions. Cell suspensions (10 mg/ml of protein) were prepared as described in Materials and Methods and incubated under $N_2$. Assays were initiated (at the arrows) by the addition of the chloride salts of sodium (traces A and D), lithium (traces B and E), or potassium (trace C) to 200 mM. Harmaline (300 μM; 30 nmol/mg) was added 5 min prior to the addition of either sodium (trace D) or lithium (trace E) salts.

was not required for sodium ion efflux (data not shown). Finally, the addition of the substrate pair $CH_3$-S-CoM-$H_2$ also led to sodium ion translocation, indicating that the extrusion of $^{24}Na^+$ was coupled to a metabolic step that took place subsequent to the formation of methylcoenzyme M.

The addition of the $CH_3$-S-CoM-$H_2$ or $CO_2$-$H_2$ substrate pair to cells grown and assayed at pH 8.2 also led to $^{24}Na^+$ efflux at a rate that was similar to that observed at pH 6.5 (Fig. 5). The addition of harmaline did not affect the extrusion of sodium ions, while the addition of TCS led to a slight stimulation of $^{24}Na^+$ efflux. While this stimulation was expected from the disruption of the $\Delta\psi$ by TCS, this result was not seen in all experiments and may reflect the observation that in this particular experiment, the rate of methanogenesis was about 25% higher in the sample that received TCS than in the sample that did not. We conclude that *M. voltae* grown and assayed at pH 8.2 has a primary sodium pump and that this pump is energized in a manner similar to that in pH 6.5-grown cells.

**Assay of $Na^+/H^+$ exchange in *M. voltae*.** To ascertain the presence of $Na^+/H^+$ antiporter activity in *M. voltae*, we prepared cell suspensions in a weak buffer. After a stable baseline (pH 7) was maintained for 5 min, sodium ions, lithium ions (an alternate substrate for $Na^+/H^+$ antiporters), or potassium ions (added as Cl− salts) were added to a final concentration of 200 mM, and the pH of the external buffer was monitored. The addition of sodium and lithium ions, but not potassium ions, led to acidification of the external buffer (Fig. 6), and this acidification was blocked by harmaline, a known inhibitor of $Na^+/H^+$ antiporters (25).

When cells were suspended in a weak buffer (pH 7.0) and equilibrated with $^{24}Na^+$, the subsequent addition of protons (pH reduction, 1 pH unit) led to harmaline-sensitive $^{24}Na^+$ extrusion (Fig. 7). In the absence of an imposed proton gradient, no significant $^{24}Na^+$ extrusion was observed. We observed similar activity in cells grown at pH 6.5 or 8.2. These data clearly indicate that *M. voltae* harbors $Na^+/H^+$ antiporter activity and define an additional mechanism by which this methanogen mediates transmembrane sodium ion translocation.

**Identification of a presumptive ATP synthase.** While we previously reported the presence of a membrane-associated P-type ATPase in *M. voltae*, our studies failed to identify an ATPase with the properties characteristic of an ATP synthase (11–13). In the present study, we used an antibody raised against the β subunit of the $H^+$-translocating ATP synthase purified from the archaea *S. acidocaldarius* (29, 30) in an attempt to identify a related ATPase in *M. voltae* membranes prepared from cells grown under the conditions

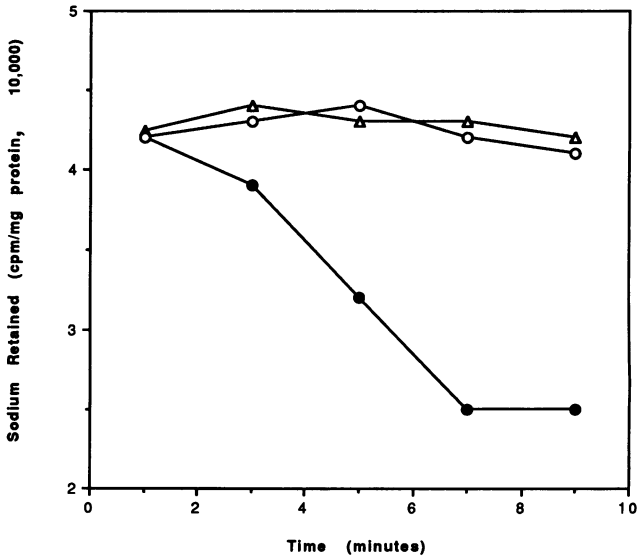


FIG. 7. Sodium ion extrusion induced by the addition of protons. Cells equilibrated with $^{24}Na^+$ as described in the legend to Fig. 5 were subjected to an acid load that reduced the pH of the cell suspensions from 7.0 to 6.0, achieved by the addition of HCl (the 1-min time point), and the amount of sodium retained by the cells was determined as described in the legend to Fig. 4. Symbols: △, control (no HCl added); ●, HCl added; ○, HCl added and 300 μM harmaline added 5 min before the HCl addition.

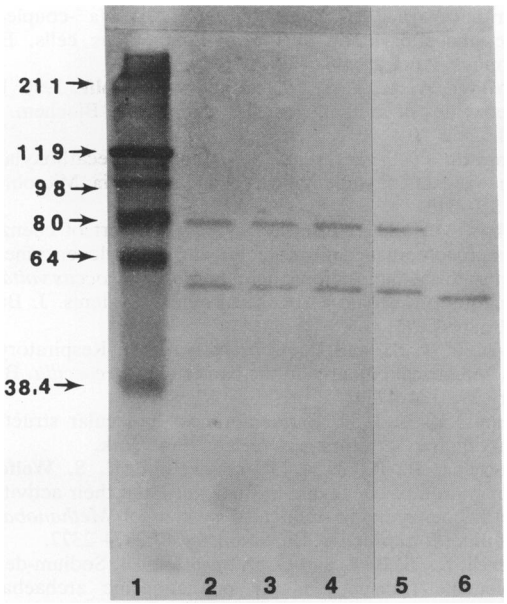


FIG. 8. Western blot (immunoblot) analysis of membranes probed with antibody directed against the *S. acidocaldarius* ATPase. All lanes contained 25 μg of membrane protein. Lanes: 1, molecular weight markers; 2, membranes from pH 6.0-grown *M. voltae*; 3, membranes from pH 7.0-grown *M. voltae*; 4, membranes from pH 8.2-grown *M. voltae*; 5, membranes from *M. voltae* grown at pH 8.2 in the presence of 5 μM (5 nmol/mg) TCS; 6, membranes from *S. acidocaldarius*.

described in Table 1. In all cases, the antibody reacted with two membrane protein components having estimated molecular masses of 51 and 65 kDa (Fig. 8). This antibody did not react with the 74-kDa membrane component that we previously identified as the P-type ATPase. Furthermore, an antibody raised against the purified P-type ATPase reacted with only a single membrane protein component, and this protein had an apparent molecular mass of 74 kDa (data not shown). In contrast to the results obtained with the *M. voltae* membranes, the antibody raised against the *S. acidocaldarius* ATPase reacted with only a single protein component present in the membranes of *S. acidocaldarius*. While we do not understand why the antibody cross-reacted with two *M. voltae* membrane protein components, we feel that our results provide presumptive evidence for a second membrane-associated ATPase in this methanogen.

## DISCUSSION

While it is known that a primary sodium ion pump operates between $CH_2O$ and $CH_3$-S-CoM in *Methanosarcina* sp. strain Gö1 (2), *M. voltae* apparently has an additional primary sodium pump that functions subsequent to the formation of $CH_3$-S-CoM. This pump is present in cells grown at pH 6.5 or 8.2. By analogy to more direct experiments carried out with methanogenic strain Gö1, in which it was shown that the reduction of the heterodisulfide of 7-mercaptoheptanoylthreoninephosphate and HS-CoM was coupled to proton translocation across the cytoplasmic membrane (8–10), we propose that in *M. voltae* such reduction is coupled to the translocation of sodium through the activity of a membrane-associated $H_2$-dependent heterodisulfide reductase.

Since ATP production in cells grown at pH 6.5 or 8.2 could be induced by the imposition of an artificial $\Delta\psi$ only in the presence of sodium ions, we believe that ATP production is mediated by a sodium ion-translocating ATP synthase. We favor a model in which ATP production is coupled to a sodium motive force regardless of the pH of growth. Nevertheless, we cannot rigorously exclude the possibility that a PMF participates, especially in cells grown at pH 6.5.

While *M. voltae* contains a membrane-associated P-type ATPase, this enzyme does not have the properties of known ATP synthases and more likely has a role in regulating the levels of intracellular sodium or hydrogen ions (11–13). While we have not as yet directly demonstrated an *M. voltae* ATPase with properties similar to those expected for an ATP synthase, the finding of a membrane component that cross-reacts with an antibody directed against the β subunit of the proton-translocating ATP synthase of *S. acidocaldarius* (29) suggests the presence of a second ATPase in *M. voltae*. This component may well be the ATP synthase.

The mechanism by which cells grown at a slightly alkaline pH maintain a lower level of intracellular sodium than cells grown in a more acidic medium remains to be elucidated. A sodium pump with a pH optimum in the alkaline range would serve this function, but other mechanisms are possible. Since the rates of sodium ion efflux observed in cells grown at pH 6.5 or 8.2 were similar, it is not likely that the mechanism was based on either the number of functional sodium ion pumps or a difference in activity. The role of the *M. voltae* P-type ATPase in this mechanism is an attractive possibility, but such a role lacks experimental support.

The capacity to utilize a primary sodium pump to generate a $\Delta\psi$ in the absence of the concomitant generation of a large transmembrane sodium gradient can be understood on the basis of the high intracellular concentration of sodium ions found in *M. voltae* (e.g., 310 mM at pH 6.0). Assuming a capacitance of $10^{-6}$ F/cm$^2$ of membrane surface (19) and a total estimated surface area of approximately $\pi \times 10^{-8}$ cm$^2$ (average cell diameter, 1.0 μm), it can be calculated that the translocation of approximately 30,000 ions will generate a $\Delta\psi$ of −150 mV. The translocation of this number of sodium ions from the cytoplasm of *M. voltae* would not significantly change the intracellular concentration of sodium ions and, thus, would not generate a sodium gradient.

While our model predicts that it should be possible to drive ATP production in whole cells by the imposition of a sodium ion gradient, we have failed in attempts to drive significant ATP synthesis by the imposition of a sodium load in the absence of a $\Delta\psi$. On the basis of the high intracellular concentration of sodium and, thus, our inability to impose a large sodium gradient across the membrane, this failure is not surprising. For example, the imposition of a 1,000-fold gradient (180 mV) would require external sodium concentrations of 370 and 37 M for cells grown at pH 6.5 and 8.2, respectively. Our results contrast those of an earlier study in which the addition of 0.5 M NaCl to *M. voltae* led to a small increase in cellular ATP levels (5). We have no explanation for the discrepancy.

Our results do not unambiguously exclude the possibility that ATP production in cells grown at pH 8.2 is based on substrate-level phosphorylation. Indeed, a few of our experimental findings are problematic in the context of a soley chemiosmotic interpretation. For example, ATP production in cells grown at pH 8.2 takes place under conditions in which the sodium motive force is less than 60 mV. Furthermore, the value of the phosphorylation potential does not vary directly with the magnitude of the electrochemical

sodium gradient. While explanations can be provided for these discrepancies, they do pose a difficulty for the scheme that we have proposed. Further experimentation is clearly required.

Since the pH of seawater ranges from 8.0 to 8.3, we believe that the energetics of *M. voltae* as determined in cells grown at pH 8.2 are physiologically significant. Furthermore, it is known that the pH of marine sediments can vary and that such fluctuations result primarily from the utilization of $CO_2$ and $HCO_3$ by the microflora in the surface sediments (33). Thus, there may well be conditions in its environment in which *M. voltae* grows at a slightly alkaline or a slightly acidic pH.

## ACKNOWLEDGMENTS

This work was supported in part by Public Health Service grant GM41587 from the National Institutes of Health.

We are grateful to both Diane Lynn and Scott Wasserstrom for providing technical assistance. We thank Mathias Lübben and Günter Schäfer for their kind gift of antibody.

## REFERENCES

1. **Avetisyan, A. V., P. A. Dibrov, A. L. Semeykina, V. P. Skulachev, and M. V. Sokolov.** 1991. Adaption of *Bacillus* FTU and *Escherichia coli* to alkaline conditions: the $Na^+$-motive respiration. Biochim. Biophys. Acta **1098**:95–104.
2. **Becher, B., V. Müller, and G. Gottschalk.** 1992. The methyltetrahydromethanopterin of *Methanosarcina* strain Göl is a primary sodium pump. FEMS Microbiol. Lett. **91**:239–244.
3. **Blaut, M., and G. Gottschalk.** 1984. Coupling of ATP synthesis and methane formation from methanol and molecular hydrogen in *Methanosarcina barkeri*. Eur. J. Biochem. **141**:217–222.
4. **Blaut, M., S. Peinemann, U. Deppenmeier, and G. Gottschalk.** 1990. Energy transduction on vesicles of the methanogenic strain Göl. FEMS Microbiol. Rev. **87**:367–372.
5. **Carper, S. W., and J. R. Lancaster, Jr.** 1986. An electrogenic sodium-translocating ATPase in *Methanococcus voltae*. FEBS Lett. **200**:177–180.
6. **Chapman, A. G., C. Fall, and D. E. Atkison.** 1971. Adenylate energy charge in *Escherichia coli* during growth and starvation. J. Bacteriol. **108**:1072–1086.
7. **Crider, B. P., S. W. Carper, and J. R. Lancaster.** 1985. Electron transport driven ATP synthesis in *Methanococcus voltae* is not dependent on a proton electrochemical gradient. Proc. Natl. Acad. Sci. USA **82**:6793–6796.
8. **Deppenmeier, U., M. Blaut, and G. Gottschalk.** 1991. $H_2$: heterodisulfide oxidoreductase, a second energy-conserving system in the methanogenic strain Göl. Arch. Microbiol. **155**: 272–277.
9. **Deppenmeier, U., M. Blaut, A. Mahlmann, and G. Gottschalk.** 1990. Reduced coenzyme F420:heterodisulfide oxidoreductase, a proton-translocating redox system in methanogenic bacteria. Proc. Natl. Acad. Sci. USA **87**:9449–9453.
10. **Deppenmeier, U., M. Blaut, A. Manlmann, and G. Gottschalk.** 1990. Membrane bound $F420H_2$-dependent heterodisulfide reductase in methanogenic bacterium strain Göl and *Methanolobus tindarius*. FEBS Lett. **261**:199–203.
11. **Dharmavaram, R., P. Gillevet, and J. Konisky.** 1991. Nucleotide sequence of the gene encoding the vanadate-sensitive membrane-associated ATPase of *Methanococcus voltae*. J. Bacteriol. **173**:2131–2133.
12. **Dharmavaram, R. M., and J. Konisky.** 1987. Identification of a vanadate-sensitive, membrane-bound ATPase in the archaebacterium *Methanococcus voltae*. J. Bacteriol. **169**:3921–3925.
13. **Dharmavaram, R. M., and J. Konisky.** 1989. Characterization of a P-type ATPase of the archaebacterium *Methanococcus voltae*. J. Biol. Chem. **264**:14085–14089.
14. **Dibrov, P. A., R. L. Lazarova, V. P. Skulachev, and M. L. Verkhovskaya.** 1986. The sodium cycle. II. $Na^+$-coupled oxidative phosphorylation in *Vibrio alginolyticus* cells. Biochim. Biophys. Acta **850**:458–465.
15. **DiMarco, A. A., T. A. Bobick, and R. S. Wolfe.** 1990. Unusual coenzymes of methanogenesis. Annu. Rev. Biochem. **59**:355–394.
16. **Dimroth, P.** 1987. Sodium ion transport decarboxylases and other aspects of sodium ion cycling in bacteria. Microbiol. Rev. **51**:320–340.
17. **Dybas, M., and J. Konisky.** 1989. Transport of coenzyme M (2-mercaptoethanesulfonic acid) and methylcoenzyme M [(2-methylthio)ethanesulfonic acid] in *Methanococcus voltae*: identification of specific and general uptake systems. J. Bacteriol. **171**:5866–5871.
18. **Efiok, B. J. S., and D. A. Webster.** 1990. Respiratory-driven $Na^+$ electrical potential in the bacterium *Vitreoscilla*. Biochemistry **29**:4734–4739.
19. **Gennis, R. B.** 1989. Biomembranes: molecular structure and function, p. 249. Springer-Verlag, New York.
20. **Gunsalus, R. P., J. A. Romesser, and R. S. Wolfe.** 1978. Preparation of coenzyme M analogues and their activity in the methylcoenzyme M reductase system of *Methanobacterium thermoautotrophicum*. Biochemistry **17**:2374–2377.
21. **Jarrell, K., S. Bird, and G. D. Sprott.** 1984. Sodium-dependent isoleucine transport in the methanogenic archaebacterium *Methanococcus voltae*. FEBS Lett. **166**:357–360.
22. **Jarrell, K. F., and G. D. Sprott.** 1985. Importance of sodium to the bioenergetic properties of *Methanococcus voltae*. Can. J. Microbiol. **31**:851–855.
23. **Jussofie, A., R. Hedderich, R. Böcher, and R. K. Thauer.** 1988. Methane formation from methanol and molecular hydrogen by protoplasts of new isolates. Arch. Microbiol. **146**:245–249.
24. **Ken-Dor, S., S. Preger, and Y. Avi-Dor.** 1986. Functional characterization of the uncoupler-insensitive $Na^+$ pump of the halotolerant bacterium BA1. Arch. Biochem. Biophys. **244**:122–127.
25. **Krulwich, T.** 1983. $Na^+/H^+$ antiporters. Biochim. Biophys. Acta **726**:245–264.
26. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
27. **Lancaster, J. R., Jr.** 1986. A unified scheme for carbon and electron flow coupled to ATP synthesis by substrate level phosphorylation in the methanogenic bacteria. FEBS Lett. **199**:12–18.
28. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
29. **Lübben, M., H. Lünsdorf, and G. Schäfer.** 1987. The plasma membrane ATPase of the thermoacidophilic archaebacterium *Sulfolobus acidocaldarius*. Eur. J. Biochem. **167**:211–219.
30. **Lübben, M., and G. Schäfer.** 1989. Chemiosmotic energy conversion of the archaebacterial thermoacidophile *Sulfolobus acidocaldarius*: oxidative phosphorylation and the presence of an $F_0$-related *N,N'*-dicyclohexylcarbodiimide-binding proteolipid. J. Bacteriol. **171**:6106–6116.
31. **MacLeod, R. A., G. A. Wisse, and F. L. Stejskal.** 1988. Sensitivity of some marine bacteria, a moderate halophile, and *Escherichia coli* to uncouplers at alkaline pH. J. Bacteriol. **170**:4330–4337.
32. **Peinemann, S., R. Hedderich, M. Blaut, R. K. Thauer, and G. Gottschalk.** 1990. ATP synthesis coupled to electron transfer from $H_2$ to the heteodisulfide of 2-mercaptoethanesulfonate and 7-mercaptoheptanoylthreonine phosphate in vesicle preparations of the methanogenic bacterium strain Göl. FEBS Lett. **263**:57–60.
33. **Pomeroy, L. R., and J. Imberger.** 1981. The physical and chemical environment, p. 21–36. *In* L. R. Pomeroy and R. G. Wiegert (ed.), The ecology of a salt marsh. Springer-Verlag, New York.
34. **Pressman, B. C.** 1976. Biological applications of ionophores. Annu. Rev. Biochem. **45**:501–530.
35. **Reed, P. W.** 1979. Ionophores. Methods Enzymol. **55**:435–454.

36. **Rottenberg, H.** 1979. The measurement of membrane potential and ΔpH in cells, organelles, and vesicles. Methods Enzymol. **55**:547–569.
37. **Tokuda, H., and T. Unemoto.** 1981. A respiration-dependent primary sodium extrusion system functioning at alkaline pH in the marine bacterium *Vibrio alginolyticus*. Biochem. Biophys. Res. Commun. **102**:265–271.
38. **Tsuchiya, T., and S. Shinoda.** 1985. Respiration-driven $Na^+$ pump and $Na^+$ circulation in *Vibrio parahaemolyticus*. J. Bacteriol. **162**:794–798.
39. **Udagawa, T., T. Unemoto, and H. Tokuda.** 1986. Generation of a $Na^+$ electrochemical potential by the $Na^+$ motive NADH oxidase and $Na^+/H^+$ antiport system of a moderately halophilic *Vibrio costicola*. J. Biol. Chem. **261**:2616–2622.
40. **Whitman, W. B., E. Ankwanda, and R. S. Wolfe.** 1982. Nutrition and carbon metabolism of *Methanococcus voltae*. J. Bacteriol. **149**:852–863.