# Osmotics of Halophilic Methanogenic Archaeobacteria

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*To Tiago, my son and friend.*

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# ABSTRACT

## Osmotics of Halophilic Methanogenic Archaeobacteria

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Halophilic methanogenic archaeobacteria populate saline and hypersaline environments, where they convert methylated compounds to methane. Methylamines are thought to be the dominant methanogenic substrates that can be utilized by halophilic methanogens. *Methanohalophilus mahii* and *Methanohalophilus halophilus*, two moderately halophilic methanogens from such environments, were the focus of this study into the physiology and osmotics of halophilic methanogens. These methanogens grew at broader salinity ranges than previously reported. In fact, *Methanohalophilus mahii* could grow in medium without added salt, contrary to published studies. Saline environments have low water-activity, and microorganisms must accumulate solutes to maintain a homeostatic level of cytoplasmic hydration. In *Methanohalophilus mahii* and *Methanohalophilus halophilus*, the cytosolic concentrations of $K^+$ and $Na^+$ were low relative to the total external solute concentration, and no other major intracellular cations were detected. This suggested that halophilic methanogens balance external osmotic pressure mainly by accumulating organic molecules, as do most halophiles. Significant concentrations of glycine betaine were observed in three moderately halophilic methanogens and in the extremely halophilic *Methanohalobium evestigatum*. Endogenous *N,N*-dimethylglycine was identified as a novel compatible solute and the predominant cytosolic osmolyte in *Methanohalophilus*. Exogenous sarcosine (monomethylglycine), dimethylglycine, and glycine betaine (trimethylglycine) stimulated growth of *Methanohalophilus mahii* in saline medium.

In some saline habitats (*e.g.*, salterns and intertidal ponds), halophilic methanogens are exposed to osmotic stress caused by rain storms or evaporation. Dilution stress must be particularly critical for many halophilic methanogens because their cell walls can not bear significant turgor pressure. The cytosolic water activity, measured in *Methanohalophilus mahii* growing in 2 M NaCl indicated the absence of a detectable turgor pressure in this archaeobacterium. *Methanohalophilus mahii* displayed unexpected capabilities to survive both hypertonic osmotic shocks and 10-fold hypotonic shocks.

# INTRODUCTION

**Background.** The physicochemical processes of life occur in aqueous solution. The actual structure of the molecules that support life results directly from the interaction with their aqueous environment, where proteins, nucleic acids, lipid membranes, and complex carbohydrates require specific physico-chemical conditions to perform their vital functions. Microbes accomplish such conditions in their internal milieu by regulating the composition of the cytosol. While the exchange of most solutes between the cytosol and external medium is subjected to cellular control, the transfer of water across the membrane is free and rapid. Osmosis keeps the activity of water ($A_w$) in the cytosol, which is lowered by the presence of solutes, equal to that in the external medium. Cells living in media with high solute concentration have a metabolic machinery adapted to function at the low $A_w$ of their cytosol. In many microbial habitats, the presence of solutes, predominantly NaCl (14, 39), reduces $A_w$ to levels not suitable for most organisms (Table 1).

Only a few specialized organisms, such as the halophilic and halotolerant bacteria, are able to tolerate low $A_w$ caused by salinity. Other organisms, specialized to grow in environments in which low $A_w$ is caused by other factors than salinity, are called xerophilic or xerotolerant (12).

**Table 1.** Lower $A_w$ limits for growth of bacteria[a].

| Bacteria | $A_w$ |
|---|---|
| Non-halophiles | 0.949 |
| *Bacillus subtilis* | 0.945 |
| *Pseudomonas aeruginosa* | 0.932 |
| *Escherichia coli* | 0.900 |
| *Lactobacillus* sp. | 0.860 |
| *Staphylococcus aureus* | |
| Moderate halophiles | 0.860 |
| *Vibrio costicola* | 0.860 |
| *Paracoccus halodenitrificans* | |
| Extreme halophiles | 0.750 |
| *Halobacterium* sp. | |

[a]After Kushner (76)

Halophiles accumulate solutes in their cytosol in order to prevent the loss of water by osmosis (178). This would occur when the external medium has lower $A_w$ than the cytosol. Because the accumulated solutes are not inhibitory to enzyme function they are called compatible solutes (11).

In some environments such as estuaries, solar salterns, shallow lakes, and intertidal ponds, microbes are subjected to osmotic stress due to fluctuations in the solute concentration caused by rain storms or evaporation. When exposed to increasing osmolalities, water moves rapidly out of the cell by diffusing through the cell membrane, causing cells to shrink (68, 97). Likewise, decreases in external osmolality leads to swelling (68, 97). Bacteria respond to these changes in a number of ways which lead to adjustments of the osmolality of the cytosol to balance that of their milieu. For instance, when exposed to high salinities they accumulate compatible solutes. When the rise in osmolality is too great, excessive dehydration may cause cells to become dormant or die (12). When decreases in the salinity are too great, cells may accumulate so much water that they burst, because the tensile strength of the cell wall is overcome by the stress of turgidity (68, 97). Dilution stress is thought

to be particularly critical for cells that, like many methanogens, have an envelope which is devoid of a significant tensile strength (61).

High and fluctuating osmolalities occur in many methanogenic habitats. Halophilic methanogenic archaeobacteria are adapted to live in saline environments and halotolerant methanogens thrive at a wide range of osmolalities. But most of the methanogens so far isolated are salt sensitive, and sodium salts represent an important inhibition problem for the anaerobic treatment of a variety of industrial waste-waters and agricultural wastes such as distillery wastes, sauerkraut production, rayon spinning, production of chemicals, tomato canning, edible-oil refining, and dairy waste (125). Moreover, in modern anaerobic treatment plants, the re-use of water in high-solids fermentations may also lead to the development of saline conditions in digestors (87).

Methanogenesis is a biological process with great environmental and biotechnological implications. It is the terminal step of the anaerobic degradation of organic matter in many environments (7). Halophilic methanogens contribute to the global carbon, nitrogen and sulfur cycles (109, 112), as well as to the ecological equilibrium of saline environments. In addition biogenic methane is a major "green-house" gas (121).

Methanogens are essential in anaerobic processes for the treatment of organic industrial, agricultural and municipal wastes, which is a globally important process used to prevent pollution and produce biogas. In addition, methanogenic archaeobacteria play an important role in the biodegradation of important hazardous wastes (45). Halophilic methanogens accumulate organic compounds which, like glycine betaine (GB), have a potential for biotechnological applications. First studies about the effect of salinity on methanogens concerned NaCl toxicity for dilute methanogenic ecosystems (91, 116). Recently, GB (79, 130), $N_\epsilon$-acetyl-$\beta$-lysine (79, 152), glutamate (79, 129, 130, 152), $\beta$-glutamate (79, 129, 130, 152), and $\beta$-glutamine (79) were identified as cytosolic osmolytes in methanogens. The cation potassium was also reported as having an osmoregulatory role in halophilic methanogens (79). Yet major compati-

ble solutes in halophilic methanogens remain to be identified, and virtually nothing is known about the abilities of methanogens to adjust to external osmolality.

Therefore understanding osmoregulatory mechanisms used by methanogenic archaeobacteria and knowledge of their capabilities to adjust to changes in medium osmolality are requirements to comprehend anoxic ecosystems, to improve the design and management of anaerobic digestors, and to develop biotechnological processes to exploit the potential of methanogenic halophiles.

**Methanogenic archaeobacteria in saline environments.** Methanogenic archaeobacteria are ubiquitous despite their strict demands in regard to ecological niches and to their relatively narrow range of catabolic substrates. Methanogens are fastidious obligate anaerobes that require an environmental redox potential ($E_h$) below -400 mV for growth (112).

Most methanogens grow only on $H_2/CO_2$ and formate (10). Other methanogens, members of the family *Methanosarcinaceae*, produce most of the methane in nature by using acetate or methyl compounds as substrate (112, 147). Members of two genera in this family, *Methanosaeta* (= *Methanothrix*) and *Methanosarcina*, utilize acetate, which is the precursor of about 70% of the methane produced in digestors (147) and fresh-water sediments (175). Many *Methanosarcina* species also can grow on $H_2/CO_2$ and methyl compounds such as methylated amines and methanol (MeOH). Apparently, methylamines, MeOH, dimethylsulfide and methanethiol are major methanogenic substrates in saline environments, where they are consumed by halophilic methanogens also in the family *Methanosarcinaceae* (108, 112). Dimethylselenate may be another substrate for methanogenesis in sites contaminated with Se (112). These methylated methanogenic substrates may be important in such environments because they have precursors that are abundant as common components of organic matter from plants, animals and microbes. Trimethylamine (TMA) can be formed through bacterial degradation of the plant constituent choline (37, 107, 119), the compatible solutes GB (66, 105) and trimethylamine oxide (160), or by chemical reduction of trimethylamine oxide. MeOH is a major product of pectin fermentation

(134, 139, 140). Dimethylsulfide and methanethiol are formed upon decomposition of dimethylsulfoniopropionate, an osmoregulatory solute of plants and algae (19, 113), from methionine (108), or from the methylation of sulfide or methanethiol by non-methanogenic methylotrophic bacteria (38).

In fresh-water environments, the major methanogenic substrates are acetate, formate and $H_2$; these are also the main natural sources of electrons for microbial sulfate reduction (173). Studies based on the inhibition of methanogenesis by acetylene or 2-bromoethanesulfonate, or by blockage of sulfate reduction through the addition of molybdate clearly indicate that sulfate reducers in the presence of sulfate can out-compete methanogens for acetate and $H_2$ (1, 4, 66, 90, 114, 132, 148). These results are in accordance both with kinetic and thermodynamic analysis of the competition by methanogens and sulfate reducers for acetate and $H_2$. The kinetic advantages of sulfate reducers over methanogens is expressed by their higher ratio specific growth rate over the saturation constant for those substrates ($\mu_{max}/K_s$) (132). Also, the reduction of sulfate with $H_2$ or acetate is more exergonic than their methanogenic conversion (112):

$$4H_2 + SO_4^{2-} + H^+ = 4H_2O + HS^- \qquad \Delta G^{0'} = -152.2 \text{ kJ} \qquad (1$$

$$4H_2 + CO_2 = CH_4 + 2H_2O \qquad \Delta G^{0'} = -139.0 \text{ kJ} \qquad (2$$

$$CH_3COO^- + SO_4^{2-} = 2HCO_3^- + HS^- \qquad \Delta G^{0'} = -47.6 \text{ kJ} \qquad (3$$

$$CH_3COO^- + H_2O = CH_4 + HCO_3^- \qquad \Delta G^{0'} = -28.0 \text{ kJ} \qquad (4$$

Because sea-water has a $SO_4^{2-}$ concentration of 20 to 30 mM, in contrast with the 0-0.2 mM in most fresh-water systems (15), methanogenesis is restricted in saline environments. However, methane detected in several anoxic saline and hypersaline environments appears to be biogenic (112, 136). The isolation of marine methanogens that can utilize only $H_2/CO_2$ or formate (Table 2) indicates that in some cases these substrates are available for methanogenesis in saline sediments, perhaps because such habitats have high concentrations of organic matter and become depleted in

sulfate. Oremland *et al.* showed that some substrates such as methylamines and MeOH are used by methanogens, and that sulfate reducers do not compete well with methanogens for these compounds (114). These methanogenic substrates accumulate in marine sediments when methanogenesis is inhibited by 2-bromoethanesulfonic acid, supporting the thesis that methylamines and MeOH are catabolized predominantly by methanogens (114). This idea was further corroborated by the later isolation of several obligatorily methylotrophic methanogens from saline environments (Table 2). Thus, TMA, MeOH, dimethylsulfide, and methanethiol are "non-competitive substrates," whereas $H_2$, formate, and acetate are competitive substrates which are used preferentially by sulfate reducers when sulfate is not limiting (114).

**Thermodynamics.** Biologists use the term water activity ($A_w$) to express the concept of availability of water for life. When differences occur between the $A_w$ of a cell's cytosol and that of the suspending medium, water movement across membrane (osmosis) is induced, with water moving to the phase with lower $A_w$. If a bacterium with its cytosol at atmospheric pressure is placed in a fresh-water environment which is also at atmospheric pressure, the higher osmolality (therefore lower $A_w$) of the cytosol induces water to move into the cell. The direct effect of this water movement is to increase the internal volume of the cell and to dilute the cytosol. This dilution may not be sufficient to accomplish equilibrium because of the large differences between $A_w$ of the cytosol and freshwater. However, the cell walls of most fresh-water bacteria have a limited elasticity and significant tensile strength. The cell walls limit the swelling of the cells, thus causing the development of significant internal pressure, called turgor pressure ($P_t$). The tensile strength of the cell wall is conferred mainly by the peptidoglycan layer (67), which allows bacteria growing in freshwater environments to develop ample $P_t$, thereby allowing thermodynamic equilibrium between the water of the cytosol and that of the environment and yet to maintain the substantial cytoplasmic concentrations of solutes necessary for metabolic function.

**Equilibria of water potential and osmotic balance.** Although $A_w$ is a useful concept in understanding osmotic balance, this balance is actually determined by the chemical potential of water ($\mu_w$). $\mu_w$ is a function of $A_w$:

**Table 2. Methanogenic archaeobacteria from saline environments.** Except *Methanomicrobium paynteri*, which is irregularly bacillar in morphology, all the isolates are coccoid (pseudosarcina-like in *Methanosarcina acetivorans*).

| Species | Strain | Source | Substrates | Optimal [NaCl] | [NaCl] tolerance | Optimal pH | Optimal Temp | References |
|---|---|---|---|---|---|---|---|---|
| *Methanococcus deltae* | ΔRC$^T$ | estuarine | formate,$H_2$ | 0.6-0.8 | 0.5-1 | ND | 37 | 27 |
| *Methanococcus voltaei* | PS$^T$ | estuarine | formate,$H_2$ | 0.3-0.7 | ND-1 | 6-7 | 38 | 57,169,172 |
| *Methanococcus vanniellii* | SB$^T$ | marine | formate,$H_2$ | 0.1 | ND | 7-9 | 35-40 | 96,155 |
| *Methanococcus maripaludis* | JJ$^T$ | estuarine | formate,$H_2$ | 0.3-0.7 | 0.09-ND | 6.5-7.2 | 36 | 60 |
| *Methanococcus jannaschii* | JAL-1$^T$ | hydrothermal marine | $H_2$ | 0.3-0.7 | 0.1-1 | 5-7 | 85 | 59 |
| *Methanococcus thermolithotrophicus* | SN-1$^T$ | marine | formate,$H_2$ | 0.3-0.7 | 0.2-1.4 | 6.5-8.0 | 65 | 50 |
| *Methanoculleus thermophilicum* | CR1$^T$ | marine | formate,$H_2$ | 0.25 | 0-0.7 | 7 | 55 | 94,128,180 |
| *Methanoculleus marisnigri* | JR1$^T$ | marine | formate,$H_2$ | 0.1 | 0-0.7 | 6.2-6.6 | 20-25 | 94,135 |
| *Methanogenium cariaci* | JR1$^T$ | marine | formate,$H_2$ | 0.5 | 0.12-0.9 | 6.8-7.3 | 20-25 | 3,135 |
| *Methanomicrobium paynteri* | G-2000$^T$ | marine | $H_2$ | 0.15 | 0-0.8 | 6.6-7.2 | 40 | 127 |
| *Methanosarcina frisia* | C 16$^T$ | marine | $H_2$,MA,Me | 0.3-0.7 | 0.34-0.19 | 6.5-7.2 | 36 | 5,6 |
| *Methanosarcina acetivorans* | C2A$^T$ | marine | Ac,$H_2$,MA,Me | 0.2 | ND-1 | 6.5-7 | 35-40 | 8,149,151 |
| *Methanococcoides methylutens* | TMA-10$^T$ | marine | MA,Me | 0.2-0.6 | 0.1-1.1 | 7.0-7.5 | 30-35 | 150 |
| *Methanolobus tindarius* | Tindari 3$^T$ | marine | MA,Me | 0.5 | 0.06-1.27 | 6.5 | 37 | 73 |
| *Methanolobus siciliae* | T4/M$^T$ | marine | DS,MA,Me | 0.4-0.6 | 0.1-1.2 | 6.5-6.8 | 40 | 157,108 |
| *Methanolobus siciliae* | HI350 | marine oil well | DS,MA,Me,MT | 0.4-0.6 | 0.1-1.2 | 6.5-6.8 | 40 | 157,108,109 |
| *Methanolobus vulcani* | | marine | MA,Me | ND | ND | ND | 37 | 157 |
| *Methanohalophilus zhilinaeae* | WeN5$^T$ | alkaline saline lake | MA,Me | 0.7 | 0.2-2.1 | 9.2 | 45 | 9,101 |
| *Methanohalophilus oregonense* | WAL1$^T$ | alkaline saline subsurface | DS,MA,Me | 0-0.7 | 0-1.5 | 8.1-9.1 | 35-37 | 88 |
| Unassigned | GS-16 | estuarine | DS,MA,Me | 0.64 | 0.1-1.2 | 8 | 37 | 65,113 |
| Unassigned | CAS-1 | saline lake | MA,Me | 0.5-1.4 | 0.51-1.4 | 6.4-7.8 | 42 | 99 |
| *Methanohalophilus mahii* | SLP$^T$ | solar saltern | MA,Me | 1.0-2.5 | 0.5-3.5 | 7.4-7.5 | 35-37 | 117,118 |
| Unassigned | SD-1 | solar saltern | MA,Me | 0.8-3.5 | 0.52-4.3 | 7.8 | 42 | 99 |
| Unassigned | FDF-1 | solar saltern | MA,Me | 2.1 | 1.3-3 | 7.1-7.4 | 37-42 | 99 |
| Unassigned | FDF-2 | solar saltern | MA,Me | 2.1 | 1.3-3 | 7.2 | 37-42 | 99 |
| Unassigned | SF-1 | solar saltern | MA,Me | 2.6 | 0.86-3.5 | 7.4 | 37 | 100 |
| Unassigned | RET-1 | saline lake | MA,Me | 2.1 | 1.3-3 | 7.1 | 37 | 99 |
| *Methanohalophilus halophilus* | Z-7982$^T$ | saline lake | MA,Me | 1.19 | 0.4-ND | 6.5-7.4 | 37 | 181,182 |
| *Methanohalobium evestigatum* | Z-7303$^T$ | saline lake | MA | 4.28 | 2.5-5.13 | 7.0-7.5 | 50 | 182,183,184 |

Abbreviations: ND: not determined; $H_2$: $H_2$ and $CO_2$; MA: methylamines; Me: methanol; DS: dimethylsulfide; MT: methanethiol.

$$\mu_w = \mu_w^0 + RT \cdot \ln(A_w) \quad (5$$

where $\mu_w^0$ is the potential of water at standard temperature and pressure, and T is the actual temperature. The small size of bacteria preclude significant temperature gradients, so for these systems T is constant and $\mu_w$ depends on $A_w$.

The bacterial plasma membrane is a selective barrier separating the cytosol from the surrounding medium. Water can freely cross the membrane, but the membrane's lipidic nature and structure make it impermeable to polar solutes and only poorly permeable to some small non-charged lipid-soluble molecules such as fatty acids and alcohols (104). Because of this selective permeability, the system "cell plus surroundings" can be considered as two aqueous phases separated by a semi-permeable membrane. In such a system, thermodynamic equilibrium exists when the potential of water in the cytosol ($\mu_c$) is equal to the potential of water in the external medium ($\mu_m$):

$$\mu_c = \mu_m \quad (6$$

**Thermodynamic basis of the effect of pressure on $\mu_w$.** At constant temperature,

$$dG = V dP \quad (7$$

where G is the Gibbs free-energy of water and P is the pressure. The potential of water ($\mu_w$) can be idealized as the free energy divided by the number of moles of water (n):

$$d\mu_w = dG/n = (V/n)dP \quad (8$$

V/n is the partial molar volume of water ($\bar{V}$), so:

$$d\mu_w = \bar{V}\, dP \quad (9$$

Integrating equation 5 gives:

$$\mu_w = \mu_w^0 + \bar{V}\, P \quad (10$$

where $\mu_w^0$ is the $\mu_w$ of a solution at standard pressure. The term $\bar{V}P$ indicates the increase in $\mu_w$ which occurs at increased pressure, and this equation allows us to calculate $\mu_w$ at any pressure when we measure $\mu_w$ at atmospheric pressure.

**Development of turgor pressure.** Because the cytoplasmic $\mu_w$ must balance $\mu_w$ of the medium (equation 6), we can use equation 10 to relate the $\mu_w^0$ and P of the cytosol and the suspending medium (156):

$$\mu_c^0 + \bar{V} P_c = \mu_m^0 + \bar{V} P_m \quad (11$$

where $\mu_c^0$ and $\mu_m^0$ are the potential of water in the cytosol and in the medium, respectively, under standard conditions of T and P. $P_c$ and $P_m$ are the pressure of the cytosol and medium, respectively. Thus, if the solute concentrations of cytosol and medium differ (which causes differences in $\mu_w^0$), $\mu_w$ must be balanced by a difference in pressure (*i.e.*, $P_t$). Bacteria growing in fresh-water have significant $P_t$ because $\mu_c^0$ is much lower than $\mu_m^0$. This leads to a $P_c$ higher than $P_m$ for bacteria in dilute environments.

**Osmotic pressure and deviations from ideality.** Osmotic pressure ($\Pi$) is another measure of $A_w$, whose use simplifies the calculations of $P_t$. Osmotic pressure is defined as the pressure which would be required to give a solution the same $\mu_w$ as that of pure water under standard conditions. Thus, $P_t$ is equal to the difference in osmotic pressure of the cytosol and the medium:

$$P_t = \Pi_c - \Pi_m \quad (12$$

The value of $\Pi$ is expressed by the van't Hoff equation:

$$\Pi = RT \sum_j \phi_j m_j \quad (13$$

where m represents the molality of the solutes and $\phi$ is the osmotic coefficient (which corrects for deviations from ideality caused by the concentration and nature of the solute, and T). Another form of equation 13 is particularly useful because it relates $\Pi$ to $A_w$. This allows $A_w$ (and therefore $\Pi$) to be determined from a comparison of the vapor pressure of a solution to that of pure water (based on the Raoult's law):

$$\Pi = -\phi RT/(\bar{V} \cdot \ln(A_w)) \quad (14$$

Because the vapor pressure, or the relative humidity of biological solutes of unknown composition can be easily measured, this expression is often used to determine $\Pi$.

**Effects of osmolality on microbial cells.** Many microbial environments have an osmolality that is significantly lower than that of the cytosol (15, 67). Since the cytosol is enclosed within a plasma membrane with selective permeability, such an

environment leads to a $P_t$ which presses the cell membrane against the semi-rigid cell wall. The $P_t$ of bacteria at steady-state with medium osmolality can be evaluated from the $A_w$ of crude extracts, from the minimum $\Pi_m$ which induces plasmolysis (158), or from the measurement of the hydrostatic pressure required to collapse extracellular or cytoplasmic gas vesicles (70, 166). It was demonstrated that $P_t$ plays a major role in maintaining cell size and shape, and there is evidence that it is required for cell growth and morphology definition in fresh-water bacteria (67, 69).

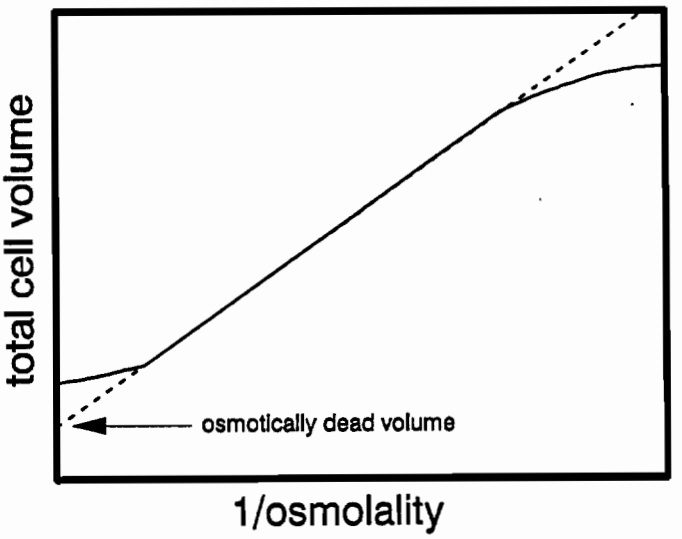


**Figure 1.** Effect of external osmolality on cell volume. The dashed line indicates changes predicted for an ideal osmometer, and the solid curve indicates the non-ideal behavior of microbial cells.

The movement of water across a cell membrane takes place rapidly, generally leading to equilibrium within a few milliseconds (68, 97). The immediate consequence of any variation of $\Pi_m$ is the passage of water between the cytosol and the medium, with the resultant change in cell volume (Fig. 1).

The swelling and shrinking of bacterial cells can be approximated (within limits) by the van't Hoff-Boyle equation for ideal osmometers (68):

$$V = V_0 \ \Pi_c / \Pi_m + b \qquad (15$$

where V is the total cell volume, $V_0$ is the initial cytosolic volume, and b is the osmotically dead volume (*i.e.*, the volume of the dry matter of the cell).

Deviations from the ideal behavior predicted by the van't Hoff-Boyle equation start soon after plasmolysis begins and at the point where swelling reaches the upper limit of the peptidoglycan-layer elasticity (68, 97).

The structural nature of the cytosol, still a matter of controversy (25, 174), is critical for organisms adapted to live at high osmolalities. These organisms accumulate solutes that must be freely mobile within the cytoplasm in order to be active osmolytes, thus maintaining the cell in osmotic balance with its surroundings (110, 174). The concept that water in cells has the properties of bulk water appears overly simplistic considering the presence of active macromolecules and, in some cases, organelles (14, 25, 174). Nuclear magnetic resonance (NMR) studies showed that glycerol accumulated by the unicellular algae *Synechococus* sp. and *Dunalliela* sp. (11) can freely move within the cytosol (110). Similar results were obtained from $^{39}$K-NMR for $K^+$ accumulation by *Halobacterium halobium* (95). However other studies suggest that, in *Halobacterium halobium*, accumulated $K^+$ ions may be fixed in "semi-crystalline cell water" (26). As Brown pointed out (14), on balance most of the evidence favors "normal" physical chemistry of internal solutes, and strong evidence to support a gel-like structure of cytosol was not produced even with an external $K^+$ and $Cl^-$ concentrations as high as 5 M. Also, in many studies of eukaryotic and procaryotic organisms, cell volume changes in response to osmotic variations are well predicted by the van't Hoff-Boyle equation for perfect osmometers (25, 68, 97). This implies that, even at relatively high solute concentrations, the cytosol behaves like a true solution.

**Microbial osmoregulation.** Since cells continue to regulate their volume even when transferred to anisosmotic media (44, 69), it may be inferred that cells at steady state with the external medium have net influx and efflux of water and osmotically active solutes in balance. Because microbes are unable to control water fluxes through their membranes (47), they must actively control $\Pi_c$ in order to maintain a homeostatic level of cytoplasmic water and regulate $P_t$ and volume.

The cytosol of bacteria and most other cells contains essential solutes with a combined osmolality of about 300 mOsm (104). Because $A_w$ of the cytosol must balance that of the medium, cells must use one of several means to adapt if they are to thrive in environments of other osmolalities. A common mechanism for adaptation to fresh-water environments (which have low osmolalities) is the presence of a cell

wall with tensile strength sufficient to withstand the $P_t$ which develops (69). In contrast, growth in environments of higher osmolalities may be accommodated by accumulation of one or more cytoplasmic solutes which lower the internal $A_w$. Many halophiles, including methanogenic species, have proteinaceous cell envelopes (S layers) devoid of significant tensile strength (72), and which therefore can not bear a considerable $P_t$. Information about $P_t$ in halophiles is scarce. However, the fact that no detectable $P_t$ occurs in one studied extremely halophilic archaeobacterium (167) denotes an adaptive mode which does not compel $\Pi_c$ to be higher than $\Pi_m$. It is not known how S-layered halophiles respond to hypotonic shock and maintain their shape.

**Microbial adaptation to high salinities.** The osmolality of most natural waters is predominantly due to NaCl concentrations (14, 39, 122), although there are some exceptions (*e.g.*, the Dead Sea contains greater amounts of $Mg^{2+}$ than $Na^+$ [122]). Microorganisms of various kinds are adapted to live and proliferate at salinities which range up to saturated NaCl (6.2 molal, $A_w = 0.75$ at 30°C). Microbes inhabiting saline environments and requiring NaCl for growth are designated as halophiles (81), and may be subdivided according to the concentration of NaCl which gives most rapid growth: non-halophiles (<0.2 M), slight halophiles (0.2 to 1.2 M), moderate halophiles (1.2 to 2.5 M) and extreme halophiles (>2.5 M [53]). Some microbes grow well at high salinities, but they have no strict requirement for NaCl and grow more rapidly at lower salinities. Such bacteria are called halotolerant (81), and they are subdivided analogously to the classification of halophiles, but based on the maximum salinity which allows growth (53): non-halotolerant (<0.2 M), slightly halotolerant (0.2 to 1.2 M), moderately halotolerant (1.2 to 2.5 M) or extremely halotolerant (>2.5 M [53]).

In order to balance $\Pi_m$, halophilic and halotolerant bacteria accumulate osmolytes, that are not otherwise required for normal cell activity, in a concentration that depends on the osmolality of the medium (178). In saline environments, cytosolic solute concentration must be very high. This could be accomplished by the accumulation of inorganic ions such as $K^+$, $Na^+$, or $Cl^-$, but such a response would require a high degree of specialization, because these ions are inhibitory to enzymes

not adapted to their presence. Indeed, most organisms actively regulate the concentrations of these ions within relatively narrow limits, independently of concentration of these electrolytes in the medium (47, 53, 178). The cation $Na^+$ is maintained at low intracellular concentrations, with many eukaryotes and prokaryotes maintaining a high cytoplasmic ratio of $[K^+]/[Na^+]$ (18, 39, 47).

**Table 3. Concentrations (mM) of the potassium and sodium cations, and their relative ratio in eukaryotic and prokaryotic cells.**

| CELL | $[K^+]$ | $[Na^+]$ | $[K^+]/[Na^+]$ | References |
|---|---|---|---|---|
| Human erythrocyte | 135 | 15 | 9 | 47 |
| *Dunaliella salina* | 150 | 20 | 7.5 | 62 |
| *Staphylococcus aureus* | 706 | 64 | 11 | 23 |
| *Escherichia coli* | 150 | 67 | 2.2 | 55 |
| *Salmonella oranienburg* | 239 | 131 | 1.8 | 24 |

Most halophiles accumulate organic osmolytes, despite the energetic costs of synthesis or uptake of such compounds (30, 31, 53, 178). These compounds are not generally deleterious to cell function, so they are called compatible solutes (11). Generally these solutes are small molecules, highly soluble in water, and with no net charge at physiological pH. However, a few bacteria, including the extremely halophilic archaeobacteria *Halobacterium* and *Halococcus*, accumulate the inorganic solutes KCl and NaCl up to 4.5 and 1.4 M respectively (24, 77). Enzymes from these archaeobacteria have an absolute requirement for salt and highest activity at a KCl concentration of 4 M (14). To achieve such a level of salt adaptation these proteins have a great proportion of aspartyl, glutamyl and weakly hydrophobic residues in comparison to the intracellular proteins of common organisms (178). KCl and NaCl were also reported to be osmoregulatory solutes in the halophilic anaerobic eubacteria *Halobacteroides halobium* and *Haloanaerobium praevalens* (115), and in a haloalkaliphilic *Bacillus* strain (170).

Common bacterial compatible solutes are amino acids (49, 162, 102), amino-acid derivatives (54), and sugars (33). In bacteria, GB is by far the most common osmo-regulatory solute (31, 53). It is also accumulated by other organisms such as marine eukaryotes and halophytic plants (28). Among eubacteria, GB is a compatible solute in a wide range of genera: *Pseudomonas* (30), *Escherichia* (80), *Rhizobium* (83), *Agrobacterium* (146), *Lactobacillus* (52), *Pediococcus* (137), *Vibrio* (30), *Erwinia* (34), *Klebsiella* (84), and *Thiobacillus* (64). GB is also widespread as a compatible solute in halophilic eubacteria (54) and halophilic methanogenic archaeobacteria (79, 130). It is ubiquitous in halophilic cyanobacteria (92) and is accumulated by the moderate halophiles *Vibrio costicola*, *Alcaligenes* strain A-514$^b$, *Alteromonas* strain A-387$^b$, *Pseudomonas* strain F-12-1$^b$, *Alcaligenes* strain F-5-7$^b$, *Acinetobacter* strain F-12-1$^b$, *Chromobacterium marismortui*, *Micrococcus halobium* (54), *Ectothiorhodospira halochloris* (43). In *Escherichia coli*, GB is formed by a two step oxidation of choline (80). Choline is oxidized to betaine aldeide, which is further oxidized with molecular oxygen (80).

Proline and glutamate also contribute to the osmotic balance of non-halophilic eubacteria (30), however these solutes have not been reported at osmotically significant concentrations. Proline occurs in members of the *Enterobacteriaceae* (85), *Pseudomonaceae* (30), *Rhizobiaceae* (83) and *Vibrionaceae* (30). Some unusual organic compounds are compatible solutes in halophilic microorganisms. The novel cyclic amino acid derivative ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid) is a major compatible solute in the halophiles *Ectothiorodospira* sp. (42, 163), *Vibrio costicola* (123), *Deleye halophila*, *Halomonas elongata*, and *Halomonas halinophila* (176). Another novel amino acid derivative $N^{\alpha}$-carbamoyl-L-glutamine 1-amide is accumulated by the halophilic *Ectothiorhodospira marismortui* (41). Trehalose is an osmoregulatory solute in *Escherichia coli* (33), *Rhizobium* sp. (35), and in the halophilic *Ectothiorodospira halochloris* (40). The unusual disaccharide mannosucrose ($\beta$-furanosyl-$\alpha$-mannopyranoside) is accumulated in *Agrobacterium tumefaciens* (146). Some exogenous compatible solutes or related compounds can

alleviate osmotic stress. Proline, GB, choline, proline betaine, betaine aldehyde, sulfo-betaine, trimethylaminobutyric acid and glycerol have osmoprotectant properties for enterobacteria (20, 21, 22, 33, 80, 84). GB stimulates growth of *Azospirillum brasiliense* (126), and *Brevibacterium lactofermentum* under salt stress (63).

The complete intracellular role of compatible solutes is not well understood and remains a matter of controversy. Such solutes are certainly osmotically active cytoplasmic constituents, but other suggested roles include the inducement of changes in water structure, or a direct stabilizing action on macromolecules, especially proteins (2, 13, 25, 29, 71, 174, 178).

Although sodium cations are essential in methanogenic bioenergetics (16, 32, 57, 141, 145) and are indispensable for growth of methanogenic bacteria (120), moderate concentrations of $Na^+$ are toxic for many methanogenic bacteria (91, 116). NaCl concentrations of about 400 mM cause 50% inhibition of the rate of methanogenesis from acetate (87, 125) and from $H_2/CO_2$ (87, 116) by adapted and non-adapted cultures. Although the observed inhibition can be attributed in part to a direct effect of the $Na^+$ cation (91, 116), the osmotic stress developed at these NaCl concentrations may also affect the activity of non-halotolerant or non-halophilic methanogens. Nevertheless some methanogens which can grow in NaCl concentrations up to 1.5 M were isolated from digestors (94). The presence in the culture medium of compounds which may serve as compatible solutes made it impossible to know if the tolerance to salinity in culture is conferred by exogenously supplied solutes or whether compatible solutes can be synthesized by the microbes as a response to osmotic stress. Yeast extract, a common component of laboratory growth media, contains GB (34); GB can alleviate salt stress in eubacteria (84) and is a compatible solute in methanogens (Table 4). GB occurs in plants (*e.g.*, wheat, barley, rye [28, 39]), along with other betaines (*e.g.*, proline betaine, $\gamma$-butyrobetaine and choline [37, 107]), compounds that also can relieve salt stress in eubacteria (80, 83). Thus, GB and other osmo-protectant compounds are likely to occur in many natural and man-made anaerobic habitats. Uptake systems for osmo-protectant

compounds have not been described for methanogens. However, such systems, widespread among eubacteria (30), may occur in methanogens as well.

Studies on methanogenic bacteria from saline environments started only a few years ago, mainly motivated by the increased interest in the biotechnological potential of organisms from extreme environments. Since then, several methylotrophic methanogens were isolated from saline environments (Table 2).

The first important osmolyte found in archaeobacteria was $K^+$ (23), which is also accumulated by other strictly halophilic anaerobic bacteria (*Halobacteroides halobium* and *Haloanaerobium praevalens*) (115). Thus, one might expect that $K^+$ is a major osmolyte methanogenic bacteria. This hypothesis was reinforced by the reported $K^+$ accumulation in *Methanobrevibacter smithii*, *Methanobrevibacter arboriphilicus* and *Methanobacterium thermoautotrophicum* at cytosolic concentrations above 1 M, even when growing in dilute media (58). Yet five independent studies with 10 different methanogens (56, 138, 142, 143, 154) found the cytosolic concentration of $K^+$ in fresh-water methanogens to be 0.15 to 0.70 M. $K^+$ was reported to have an osmoregulatory role in *Methanohalophilus* strain FDF-1 (79). However the cytosolic concentration measured indicated that it was not a major osmolyte, and other methanogenic archaeobacteria accumulate mainly organic osmolytes, including $\beta$-amino acids and other amino acid derivatives (79, 129, 130, 152) (Table 4).

Among the compatible solutes described for methanogens, glutamate is by far the most common. It occurs in all studied methanogens (79, 129, 130, 152). $\beta$-Glutamate is accumulated by thermophilic species, slightly halophilic strain CAS-1 and moderately halophilic *Methanohalophilus halophilus* (79, 129, 130, 152). $\beta$-Glutamine is accumulated by halophilic methanogenic species (79). GB was reported for *Methanococcus voltaei,* halotolerant *Methanogenium* sp. and moderately and extremely halophilic methanogens, with striking exceptions in *Methanohalophilus halophilus* and *Methanohalophilus* strains FDF2 and RET-1 (79). $N_\epsilon$-Acetyl-$\beta$-lysine is a compatible solute in *Methanosarcina thermophila*, *Methanogenium cariaci*, and in halophilic methanogens (79, 152). The concentrations of compatible solutes were

**Table 4. Osmoregulatory osmolytes in methanogenic archaeobacteria.**

| OSMOLYTE | METHANOGEN | ORIGIN | REFERENCE |
|---|---|---|---|
| $K^+$ | | | |
| | *Methanohalophilus* strain FDF-1 | exogenous | 79 |
| Glutamate | | | |
| | *Methanosarcina thermophila* | endogenous | 152 |
| | *Methanogenium* strain AN9 | | 129,130 |
| | *Methanogenium cariaci* | endogenous | 129,130,152 |
| | *Methanococcus thermolithotrophicus* | | 129 |
| | *Methanococcus voltaei* | | 129,130 |
| | Strain CAS-1 | endogenous | 79 |
| | Strain GS-16 | endogenous | 79 |
| | *Methanohalophilus zhilinaeae* | | 129,130 |
| | *Methanohalophilus halophilus* | endogenous | 79 |
| | *Methanohalophilus mahii* | endogenous | 79,129,130 |
| | *Methanohalobium evestigatum* | endogenous | 79 |
| β-Glutamate | | | |
| | *Methanococcus thermolithotrophicus* | endogenous | 129,130 |
| | *Methanococcus jannaschii* | endogenous | 129,130 |
| | *Methanogenium cariaci* | endogenous | 129,130,152 |
| | Strain CAS-1 | endogenous | 79 |
| | *Methanohalophilus halophilus* | endogenous | 79 |
| β-Glutamine | | | |
| | Slightly halophilic strain GS-16 | endogenous | 79 |
| | Slightly halophilic strain CAS-1 | endogenous | 79 |
| | *Methanohalophilus* sp. | endogenous | 79 |
| | *Methanohalobium evestigatum* | endogenous | 79 |
| Glycine betaine | | | |
| | *Methanogenium* strain AN9 | | 129,130 |
| | *Methanogenium cariaci* | endogenous | 129,130 |
| | *Methanococcus voltaei* | | 130 |
| | *Methanohalophilus zhilinaeae* | | 130 |
| | *Methanohalophilus* sp. | endogenous | 79 |
| | *Methanohalobium evestigatum* | endogenous | 79 |
| Nε-Acetyl-β-lysine | | | |
| | *Methanosarcina thermophila* | endogenous | 152 |
| | *Methanogenium cariaci* | endogenous | 152 |
| | Slightly halophilic strain GS-16 | endogenous | 79 |
| | Slightly halophilic strain CAS-1 | endogenous | 79 |
| | *Methanohalophilus* sp. | endogenous | 79 |
| | *Methanohalobium evestigatum* | endogenous | 79 |

determined in *Methanosarcina thermophila* (152) and in *Methanohalophilus* strains FDF-1 and Z-7401 (79). Their total osmolalities only attain about 25% and 50% of that required to balance $\Pi_m$, thus indicating that knowledge of osmoregulatory limits the magnitude of the $\Pi$ gradient which the cell can withstand. Knowledge of cyto-

solic solutes in halophilic and halotolerant methanogens is still incomplete and that they have compatible solutes yet to be identified.

**Microbial adaptation to low-salt environments.** The ability of bacterial cells to grow in dilute environments is widespread. In such environments, the osmolality of the cytosol is generally much higher than that of the menstruum, so $P_t$ is significant. Eubacterial cell walls have a peptidoglycan layer which resists the $P_t$ by its tensile strength (69). The elasticity of the cell wall determines the extent of swelling, and the wall's tensile strength Gram-positive bacteria have a thick peptidoglycan layer (*ca.* 20 nm) and generally have a high $P_t$ (15-20 atm [70, 167]). Gram-negative cells have a thinner peptidoglycan layer (*ca.* 4.5 nm) and lower $P_t$ (1-4 atm [70, 167]). In contrast with the need of the halophiles for the accumulation of solutes in the cytosol, fresh-water bacteria have a cytosolic solute concentration that is limited by the tensile strength that the cell envelope can bear.

Fresh-water methanogens must also have a cytosolic osmolality of vital solutes that is higher than that of the menstruum. So, a significant $P_t$ is expected to occur in these archaeobacteria. Studies with spheroplasts from *Methanospirillum hungateii* (153) provide some useful information concerning $P_t$ in fresh-water methanogens. *Methanospirillum hungateii* has a protein S-layer and is contained in a sheath which confers rigidity and resists the cell's turgidity (61, 72). Spheroplasts are formed by treatment with dithiothreitol, which removes the sheath (153). These spheroplasts require a sucrose concentration of about 0.5 M to prevent hypotonic lysis, a concentration similar to the minimum concentration which causes plasmolysis in the same archaeobacterium (153). This osmolality indicates a $P_t$ of about 12 atmosphere for cells in dilute medium.

Methanogens which stain gram-positive have cell walls composed of pseudomurein, methanochondroitin, or heteropolysaccharide (72) that presumably constitutes structures with osmoadaptive features similar to the peptidoglycan layer of eubacteria. *Methanospirillum* and *Methanosaeta* sp. have a cell wall composed of a protein cell wall and an outer sheath that exhibits an unusual resistance to chemical agents (72).

*Methanobacteriales* strains have a dense cell wall composed of a pseudomurein layer with a thickness of about 15 nm (72). *Methanosarcina barkeri* has a protein cell wall and a rigid heteropolysaccharide outer layer with a thickness up to 200 nm (72). By the same token that thick-walled fresh-water eubacteria can resist fairly large internal hydrostatic pressures (17), it may be expected that methanogens inhabiting dilute environments would be the type which possess strong cell walls, such as thick pseudomurein walls, able to resist significant $P_t$. However about 50% of the described species of fresh-water methanogens have only a proteinaceous cell wall (10, 72). Even *Methanosarcina mazeii* and other *Methanosarcina* often lack any cell envelope other than an S-layer (89, 177). *Methanogenium*, *Methanocorpusculum*, *Methanococcus*, and *Methanoculleus* sp., occur in dilute environments (94). In the absence of any information related to the $\Pi_c$ of such organisms, their prevalent morphology (very irregular coccus with a lobulated morphology) suggests absence of any $P_t$. It is intriguing and remains to be explained whether or how such methanogens maintain a homeostatic cytoplasmic $A_w$. These ubiquitous methanogens, which are sensitive to isolation procedures, may be even more predominant than described, because isolation attempts may not detect the presence of these bacteria as easily as the larger, faster-growing, rigid-wall cells (177).

**Microbial response to increasing salt concentration.** When exposed to a sudden increase in external osmolarity, bacteria lose water and begin to plasmolyze before they can build up a sufficient concentration of compatible solutes. *Escherichia coli* requires more than 0.5 h to take up exogenous osmoprotectants or more than 1 h to synthesize an effective compatible solute concentration to counteract an instantaneous increment of 500 mOsm (33). These times are consistent with the requirement for *de novo* enzyme synthesis, either for transport proteins or for the synthesis of the compatible solutes themselves (33, 168). Thus, the initial cellular change which accompanies increasing environmental salinity is a loss of water and decrease in cell volume. This passive alteration of cell volume does not represent a satisfactory, long-term response to osmotic stress or an effective means of osmoadaptation. Plasmolysis

alone would lead to an elevated concentration of various toxic intracellular molecules, especially specific enzymatic inhibitors and electrolytes, which lead to inhibition of nutrient uptake (168) and DNA replication (103); this compromises cell viability.

Little is known about the immediate responses of bacteria to increases in salinity, but the response of *Escherichia coli* has been examined. The very first observed reaction to increased medium osmolality is a relatively rapid accumulation of $K^+$ (33, 53, 171). In order to maintain the membrane potential, that increment in $K^+$ concentration must be balanced by the equivalent accumulation of anions, which is only partially achieved by an increase in intracellular glutamate levels (33, 171). The observed glutamate concentrations are systematically lower than that expected to balance $K^+$ (33). The cytosolic pH of *Escherichia coli* (33) and of the alga *Dunaliella tertiolecta* (75) increases after exposure to saline media. In cells under hypertonic stress, $K^+$ influx is stimulated whereas the rate of efflux is probably unchanged (36). Apparently, the net ratio $K^+_{influx}/K^+_{efflux}$ is under control of the membrane tension (36). A mechano-sensitive ion channel with high selectivity for $K^+$ ions was found in *Escherichia coli* membranes (98).

The accumulation of $K^+$ and glutamate constitutes only a transient means for the relief of plasmolysis. Presumably $K^+$ accumulation is used because it is a more rapid response than the synthesis of organic compatible solutes, which is otherwise the preferred mechanism. Soon after hypertonic stress starts, trehalose over-production begins and within 1 h is the main compatible solute in *Escherichia coli* (33, 171). Before trehalose synthesis can begin, the biosynthetic enzymes must be synthesized *de novo* (33, 171). But, when the osmoprotectant proline or GB is present in the hypersaline medium, no significant $K^+$ accumulation is observed and trehalose over-production is not triggered (33). This indicates the existence of a constitutive system for the transport of GB, compound which is known to alleviate hypertonic shock in *Escherichia coli* (84). The mechanisms that methanogens use to respond to hyperton-ic stress are not known.

**Bacterial osmoresponse to decreasing salt concentration.** Just as water efflux is the immediate result of a rapid increase in environmental salinity, water influx is the initial result of decreasing salinity. The active excretion of solutes is as slow in these systems as is their uptake when external salinity increases. Thus, cell volume increase and dilution of the cytoplasm is the immediate consequence of hypotonic shock. This increases $P_t$ and stresses the cell wall. Excessive swelling and consequent stretching of the plasma membrane and cell wall may jeopardize membrane integrity. Therefore, to overcome osmotic stress cells must activate effective mechanisms of osmoresponse before cell structure and function are at risk. Many archaeobacteria are particularly at risk because their protein cell walls have little tensile strength.

When *Escherichia coli* is subjected to hypotonic shock, a transient breakdown of the permeability barrier of the membrane occurs. This allows the immediate excretion of small molecules and thereby lowers the $P_t$ (36, 102, 164). The loss of cations is subjected to the restrictions of electroneutrality, hence a significant retention of $K^+$ is observed (36). Apparently, a reduction in the cell pool of amino acids, presumably through excretion or polymerization, is the primary mechanism by which bacteria adjust to hypotonic media (162). However, because availability of amino acids should not limit protein synthesis in growing cells, it is likely that such a mechanism may only be adequate for relatively small drops in media osmolality. The extremely halophilic bacterium *Ectothiorhodospira halochloris* reacts to dilution stress primarily by excretion of GB, its major compatible solute (165). GB excretion occurs within minutes (165), yet this response is unlikely to be as fast as it would be required to prevent $P_t$ to be boosted. A similar situation may occur in methanogens. However studies on osmoregulation in methanogenic archaeobacteria are very recent and the mechanisms that methanogens use to respond to dilution stress are not known.

**Microbial growth in saline habitats.** Methanogenic archaeobacteria inhabit saline and hypersaline environments such as saline lakes (99, 117, 179), solar salterns (99, 100), intertidal lagoons (182), estuaries (65, 113), and saline ground water (88). Among the described moderately or extremely halophilic methanogens which grow

well at neutral pH, one species is extremely halophilic (*Methanohalobium evestigatum*) and two species are moderately halophilic (*Methanohalophilus halophilus* and *Methanohalophilus mahii*). One other species of neutrophilic, halophilic methanogens (*Halomethanococcus doii*) was reported (179) but the availability of its type strain and its distinction from *Methanohalophilus halophilus* and *Methanohalophilus mahii* are in doubt (8).

All the halophilic methanogens so far isolated are obligate methylotrophs, whose catabolic substrates include MeOH, methyl amines (including monomethylamine [MMA], dimethylamine, and TMA), and methyl sulfides (dimethylsulfide and methanethiol [88, 99, 101, 108, 109, 117, 182]). Thus, methanogens may be an important source of methane in saline lakes, and the trophic niches for methanogens in saline and hypersaline ecosystems must include one or more of these methyl compounds as catabolic substrate. TMA, which is catabolized by all halophilic methanogens, is thought to be the dominant methanogenic precursor in saline environments (184). It may be produced from various organic solutes which cells synthesize as cytosolic osmoregulators during growth in saline environments. These compatible solutes include trimethylamine oxide and betaines; they are dissimilated by various bacteria to trimethylamine (66, 105). Although the MeOH precursor pectin (134, 139, 140), a plant constituent, may be abundant in anaerobic sediments, the importance of MeOH as a methanogenic substrate in natural environments is not established. The extreme halophile *Methanohalobium evestigatum* was reported as unable to catabolize MeOH (183), thus suggesting that MeOH may not be important in hypersaline habitats (184). However, in this studies, *Methanohalobium evestigatum* could grow on MeOH, therefore supporting the possibility that MeOH is a methanogenic substrate in such environments.

Growth data for *Methanohalophilus* sp. and *Methanohalobium evestigatum* in defined media are not available. Published growth parameters for halophilic methanogens were from studies with cultures in media with yeast extract (117, 118, 181, 182). Yeast extract has GB, compound that affects microbial growth in saline media

(34, 86). In addition, in those studies only NaCl was used as medium osmolyte (99, 100, 117, 118, 181, 182), so it is not known if halophilic methanogens have a specific requirement for this salt.

Rain storms and evaporation cause fluctuations in the $A_w$ of many saline habitats (*e.g.*, salterns, intertidal ponds). Because the equilibration of cytosolic and environmental $A_w$ is almost instantaneous, thus fastest than any active cellular osmoresponse, such fluctuations may compromise cell viability. Significant decreases in external $A_w$ may lead to inhibition of cell metabolism due to excessively high concentrations of cytosolic solutes, including specific enzymatic inhibitors; an excessively low cytosolic $A_w$ may compromise the structure and function of vital cytoplasmic macromolecules. In contrast, elevated external $A_w$ causes water to diffuse into the cell, thereby increasing the $A_w$ of the cytosol. The immediate effect of this water movement is an increase in cell volume and, depending on the elasticity and tensile strength of the cell wall, development of $P_t$. But most halophilic methanogens are expected to be very sensitive to hypotonic stress because they have weak cell walls (117, 181), so they cannot develop significant $P_t$. In the absence of $P_t$, the cytosolic $A_w$ can be rapidly raised only by dilution (and expansion of cell volume) or release of cytosolic osmolytes. Release of cytosolic osmolytes other than by lysis has not been described for methanogens, but possible mechanisms include pressure-sensitive channels, and temporary breaches in the cell membrane which quickly re-seal (36).

Because water moves freely across microbial cell membranes, microbes inhabiting environments of low $A_w$, such as saline lakes and solar salterns, must have cytosol with $A_w$ as low as that of the environment. $Na^+$ is often the major cation in these environments, but it may be inhibitory at high cytosolic concentrations and many microbes exclude it actively (23, 47). The extremely halophilic archaeobacteria of the genera *Halobacterium* and *Halococcus* and the moderately halophilic eubacteria *Halobacteroides halobium* and *Haloanaerobium praevalens* accumulate $K^+$ salts (23, 115). But in most halophiles predominant cytosolic osmolytes are small organic molecules, highly soluble in water and with a net charge of zero within the physiolog-

ical pH range (30, 31, 178). In addition to lowering $A_w$ of cytosol, some compatible solutes stabilize the structure of proteins and other cytosolic macromolecules (2, 13, 25, 29, 71, 174, 178). Examples of compatible solutes in bacteria are betaines, amino acids, and sugars (30, 31, 178). Glutamate and GB are particularly common among prokaryotes (30, 31). In saline media, methanogenic archaeobacteria also accumulate organic osmolytes, including GB, $\beta$-amino acids and derivatives (Table 4). Glutamate is ubiquitous in methanogens and $\beta$-glutamate is common. Methanogens from saline environments or that tolerate salinity (*Methanococcus voltaei*, *Methanogenium cariaci*, *Methanogenium* strain AN9, *Methanohalophilus zhilinaeae*, and *Methanohalophilus mahii*) have significant cytosolic concentrations of GB when grown in saline medium (79, 130). However, GB was recently reported as not important in *Methanohalophilus* strain RET-1 and *Methanohalophilus halophilus* (79). This report also stated $K^+$ as a predominant cytosolic osmoregulator in moderately and extremely halophilic methanogens.

Studies concerning osmoregulation in methanogens are very recent. As previously referred, knowledge of their compatible solutes is still incomplete and their response to changes in osmotic pressure remains to be studied. In addition, their osmophilia and osmotolerance are not known for osmolytes other than NaCl (99, 100, 118, 117, 181, 182). Moreover, published osmotic parameters for *Methanohalophilus* (99, 100, 118, 117, 181, 182) were obtained from studies in complex media.

This experimental work was designed for the study of the osmotic behavior (osmophilia, osmotolerance, halophilia, halotolerance, and resistance to osmotic stresses) of halophilic methanogens, and the mechanisms that they use for osmoadaptation and osmoresponse. First, growth requirements for organisms representative of moderately and extremely halophilic methanogens were determined in order to define experimental conditions. Specific growth rates of *Methanohalophilus mahii*, *Methanohalophilus halophilus*, and *Methanohalobium evestigatum* in defined saline media, on TMA, MMA and MeOH, were determined, and the effect of yeast extract examined. Unexpected broad osmoadaptive features of the methanogenic archaeobac-

teria *Methanohalophilus mahii* and *Methanohalophilus halophilus*, and a notable ability of *Methanohalophilus mahii* to withstand both hypotonic and hypertonic osmotic shocks were found. *N,N*-Dimethylglycine (DMG) was detected as an important new compatible solute in three methylotrophic and moderately halophilic methanogens, *Methanohalophilus mahii*, *Methanohalophilus halophilus*, and *Methanohalophilus* strain RET-1. The cytosol of these strains also contained GB and $K^+$, but at concentrations that were significantly lower than that of DMG.

# MATERIALS AND METHODS

**Microbial strains.** *Methanohalophilus mahii* SLP (OCM 68), *Methanohalophilus halophilus* Z-7982 (OCM 160), *Methanohalophilus* strain RET-1 (OCM 57), and *Methanohalobium evestigatum* Z-7303 (OCM 161), were obtained from the Oregon Collection of Methanogens.

**Culture methods.** Media preparation, culture procedures and anoxic manipulations were adapted from the Hungate technique (51). Basal culture medium (ZV medium) contained (per liter): 4 g of NaOH; 5 g of $MgCl_2 \cdot 6H_2O$; 1.5 g of KCl; 1 g of $NH_4Cl$; 0.4 g of $K_2HPO_4$; 0.25 g of $Na_2S \cdot 9H_2O$; 0.05 g of $CaCl_2 \cdot 2H_2O$; 5 mg of $Na_2$-EDTA$\cdot 2H_2O$; 1.5 mg of $CoCl_2 \cdot H_2O$; 1.0 mg of $MnCl_2 \cdot 4H_2O$; 1.0 mg of $FeSO_4 \cdot 7H_2O$; 1.0 mg of $ZnCl_2$; 0.4 mg of $AlCl_3 \cdot 6H_2O$; 0.3 mg of $Na_2WO_4 \cdot 2H_2O$; 0.2 mg of $CuCl_2 \cdot 2H_2O$; 0.2 mg of $NiSO_4 \cdot 6H_2O$; 0.1 mg of $H_2SeO_3$; 0.1 mg of $H_3BO_3$; 0.1 mg of $NaMoO_4$; 0.5 g of mercaptoethanesulfonic acid (coenzyme M); and 2.5 ml of a vitamin solution (3). Media of various osmolalities were prepared by varying the amount of NaCl added, or by replacing it with KCl or sucrose (Table 5), and empirically adjusting the amount of NaOH to obtain a final pH of 7.25. Osmolality was calculated according to the formula:

$$\text{osmolal} = \sum_j \phi_j m_j \qquad (16$$

where values for $\phi$, the osmotic coefficient, were from published values (133). Media were prepared by dissolving NaOH in distilled water and equilibrating with an $O_2$-free mixture of $N_2$ and $CO_2$ (7:3). Residual $O_2$ was removed by bubbling the gas mixture for 10 min with stirring, while medium constituents other than sodium sulfide and vitamin solution were added, with coenzyme M added last. The medium was dispensed into individual vessels, sealed with butyl rubber stoppers, and autoclaved at 121°C. Cells were grown in 27-ml serum tubes containing 5 ml of medium, and 70-

**Table 5. Concentration of osmolytes in culture media.**

| MEDIUM | NaCl | KCl | sucrose | $Na^+$ | osmolality | osmosity[a] |
|---|---|---|---|---|---|---|
| ZV-0 | 0.00 | 0.02 | 0.00 | 0.10 | 0.18 | 0.09 |
| ZV-0.1 | 0.10 | 0.02 | 0.00 | 0.20 | 0.36 | 0.19 |
| ZV-0.5 | 0.50 | 0.02 | 0.00 | 0.61 | 1.12 | 0.60 |
| ZV-1 | 1.00 | 0.02 | 0.00 | 1.15 | 2.17 | 1.14 |
| ZV-1.5 | 1.50 | 0.02 | 0.00 | 1.68 | 3.27 | 1.67 |
| ZV-2 | 2.00 | 0.02 | 0.00 | 2.17 | 4.40 | 2.16 |
| ZV-2.5 | 2.50 | 0.02 | 0.00 | 2.69 | 5.66 | 2.68 |
| ZV-3 | 3.00 | 0.02 | 0.00 | 3.19 | 7.03 | 3.18 |
| ZV-3.5 | 3.50 | 0.02 | 0.00 | 3.68 | 8.49 | 3.67 |
| ZV-4 | 4.00 | 0.02 | 0.00 | 4.12 | 9.99 | 4.11 |
| ZV-4.5 | 4.50 | 0.02 | 0.00 | 4.65 | 11.8 | 4.64 |
| ZV-5 | 5.00 | 0.02 | 0.00 | 5.17 | 13.8 | 5.16 |
| S-1 | 0.00 | 0.02 | 1.40 | 0.13 | 2.58 | 1.33 |
| S-1.5 | 0.00 | 0.02 | 1.89 | 0.09 | 4.32 | 2.09 |
| S-2 | 0.00 | 0.02 | 2.31 | 0.07 | 6.61 | 2.97 |
| Sa-0.5 | 0.00 | 0.02 | 0.73 | 0.12 | 1.14 | 0.61 |
| Sa-1 | 0.50 | 0.02 | 0.73 | 0.64 | 2.11 | 1.10 |
| Sa-1.5 | 1.00 | 0.02 | 0.73 | 1.16 | 3.11 | 1.57 |
| Sa-2 | 1.50 | 0.02 | 0.73 | 1.65 | 4.14 | 2.02 |
| Sb-1.5 | 0.50 | 0.02 | 1.27 | 0.65 | 3.24 | 1.63 |
| Sb-2 | 0.50 | 0.02 | 1.64 | 0.65 | 4.35 | 2.11 |
| ZK-0.5 | 0.00 | 0.53 | 0.00 | 0.13 | 1.12 | 0.60 |
| ZK-1 | 0.00 | 1.05 | 0.00 | 0.16 | 2.17 | 1.14 |
| ZK-1.5 | 0.00 | 1.59 | 0.00 | 0.14 | 3.27 | 1.67 |
| ZK-2 | 0.00 | 2.11 | 0.00 | 0.14 | 4.38 | 2.16 |
| ZK-2.5 | 0.00 | 2.67 | 0.00 | 0.14 | 5.63 | 2.68 |

[a]osmosity of a solution is the molar concentration of NaCl which would have the same osmolality.

and 3500-ml bottles containing 20 and 2000 ml of medium, respectively. Serum tubes and 70-ml bottles were autoclaved for 20 min, and 3500-ml bottles for 45 min. After the medium cooled and the gas re-equilibrated, the pH was 7.25. Growth substrates, vitamin solution, amendments, and sodium sulfide were added prior to inoculation, from separate, sterile, anoxic, stock solutions. *Methanohalophilus* strains were incubated statically at 37°C, and *Methanohalobium evestigatum* at 50°C.

**Determination of specific growth rates.** Growth rates were measured in 20-ml cultures contained in 70-ml serum bottles inoculated at 5% (v/v). Inocula were cultures in exponential phase that had been transferred at least 4 times (1% v/v) in media with the same composition of that used in the experiments. Growth rate was calculated from $CH_4$ accumulation during the exponential phase (10).

**Preparation of cell suspensions.** Concentrated cell suspensions for osmotic shock experiments were obtained by centrifuging tubes with late-logarithmic-phase cultures (7,000 × *g* for 20 min at room temperature) and discarding 75% of the supernatant.

**Cell harvesting.** Cultures in late exponential phase were centrifuged at 4°C for 45 min at 7,000 × *g* to form a soft pellet, which was washed 3 times in 5 ml of isotonic phosphate buffer (50 mM, pH 7.2, with the $Mg^{2+}$ and $Ca^{2+}$ concentrations adjusted to that of the culture medium, cellobiose at 10 mM, and with the osmolality adjusted to that of the medium with NaCl). When cytosolic $Na^+$ was measured, cells were washed in buffer with $K^+$ salts replacing $Na^+$ salts. After the last centrifugation the supernatant was removed with a Pasteur pipet, and a strip of filter paper was inserted into the pellet for 5 min to withdraw residual buffer.

*Methanohalobium evestigatum* cells did not form a pellet under these conditions, so cells were obtained after first diluting the culture by adding 250 ml of deionized water per liter of culture medium prior to centrifugation.

**Preparation of cell lysates.** Cell lysates for $A_w$ measurements were made by sonicating (30 W, 30 s) cell pellets of about 1 g, previously washed with equiosmolal ZV in an ice bath.

**Preparation of cell aqueous-extracts.** Cell pellets were diluted 10 times (w/v) with deionized water (18 MΩ $cm^{-1}$), and stirred at 100°C for 10 min (161). The lysate was centrifuged at 7,000 × *g* and 4°C for 30 min, and the supernatant was stored at -20°C.

**Microscopy and detection of lysis.** Morphology, size and integrity of cells were examined in fresh wet mounts, with a Zeiss Axioskop equipped with phase-

contrast, epifluorescence and microphotography. Phase-contrast microphotographs were taken from fresh wet mounts of exponentially growing cultures.

Cells were counted in a Petroff-Hausser chamber.

Lysis of cells by detergents was determined by visual changes in turbidity (10) and by microscopic examination of wet-mounts. Cell integrity was indicated by retention of epi-fluorescence.

**Cell-volume measurements.** Cytosolic volumes were calculated assuming that the volume of the particulate cell fraction was negligible, so the cytosolic volume in cell pellets was the total volume of each cell pellet minus the volume of buffer retained in the intercellular space. The total volume of cell pellets was found from the mass of the pellet and its density, which was determined by using a 10-ml volumetric flask as a pycnometer, at 25°C. The intercellular volume was determined by including cellobiose in the washing buffer and measuring the amount of cellobiose retained in the pellet.

**Analytical methods.** Methane was analyzed by gas chromatography with flame ionization detection (93).

Media pH were measured with a combination electrode Orion model 91-04 (Boston, Mass.), which was found to be relatively insensitive to interference by $Na^+$. The signal from the pH electrode in saline media and buffers stabilized within about 30 s. Longer exposure of the electrode to saline solution necessitated extended equilibration of the electrode in low-salt buffer before it could be used again.

$A_w$ was measured with a Decagon model SC-10 psychrometer (Pullman, Wa.). In order to avoid effects of temperature variations on $A_w$ measurements, the psychrometer was loaded with samples from the culture medium, cell lysate, and standards; after 1 h equilibration, 3 sets of readings were made at 30 min intervals.

Absorbance ($A_{450}$) of cell suspensions was measured with a Spectronic 21 colorimeter (Milton Roy, Rochester, NY).

Cellobiose was determined colorimetrically by using cellobiose oxidase (124).

$K^+$ and $Na^+$ were determined with an atomic absorption spectrophotometer (Perkin-Elmer model 603, Norwalk, Conn.) with an acetylene and air flame.

DMG and GB were analyzed by cation-exchange high-pressure liquid chromatography with a column of Partisil 10-SCX (Whatman, Hillsboro, Ore.) and detected by UV absorbance at 195 nm (46). The mobile phase was phosphate buffer (50 mM $KH_2PO_4$, pH 4.6) with 5% MeOH (v/v). The presence of these compounds was confirmed by thin-layer chromatography on 0.2-mm silica gel with various solvent systems; best separation of GB, DMG, and sarcosine was obtained with ethanol, n-butanol, and 5% (v/v) ammonia (10:5:1) or with ethanol and water (5:1). Chromatograms were developed in iodine vapor.

# RESULTS

**Catabolic substrates.** *Methanohalophilus mahii* and *Methanohalophilus halophilus*, the two moderate halophiles in this study, grew in ZV-2 media on TMA, MeOH, or MMA. *Methanohalophius mahii* grew rapidly on TMA (0.036 $h^{-1}$) or MeOH (0.034 $h^{-1}$) and more slowly on MMA (0.019 $h^{-1}$) (Fig. 2). A similar pattern was observed for *Methanohalophilus halophilus* (0.028 $h^{-1}$ on TMA; 0.021 $h^{-1}$ on MeOH; and 0.019 $h^{-1}$ on MMA).

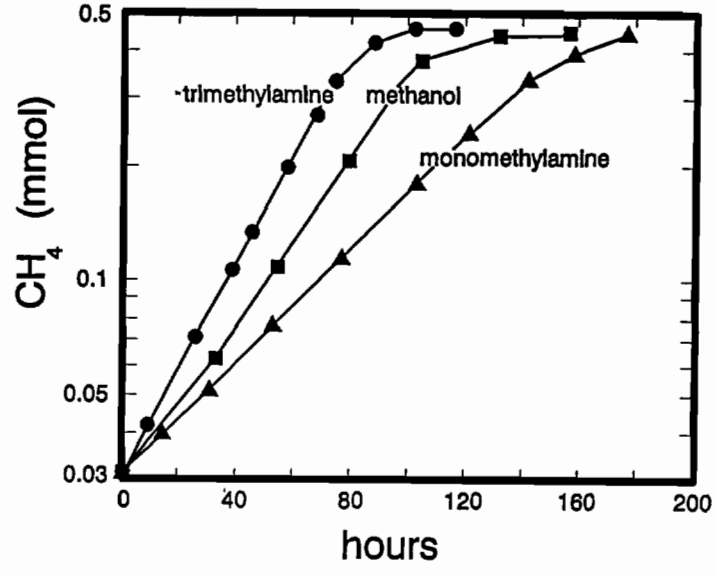


**Figure 2.** Growth rates of *Methanohalophilus mahii* in ZV-2.

The extremely halophilic *Methanohalobium evestigatum* grew more slowly than the two moderate halophiles. Growth in ZV-4 medium was fastest on TMA (0.013 $h^{-1}$), and slower on MeOH (0.006 $h^{-1}$) or MMA (0.003 $h^{-1}$) (Fig. 3). *Methanohalobium evestigatum* grew reproducibly on 15 mM MeOH, but it grew erratically on 20 mM MeOH. Cells did not grow in ZV-4 medium when the initial MeOH concentration was 30 mM or higher, even if TMA was present as a co-substrate.

**Stimulation by yeast extract.** The presence of yeast extract (1 g per liter) in the medium stimulated the growth of *Methanohalophilus mahii* (see Fig. 9). It also strongly stimulated growth of *Methanohalobium evestigatum* on TMA (0.045 $h^{-1}$),

MMA (0.024 $h^{-1}$) or MeOH (0.011 $h^{-1}$) (Fig. 3). In addition, *Methanohalobium evestigatum* grew well in media with 30 mM MeOH only when yeast extract was present.

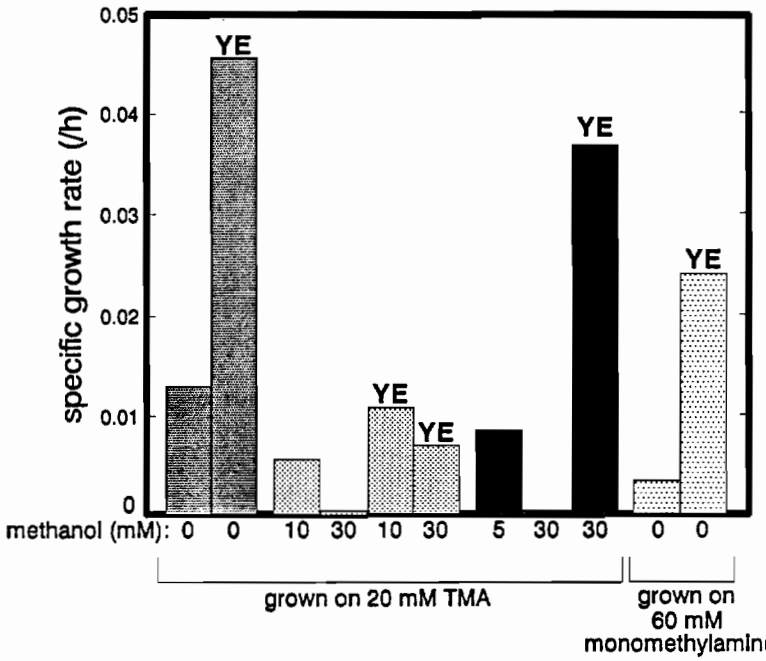


**Figure 3.** Growth of *Methanohalobium evestigatum* in ZV-4 medium, and effect of YE on growth.

**Halophilia and halotolerance.** *Methanohalophilus mahii* grew rapidly in medium with osmolality of 2.2 to 4.4 osmol (equiosmolal to 1.1 and 2.2 M NaCl) when NaCl was the major solute in the medium, with fastest growth (0.043 $h^{-1}$) at 3.3 osmol (equiosmolal to 1.7 M NaCl) (Fig. 4). *Methanohalophilus halophilus* was slightly less halophilic than *Methanohalo-*

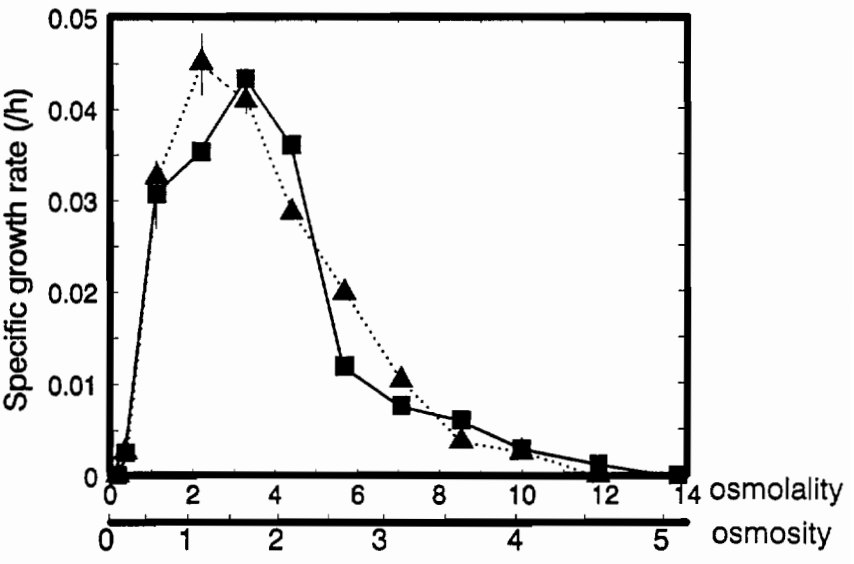


**Figure 4.** Effect of NaCl osmolality on the growth rate of *Methanohalophilus mahii* and *Methanohalophilus halophilus*.

*philus mahii*, growing rapidly at 2.2 to 3.3 osmol (equiosmolal to 1.14 M to 1.67 M NaCl), with fastest growth (0.045 $h^{-1}$) also at a slightly lower osmolality (2.2 osmol, equiosmolal to 1.14 M NaCl) than was found for *Methanohalophilus mahii* (Fig. 4). Both of these methanogens grew (though slowly) in medium with an osmolality as low as 0.36 osmol (equiosmolal to 0.19 M NaCl). Not only was *Methanohalophilus mahii* slightly more halophilic than *Methanohalophilus halophilus*, but it was also more halotolerant, growing at osmolalities up to 11.8 osmol (equiosmolal to 4.6 M NaCl). *Methanohalophilus halophilus* did not grow above 10 osmol (equiosmolal to 4.1 M NaCl).

**Sucrose as the dominant extracellular osmolyte.** When NaCl was omitted from culture medium and replaced by sucrose as the main osmolyte, rapid growth occurred at lower osmolalities (Fig. 5). Growth was faster in sucrose medium than NaCl medium at 1.1 osmol and slower at 4.3 osmol. The growth of *Methanohalophilus mahii* in sucrose media was as rapid at osmolalities of 1.1 osmol to 2.6 osmol (250.5 g to 518.8 g of sucrose per liter) as the most rapid growth in NaCl media. Media without added NaCl contained 70 mM to 120 mM $Na^+$ from other medium salts. The specific growth rate of *Methanohalophilus mahii* was not greatly affected when NaCl partially replaced sucrose as medium osmolyte (Fig. 5).

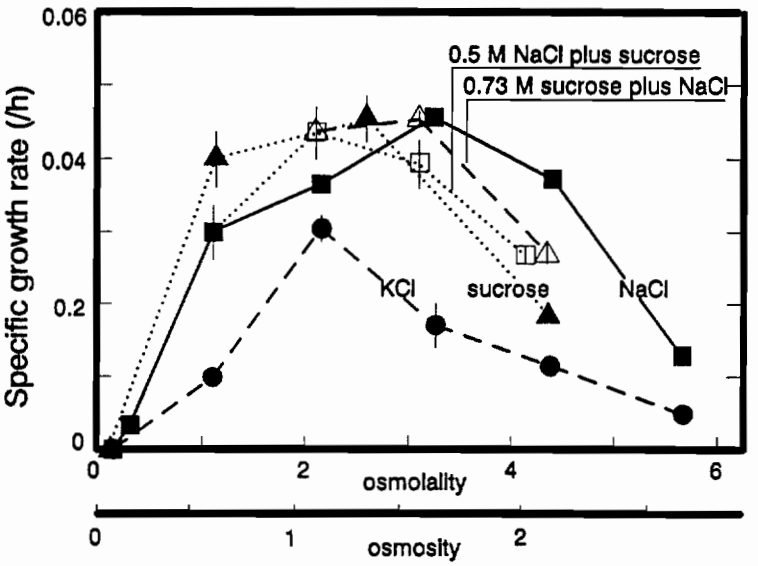


**Figure 5.** Growth rates of *Methanohalophilus mahii* with various osmolytes.

**KCl as the dominant extracellular osmolyte.** *Methanohalophilus mahii* also grew in media having KCl as the major osmolyte (Fig. 5). These media had 120 to 138 mM $Na^+$ from other salts. Growth was slower than in equiosmolal media with NaCl at all concentrations tested. Growth occurred in media with 0.51 M to 2.65 M KCl. Fastest growth occurred at lower osmolality (2.2 osmol [1.03 M KCl]) with KCl as the predominant medium osmolyte than when NaCl was the major osmolyte (Fig. 5).

**Effect of external osmolality on cell morphology.** The morphology of *Methanohalophilus mahii* and *Methanohalophilus halophilus* varied according to salinity (Fig. 6). When these two species grew at osmolalities of 2.2 osmol or higher, they appeared microscopically as very irregular cocci, but when they grew at 0.36 osmol they were large and spherical, appearing to be turgid. Changes in morphology have been reported for other halophilic bacteria as well. These cells tend to be larger and rounder at lower osmolalities (13). At osmolalities below 1.14 (equiosmolal to 0.5 M NaCl), *Methanohalophilus mahii* did not grow when incubated with agitation, presumably because it was sensitive to shear stress.

**$P_t$.** In *Methanohalophilus mahii* $P_t$ was determined by measuring the $A_w$ of the culture medium and comparing it to the $A_w$ of the cytosol of lysed cells. During growth, the $A_w$ equilibrates across the cell membrane, so the cytoplasmic $A_w$ is equal to that of its environment. A change in cytosolic $A_w$ upon lysis would indicate that a significant component of this $A_w$ was derived from $P_t$. *Methanohalophilus mahii* was grown in medium with 2 M NaCl and cell pellets collected. The volume of extracellular water in these pellets was about 3% (v/w). Cells were lysed by sonication, the $A_w$ of the lysate measured and compared with the $A_w$ of the culture medium. Two independent measurements of $P_t$ in *Methanohalophilus mahii* indicated $0 \pm 0.5$ atm and $0.6 \pm 0.4$ atm.

**Susceptibility to detergents.** *Methanohalophilus mahii* was tested for susceptibility to sodium dodecylsulfate and Triton X-100, at 0.5% and 0.05% (w/v), under iso- and hypotonic conditions. A 20-ml culture was grown to late exponential

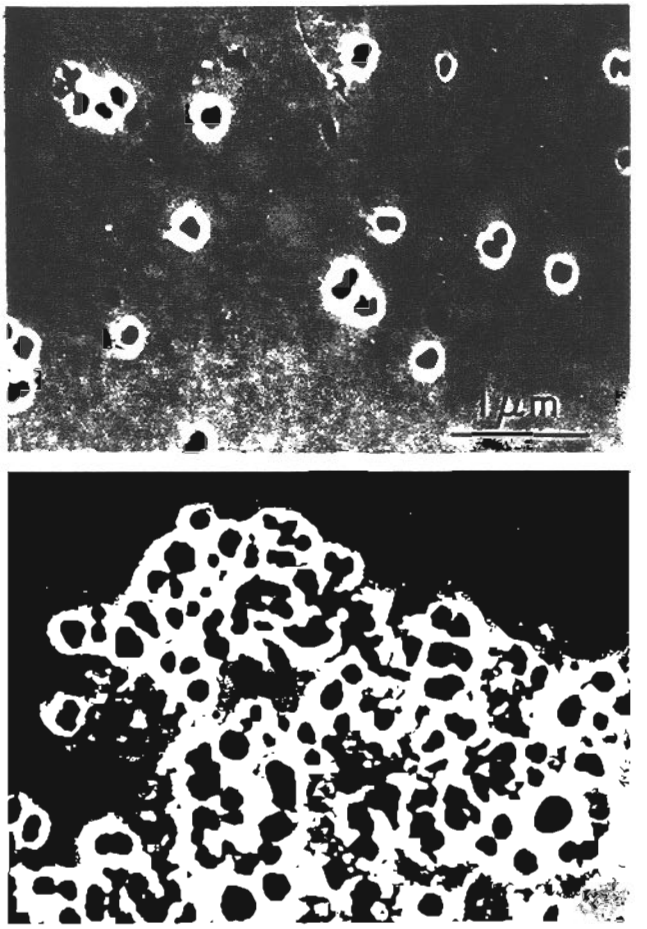

**Figure 6.** Photomicrographs of *Methanohalophilus mahii* growing in media with 4 M (top half) and 0.1 M (bottom half) NaCl.

phase in medium with 2 M NaCl, the cells were collected by centrifugation and resuspended in 2 ml of the same medium. This suspension (0.1 ml) was added to 0.9 ml of: (a) isotonic medium; (b) water; (c) isotonic medium plus detergent; or (d) water plus detergent. In the absence of detergent, cell suspensions diluted into water or into isotonic medium remained turbid. Some lysis may have occurred, but a substantial fraction of cells remained intact and retained their epi-fluorescence. The effect of detergents was the same at either concentration (0.5% or 0.05%). Triton X-

100 lysed cells in both water and in isotonic medium, as determined by loss of turbidity and microscopic examination. However, sodium dodecylsulfate lysed cells diluted into water, but not those in isotonic medium.

**Hypertonic and hypotonic shock.** *Methanohalophilus mahii* cultures were grown to late exponential phase in media with 0.5 M NaCl, and inoculated (10% v/v) into media with various NaCl concentrations ranging from 0.5 to 4 M NaCl. Within 3 weeks all of these cultures grew to completion, as indicated by accumulated methane approximating the amount stoichiometrically expected from the catabolic substrate. These results indicated that a significant number of *Methanohalophilus mahii* cells could survive large, rapid shifts to higher osmolality.

*Methanohalophilus mahii* was grown in media with various concentrations of NaCl (1 to 3 M) and exposed to osmotic shock by a 1:10 dilution in media without NaCl. For each initial concentration of NaCl tested, two diluted cultures were mixed by gently inverting the dilution tube, and two others were immediately stirred with a vortex-mixer for 15 s. After 3 weeks of incubation, growth was complete in all samples. *Methanohalophilus mahii* growing in medium with 4 M NaCl gave erratic results when diluted in this way, with some cultures becoming oxidized (indicated by resazurin turning pink) before growth occurred. The experiment was modified to give a higher number of cells in the inoculum and allow a faster growth of the shocked cells: a culture was grown in 4 M NaCl, concentrated by centrifugation, resuspended in the same medium, and 10 10-fold dilutions were made in medium without NaCl. Two of these were vortexed immediately, all were incubated 30 min at room temperature, and then the NaCl concentration of each was adjusted to 1.5 M. Near complete growth (>50% of expected methane) occurred within 1 week of incubation in four samples, within 6 weeks in four other samples (including the two vortexed samples), but no methane was formed in the other two samples.

The time-course of cell swelling after hypotonic shock was investigated by using optical density (Fig. 7). Light scattering of a cell suspension is a function of cell size, with larger cells having lower turbidity (68). A 20-ml culture grown in

medium with 4 M NaCl was centrifuged to form a dense cell suspension of about 1 ml. This suspension was diluted 1:10 under non-anoxic conditions, either with isotonic medium or with water. Microscopic observation indicated that cells swelled and many lysed within 30 s when the culture was diluted with water. During the next hour, the unlysed cells appeared to swell and a few cells were seen to lyse. No swelling or lysis occurred in isotonic medium during the same time period.

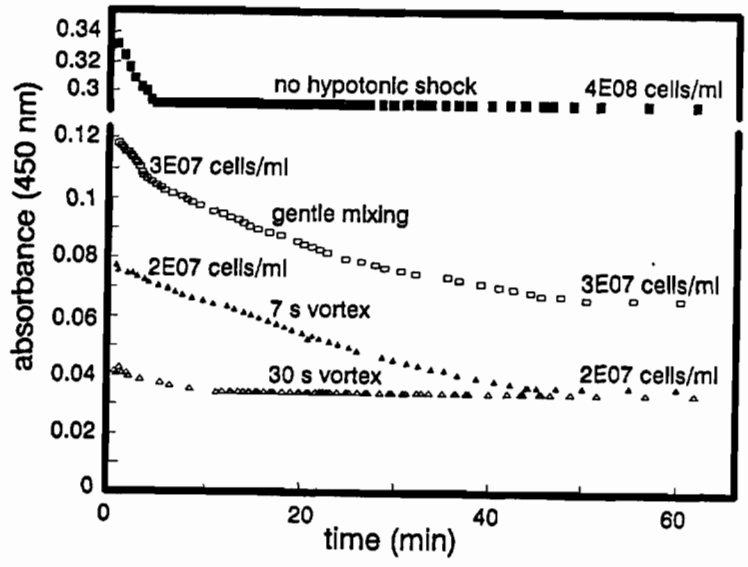


**Figure 7.** Effect of hypotonic shock on absorbance and numbers of *Methanohalophilus mahii*. Cells were grown in medium with 4 M NaCl were diluted 10× (final NaCl concentration was 0.4 M).

The experiment was repeated, but this time cells were counted after 30 s and after 1 h (about 10 min was required to count the cells) and $A_{450}$ was determined (Fig. 7). These results indicate that minimal lysis occurred after 30 s, and that the cells continued to swell for about 45 min. In no case were the counts of a culture significantly different (95% confidence) when measurement began 30 s or 1 h after hypotonic shift. This slowness of the swelling was unexpected, so the hypotonically shocked cells were vortexed for 7 s and for 30 s. This treatment caused slightly greater lysis of cells during the first 30 s than in gently mixed suspensions. Those cells vortexed for 7 s continued to swell for 45 min, but cells vortexed for 30 s did not swell further. To determine whether enzymatic activity was necessary for cells to withstand 10-fold hypotonic shock, the cell suspensions were chilled on ice, and diluted with cold water. Microscopic observations indicated a similar pattern of rapid lysis of

some cells during the first 30 s to 1 min, and afterward continued slow swelling, and little additional lysis.

**Major cytosolic cations.** The cytosolic concentrations of $K^+$ and $Na^+$ in *Methanohalophilus* strain RET-1, *Methanohalophilus mahii*, and *Methanohalophilus halophilus* were determined. The cytosolic $K^+$ concentration never exceeded 0.35 M when *Methanohalophilus mahii* and *Methanohalophilus halophilus* were grown in media with a total osmotic strength of 2.2, 4.4, and 7.0 osmol (1, 2, and 3 M NaCl added) (Fig. 8). Intracellular $Na^+$ concentrations were 0.025 $\pm$0.008 M in both of these two strains grown at these osmolalities (Fig. 8). When grown in medium with 1.5 M NaCl, *Methanohalophilus* strain RET-1 had intracellular concentrations of 0.12 M $K^+$ and 10 mM $Na^+$.

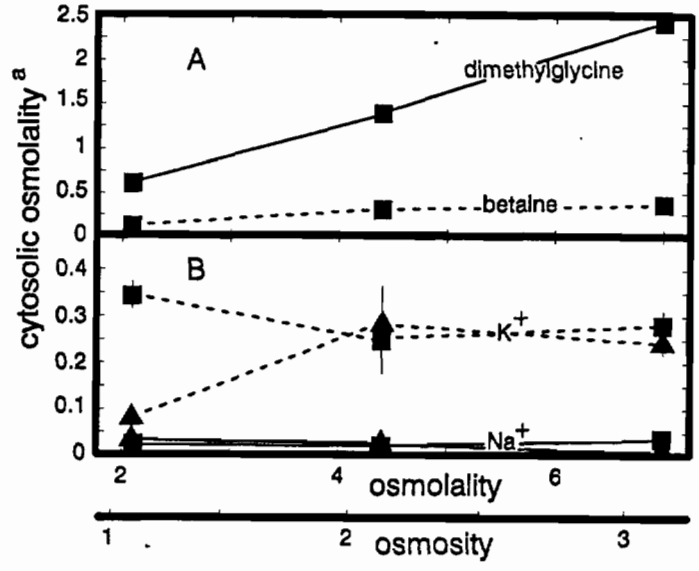


**Figure 8.** (A) Cytosolic GB and DMG in *Methanohalophilus mahii*, and (B) $K^+$ and $Na^+$ in *Methanohalophilus mahii* (■) and *Methanohalophilus halophilus* (▲).

**Cytosolic compatible solutes.** GB was detected in aqueous extracts from all four halophilic methanogens. When *Methanohalophilus* was grown at 4.4 osmol, cytosolic GB levels (0.24 $\pm$0.07 M) did not significantly vary among strains or with TMA or MeOH as catabolic substrate. *Methanohalobium evestigatum* grown in 4 M NaCl had similar concentrations of cytosolic GB (approximately 0.29 M). For *Methanohalophilus mahii*, cytosolic GB concentration was higher when cells were grown in 4.4 osmol than in 2.2 osmol, but the concentration was not much higher in cells grown in medium of 7.0 osmol (Table 6).

Thus, it appears that cells used increased GB to raise cytosolic osmolality at the lower range, but did not further increase GB concentrations at higher media osmolalities.

DMG was detected by liquid chromatography of aqueous extracts from each of the 3 *Methanohalophilus* strains. Similar cytosolic levels of DMG (1.3 $\pm$0.3 M) were found in all strains in medium with 4.4 osmol, whether they were grown on TMA or MeOH. In *Methanohalophilus mahii* intracellular DMG concentration increased with media osmotic strength, especially at higher medium osmolalities (Fig. 8 and Table 6). GB but not DMG was detected in *Methanohalobium evestigatum.*

**Table 6. Cytosolic DMG and GB in *Methanohalophilus mahii*: concentrations and percent of total medium osmolality.**

| | GLYCINE BETAINE | | DIMETHYLGLYCINE | |
|---|---|---|---|---|
| Medium osmol. | Cytosolic (M) | % medium[a] osmol. | Cytosolic (M) | % medium[a] osmol. |
| 2.2 | 0.11 | 5.3 | 0.58 | 28 |
| 4.4 | 0.30 | 7.0 | 1.24 | 31 |
| 7.0 | 0.36 | 5.4 | 2.00 | 34 |

[a] Osmotic coefficients for GB and DMG assumed as 1.

Aqueous cell extracts of *Methanohalophilus mahii*, *Methanohalophilus halophilus*, and *Methanohalobium evestigatum* were examined by thin-layer chromatography. GB was found in all three extracts, but DMG was found only in the *Methanohalophilus* strains. Sarcosine could be separated and detected in standards, but it was not found in any of these cell extracts.

**Effect of exogenous compatible solutes on growth.** The effect of various potential compatible solutes and related compounds on the growth rate of *Methanohalophilus mahii* was tested. Growth media were amended with 5 mM of DMG, GB, sarcosine, glycolate, oxalate, acrylate, choline, glycine, urea, proline, glutamate, $\beta$-glutamate, glycerol, trehalose, dimethylsulfoniopropionate, acetate, $\gamma$-aminobutyrate,

putrescine, spermine, or spermidine (at 5 mM), or yeast extract, Trypticase peptone (BBL Microbiology Systems, Cockeysville, Md.), or casein hydrolysate (Difco, Detroit, Mich.) at 2 g per liter of medium. Only DMG, GB, sarcosine, and yeast extract stimulated *Methanohalophilus mahii* (Fig. 9).

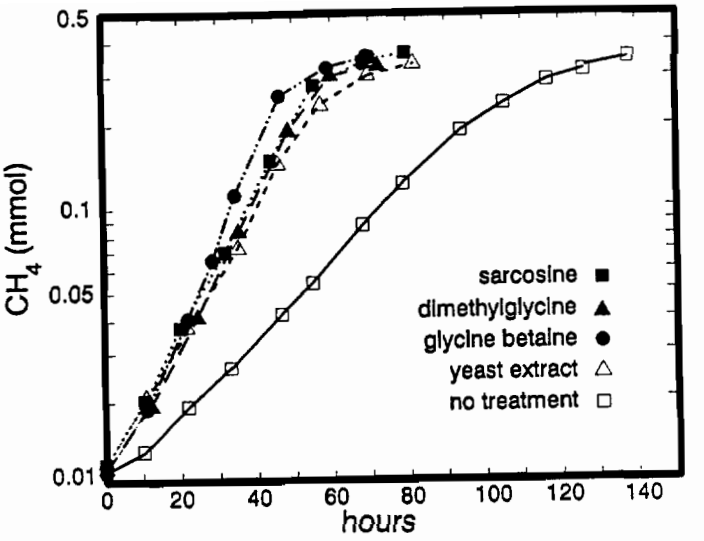


**Figure 9.** Effect of DMG, GB, sarcosine, and yeast extract on the growth rate of *Methanohalophilus mahii* in ZV-2.

The effect of GB on growth of *Methanohalophilus mahii* was examined throughout the entire range of salinity that allows its growth (Fig. 10). At higher salinities (from 1.5 to 4 M NaCl) the addition of GB (5 mM) stimulated growth. However, no growth stimulation occurred at the upper limit of salinity (4.5 M NaCl), neither the presence of GB increased its salt tolerance (up to 4.5 M NaCl). At lower salinities GB had no appreciable effect (1 M NaCl) or was even inhibitory; in ZV-0.5 (0.5 NaCl) the addition of GB lowered the specific growth rate of *Methanohalophilus mahii* by about 65%.

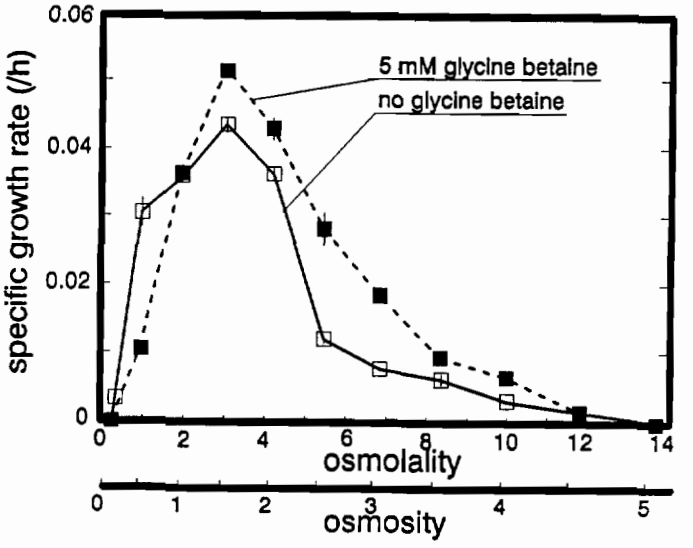


**Figure 10.** Effect of glycine betaine on the growth rates of *Methanohalophilus mahii* within a range of NaCl osmolalities.

## DISCUSSION

**Growth substrates and effect of yeast extract.** Halophilic methanogens synthesize and accumulate high concentrations of compatible solutes (79), whose biosynthesis requires an energy expenditure. TMA, MeOH, and MMA substrates may enter at different levels or follow distinct pathways for biosynthesis of compatible solutes. Thus, the energy required for the synthesis of osmoregulatory solutes may depend on the type of the available raw material. *Methanohalophilus* sp. and specially *Methanohalobium evestigatum* grew faster on TMA than on methanol. Growth stimulation by exogenous GB, a compatible solute in eubacteria (31) and methanogens (79, 131), is well documented for eubacteria under saline stress (20, 34, 86, 126), and was demonstrated for moderately halophilic methanogens (Figs. 9 and 10). The observed growth stimulation of *Methanohalophilus* by yeast extract may have resulted from the uptake of GB, which can be provided by yeast extract (34). Ultimately, observed growth stimulation by yeast extract may reflect that less energy was necessary for the biosynthesis of compatible solutes, so more energy was available for growth.

Although inhibition of methanogens by MeOH has not been previously reported, MeOH at 20 mM or higher concentration inhibited growth of *Methanohalobium evestigatum* (Fig. 3). Some *Methanosarcina* sp. can even grow on MeOH at concentrations higher than 1 M (unpublished results). When used as a substrate for culturing methanogens, MeOH is commonly added to media at concentrations much higher than 20 mM, in order to obtain substantial growth. It may be that the reported inability of *Methanohalobium evestigatum* to use MeOH (184) was observed in cultures with MeOH concentrations that are inhibitory. MeOH utilization by *Methanohalobium evestigatum* was demonstrated, thus suggesting MeOH as a possible methanogenic substrate in hypersaline environments.

**Osmophilia and osmotolerance.** *Methanohalophilus* species grew well in medium with low NaCl concentration when another osmotic agent (sucrose) was present. Thus, when *Methanohalophilus* species are tested in media with NaCl as the major osmolyte they are found to be moderately halophilic, although they are not strictly halophilic because they can grow well with osmolytes other than salt (Fig. 5).

*Methanohalophilus mahii* and *Methanohalophilus halophilus* grew over a wider range of osmolarities than previously published ranges (117, 181). Possibly, growth at lower salinities was allowed by a step-wise adaptation of the inocula to NaCl concentrations below 0.5 M NaCl, and by the absence of GB in the culture media. Yeast extract, which was a component of the culture medium used in the initial characterization of *Methanohalophilus* (117, 181), contains GB. At low salinity, GB inhibited growth of *Methanohalophilus mahii* (Fig. 10), possibly because it is taken up even after osmotic balance is established.

**Resistance to hypo-osmotic stress.** The survival of intact cells of *Methanohalophilus mahii* after a 10-fold hypotonic shift was unexpected, given that osmotic equilibrium of water across cell membranes occurs within a few milliseconds (68, 97). This influx of water results in cell bursting unless the influx is managed in one of three ways: swelling, increased $P_t$, or loss of osmolytes from the cytoplasm (31).

Cell swelling is certainly one of the mechanisms by which *Methanohalophilus mahii* dealt with hypotonic shock, as was observed microscopically. The highly irregular morphology of halophilic methanogens may allow significant increases in cell volume as the cells swell and become more spherical. However, cells would have had to increase 10-fold in volume (approximately 2-fold in diameter) to achieve equilibrium if swelling were the only adaptive response.

The development of a significant $P_t$ in *Methanohalophilus mahii* is unlikely to be an important factor in tolerance of hypotonic shock. *Methanohalophilus mahii*'s cell wall appears to be a single glycoprotein layer (S layer), like that of *Methanohalophilus halophilus* (181) and many other methanogens (61, 72), and S layers lack the tensile strength to withstand significant $P_t$ (61, 144). Also, *Methanohalophilus mahii*

was fragile when grown in media of low osmolarity, and the sheer stress of agitation was probably much less than the $P_t$ which would result from a 10-fold hypotonic shift (approximately 200 atm of $P_t$).

The loss of osmolytes from the cytoplasm might be an adaptive response of these methanogens. Examples of this response in other cells includes the presence of pressure-sensitive membrane channels in *Escherichia coli* or even a breach of the cell membrane which rapidly recloses (36). However, epi-fluorescence of shocked cells did not decrease appreciably, so no apparent release of cytosolic solutes occurred. Other responses such as polymerization of cytoplasmic constituents is unlikely to be swift enough to respond to the rapid influx of water.

**Compatible solutes.** When *Methanohalophilus mahii* grew in medium with 2.2, 4.4, and 7.0 osmol NaCl, the cytosolic DMG concentrations were about 5 times higher than those of GB (Table 6). Osmotic coefficients for DMG are not available, so it was not possible to compute its actual cytosolic osmolalities, but table 6 shows that if the osmotic coefficients of GB and DMG were similar, DMG would be a much more important intracellular solute.

This study corroborated published results showing that GB is a compatible solute in moderately and extremely halophilic methanogens (79, 130), including three strains (*Methanohalophilus mahii*, *Methanohalophilus halophilus*, and *Methanohalophilus* strain RET-1) in which previous studies were unable to detect cytosolic GB (79). In this work, larger cell pellets and aqueous (rather than ethanolic) extractions were used, which may have allowed detection of GB. Also, the use of UV detection rather than periodate or NMR was used. Finally, it was found that, compared to the methods of Lai *et al.* (79), longer centrifugations at higher gravitational forces were necessary to obtain suitable pellets of halophilic methanogens.

Another important difference between these results and those of Lai *et al.* (79) was that they found substantially higher cytosolic concentrations of GB and $K^+$ in cells grown in medium with 2.7 M NaCl than in cells grown at lower concentrations, while in this study only a small positive relationship between the concentration of

cytosolic GB and extracellular NaCl when the concentration of NaCl was above 2 M. Cell volumes are difficult to measure accurately (78), and differences in methods could account for these differences in results if Lai *et al.* (who measured tritiated water as an indication of extracellular water volume) overestimated extracellular volume or here (by using extracellular cellobiose concentration) they were underestimated. In this study, measurements consistently indicated that about 3% of cell pellets were extracellular fluid.

This work supported the importance of $K^+$ as an intracellular solute of halophiles, although it was not a major solute for the moderately and extremely halophilic methanogens tested. Lai *et al.* suggest that $K^+$ salts are a major cytosolic osmolyte in halophilic methanogens (79), based on concentrations they measured in *Methanohalophilus* strain FDF-1. That strain was not examined in this study, but $K^+$ was not a major solute in any of the halophilic methanogens which were examined. In this study, the combined concentration of organic compatible solutes, but not of $K^+$, increased at an almost constant rate with respect to media salinity (Fig. 8). Because of the absence of published results on the subject, a possible role of $K^+$ as an osmoregulator in extremely halophilic methanogens (*Methanohalophilus evestigatum*) is not known. The observed cytosolic concentrations of $K^+$ and $Na^+$ in moderately halophilic methanogens were more similar to their concentration in non-halophilic cells (23, 24, 47, 55) than to cytosolic concentrations found in those exceptional microbes that use electrolytes as cytosolic osmoregulators.

Osmotically important concentrations of DMG occurred in *Methanohalophilus,* and GB is accumulated by halophilic methanogens (79, 130). It is likely that glycine derivatives have a central role in the adaptation of these organisms to high osmotic pressures. The observed growth stimulation of *Methanohalophilus mahii* by sarcosine, DMG, and GB (Fig. 9) supports this suggestion. This stimulation provided evidence for its natural availability in saline environments, as well for the presence of transport systems for these compounds in this archaeobacterium. Sarcosine occurs in ray (178) starfish (74), sea urchins, and rock lobsters (111), GB is a constituent of

many halophilic bacteria (43, 54) and plants (159), and DMG may be formed in anaerobic environments by demethylation of GB (48, 82, 105, 106). GB promotes the survival of eubacteria under hypertonic shock (34, 86). DMG is much less effective, and sarcosine shows no appreciable effect (34, 86). Choline, which in aerobic eubacteria has an osmoprotective effect similar to that of GB (86) and is an intermediary in GB synthesis in *Escherichia coli* (80), did not stimulate growth of *Methanohalophilus mahii* in 2 M NaCl. In *Escherichia coli*, the synthesis of GB requires molecular $O_2$ (80), so this pathway is not likely to occur in methanogens. A different pathway for the synthesis of methylated derivatives of glycine must occur in methanogenic archaeobacteria.

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# BIOGRAPHICAL SKETCH

José António Gomes Ferreira Menaia was born in Abrantes, Portugal, in 1950, on September the 8th. He attended the School of Veterinary Medicine of the Technical University of Lisbon, earning the DVM since 1983. José started his professional experience in environmental microbiology in 1974 at the National Laboratory of Engineering and Industrial Technology (LNETI, Lisbon), where he worked on microbial degradation of organic matter, first as laboratory technician and in 1984 as a research assistant. At LNETI he studied the degradation of lignocellulosics by white-rot fungi, and became interested in the microbiology of methanogenesis. He brought the study if the ecology of methanogenic archaeobacteria to Portugal, and in 1985 he studied the physiology of acetoclastic methanogenesis by *Methanosarcina barkeri* at the University of California, Los Angeles, under the supervision of Profs. Robert A. Mah and David R. Boone. These studies developed into interests in the microbiology of methanogens from thermophilic and saline environments. In 1987, he started doctoral studies at the Oregon Graduate Institute of Science & Technology, under the supervision of the Prof. David R. Boone. From 1989 to 1992 José was a graduate student and a member of the staff of the Oregon Collections of methanogens.

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