

# Glycine Betaine and Potassium Ion Are the Major Compatible Solutes in the Extremely Halophilic Methanogen *Methanohalophilus* Strain Z7302

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*Methanohalophilus* strain Z7302 was previously isolated from a hypersaline environment and grows over a range of NaCl concentrations from 1.7 to 4.4 M. We examined the relationships between cell growth rate, cell volume, and intracellular solute concentrations with increasing salinity. This extremely halophilic methanogen synthesized three zwitterionic compounds,  $\beta$ -glutamine,  $N_e$ -acetyl- $\beta$ -lysine, and glycine betaine, and also accumulated potassium ion as compatible solutes to balance the external and internal osmotic pressures. Potassium and glycine betaine were the predominant compatible solutes when *Methanohalophilus* strain Z7302 was grown at high external NaCl concentrations and approached intracellular levels of 3 and 4 M, respectively.

Microorganisms accumulate cations and/or low-molecular-weight organic compounds, known as compatible solutes, when the extracellular solute concentration exceeds that of the cell cytoplasm. These compounds enable the cell to minimize water loss and maintain cell turgor pressure by reducing the osmotic potential between the cell and the environment. They also protect enzymes from the low water activity (41). With growing interest in bacterial osmoregulation, there are a number of recent reviews (2, 3, 5, 6, 22, 35) describing the various aspects of bacterial osmoregulation.

Changes in the extracellular osmolarity have similar physicochemical effects on cells from all biological kingdoms, and thus their responses to osmotic shifts have considerable similarities (6). Nonelectrolytes such as carbohydrates and polyols are widely distributed among algae, fungi, and cyanobacteria (1, 3, 16, 21, 36). Eubacteria contain potassium ion as an electrolyte (38) plus a broad spectrum of nitrogenous compounds, including proline, glutamic acid, glutamine, aminobutyric acid, ectoine, glutamate betaine, and glycine betaine (3, 5, 9, 15, 16, 37, 40). Some eubacteria also accumulate carbohydrates (e.g., trehalose) in response to salt stress (37).

Several compatible solutes in archaeobacteria have been identified. The predominant compatible solute in extremely halophilic archaeobacteria, such as *Halobacterium* and *Halo- bium* species, is potassium (3, 11, 35). The compatible solutes of methanogenic archaeobacteria were recently examined and include several unusual organic compounds. The accumulation of  $\beta$ -amino acids,  $\beta$ -glutamate in thermophilic *Methanococcus* strains (27, 28) and  $N_e$ -acetyl- $\beta$ -lysine in *Methanosarcina thermophila* and *Methanogenium cariaci*, was recently reported (30).  $N_e$ -Acetyl- $\beta$ -lysine,  $\beta$ -glutamine, and the quaternary amine glycine betaine are also synthesized and/or accumulated as compatible solutes by the halotolerant and halophilic methanogens (13).

Halophilic methanogens have been isolated from a variety of environments that represent a wide range of solute concentrations (7, 12, 18-20, 24, 42-44). According to their salt requirement, these halophilic methanogens, including

*Methanohalophilus* and *Methanohalobium* species, can be separated to three groups: halotolerant species, moderate halophiles, and extreme halophiles. Zhilina described two extremely halophilic methanogens, strains Z7302 and Z7404, that were isolated from the black mud of Chokrack lake in the Kerchian peninsula; the mud contained 300 g of salt per liter (43). Both strains grow in defined media containing NaCl ranging from 1.7 to 4.5 M in final concentration and use trimethylamine as a sole substrate for methanogenesis (43, 44).

Cells were grown in defined media using a trimethylamine substrate that contained a graded series of NaCl concentrations ranging from 2.0 to 4.1 M. Medium composition and preparation were as previously described (13). The growth rates of the cultures were monitored by  $A_{540}$ . The specific growth rate of *Methanohalophilus* strain Z7302 is shown in Fig. 1. Maximum growth occurred at 2.4 M NaCl with a doubling time of 10 h, and growth decreased gradually with increasing medium salinity to a cell doubling time of 50 h at 4.1 M NaCl. A related strain, Z7404, grew over the same NaCl concentrations with similar growth rates (data not shown).

Both *Methanohalophilus* strains exhibit a irregular coccoid morphology. The cell volume of strain Z7302 at the midlog phase was determined after growth at the indicated NaCl concentrations by measuring the differential retention of [ $^{14}$ C]glucose and  $^3$ H $_2$ O in cell pellets (4, 13, 30). The cell volume remained relatively constant within the salt concentration range tested from 2.4 to 3.8 M (Fig. 1). This result was consistent with the microscopic observations and demonstrated the ability of bacterial cells to maintain turgor pressure over a broad range of osmotic pressures.

To examine the composition of intracellular organic compatible solutes, ethanol extracts were prepared from cultures grown in a defined medium containing various concentrations of NaCl. The ethanol extraction of cell material was performed as previously described (13, 30). The primary amines were detected and quantitated by ion-exchange high-performance liquid chromatography (13, 30), and glycine betaine was measured as the periodate derivative by spectrophotometry (8, 13, 31).

The major organic compatible solutes found in the ex-

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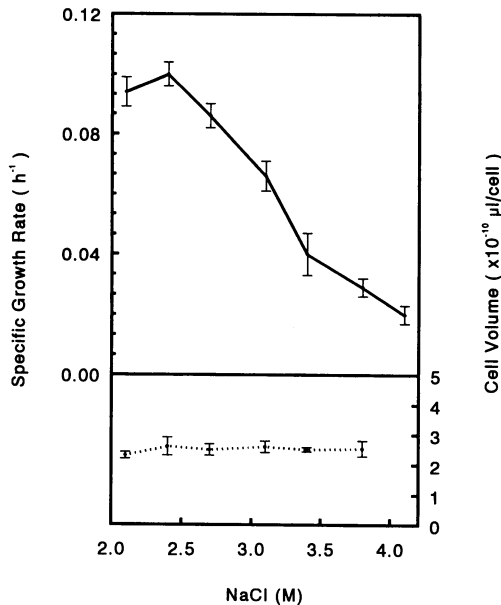


FIG. 1. Specific growth rate (—) and cell volume (---) of *Methanohalophilus* strain Z7302 grown in media containing increasing concentrations of NaCl.

tremely halophilic methanogen strain Z7302 were the three zwitterionic compounds  $\beta$ -glutamine,  $N_e$ -acetyl- $\beta$ -lysine, and glycine betaine. These solutes were the same as those found in the moderately halophilic methanogen *Methanohalophilus* strain FDF1 (13), although the concentrations of each varied considerably. Within the NaCl range of 2.1 to 4.4 M, the intracellular concentrations of glycine betaine,  $\beta$ -glutamine, and  $N_e$ -acetyl- $\beta$ -lysine increased in strain Z7302 as the extracellular NaCl concentration increased (Table 1). The intracellular level of glycine betaine increased from 0.5 M to just over 4 M.  $\beta$ -Glutamine and  $N_e$ -acetyl- $\beta$ -lysine also increased and reached final concentrations of 0.4 and 0.7 M, respectively, which were each well below that observed for glycine betaine. The concentration of  $\alpha$ -glutamate did not exceed 0.2 M. No other amino acids were detected at similar levels. Nearly identical findings were observed for *Methanohalophilus* strain Z7404. The three zwitterionic compounds, glycine betaine,  $\beta$ -glutamine, and  $N_e$ -acetyl- $\beta$ -lysine, were the predominant organic solutes for balancing the external osmotic pressure; their zwitterionic nature appears to make them desirable as compatible solutes.

TABLE 1. Intracellular compatible solutes of *Methanohalophilus* strain Z7302

NaCl concn (M) in medium	Intracellular solute concn (M)			
	$N_e$ -Acetyl- $\beta$ -lysine	$\beta$ -Glutamine	Betaine	$K^+$
2.05	0.04	0.04	0.32	1.22
2.39	0.03	0.08	0.75	1.38
2.74	0.07	0.10	1.01	1.17
3.08	0.10	0.15	1.26	1.67
3.42	0.15	0.29	2.43	2.50
3.71	0.16	0.33	2.85	2.97
4.10	0.25	0.40	3.89	3.09
4.44	0.45	0.70	4.13	ND <sup>a</sup>

<sup>a</sup> ND, not determined.

The quaternary amine glycine betaine, which is the dominant organic compatible solute in both extremely halophilic methanogen strains approached about 4.0 M when the strains were grown in defined media containing 4.4 M NaCl (Table 1). The ratio of glycine betaine to  $N_e$ -acetyl- $\beta$ -lysine was about 20:1 in strain Z7302, compared with a ratio of 1:1 in the moderately halophilic methanogen *Methanohalophilus* strain FDF1 (26). The pathway for synthesis of glycine betaine in *Methanohalophilus* strain FDF1 was recently deduced from nuclear magnetic resonance analysis by methylation of glycine generated from serine (via serine hydroxymethyltransferase) (25).

The intracellular concentration of potassium in *Methanohalophilus* strain Z7302 was also examined after cell growth at different NaCl concentrations. Cells were harvested at the midlog phase by centrifugation, and the cell pellets were washed with an equiosmotic solution of LiCl and extracted with butanol at 90°C (33, 34). Potassium analysis was performed with a multichannel inductively coupled argon emission spectrometry system equipped with an ultrasonic nebulizer (13, 29, 30). The internal potassium concentration increased as the external NaCl concentration increased and approached a maximum of 3.1 M when the external NaCl concentration was 4.1 M (Table 1). The three zwitterionic compounds, glycine betaine,  $\beta$ -glutamine, and  $N_e$ -acetyl- $\beta$ -lysine, are net neutral in charge, whereas potassium ions have a net positive charge. Since  $\alpha$ -glutamate can only contribute a minor amount of negative charge to balance this, theoretically there is a considerable charge that is unaccounted for. We have tested for the presence of 18 different inorganic ions in the cell. However, no significant accumulation was detected. In addition, no other low-molecular-weight organic compounds were detected. Thus, it is unknown what anions serve as counterions for potassium in *Methanohalophilus* spp. Perhaps the missing charge is due to chloride ion, by analogy to the other group of halophilic archaeobacteria (*Halobium* and *Halobacterium* spp.). The acidic ribosomal proteins of halophilic methanogens may also contribute in part to the charge difference, as proposed for the aerobic halophilic archaeobacteria (10).

In other extremely halophilic archaeobacteria, such as *Halobacterium* and *Halobium* species, potassium is the major compatible solute and can accumulate to a ratio of 1:1 relative to the external NaCl concentration (3, 11). An intracellular potassium content of 0.8 M in several marine and moderately halophilic methanogens (10, 13, 32) and in some anaerobic moderately halophilic eubacteria (17, 23) has been reported. As documented in this study, the extremely halophilic methanogen *Methanohalophilus* strain Z7302 contains potassium and the three zwitterionic compounds ( $\beta$ -glutamine,  $N_e$ -acetyl- $\beta$ -lysine, and glycine betaine) at high concentrations as compatible solutes (Table 1). Interestingly, none of several halobacterial species screened contained detectable amounts of these three zwitterionic compounds (13a).

The aerobic extreme halophiles and the methanogenic bacteria are both members of the domain *Archaea* (39). The methanogens and the halophiles appear to be more closely related to each other than to the third group within this domain, the *Crenarchaeotes* (14, 39). However, potassium is the dominant compatible solute in aerobic extreme halophiles (11), and organic compatible solutes do not accumulate to a large extent in these organisms. Eubacteria and eukaryotes employ mostly the nonelectrolyte compatible solutes (3). Only the extremely halophilic methanogens appear to synthesize nonelectrolyte nitrogenous compounds

as well as accumulate potassium in large amounts as compatible solutes.

The extremely halophilic methanogens examined in this study accumulate potassium and synthesize high concentrations of glycine betaine,  $\beta$ -glutamine, and  $N_{\epsilon}$ -acetyl- $\beta$ -lysine to balance the external osmotic pressure. These findings suggest that this group of methanogens has evolved a sophisticated ability to regulate the accumulation of compatible solutes to survive and grow in habitats with extremely high saline concentrations.

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