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The Methanogenic Archaeon *Methanosarcina thermophila* TM-1 Possesses a High-Affinity Glycine Betaine Transporter Involved in Osmotic Adaptation

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Methanogenic *Archaea* are found in a wide range of environments and use several strategies to adjust to changes in extracellular solute concentrations. One methanogenic archaeon, *Methanosarcina thermophila* TM-1, can adapt to various osmotic conditions by synthesis of α-glutamate and a newly discovered compatible solute, N^{ε}-acetyl-β-lysine, or by accumulation of glycine betaine (betaine) and potassium ions from the environment. Since betaine transport has not been characterized for any of the methanogenic *Archaea*, we examined the uptake of this solute by *M. thermophila* TM-1. When cells were grown in mineral salts media containing from 0.1 to 0.8 M NaCl, *M. thermophila* accumulated betaine in concentrations up to 140 times those of a concentration gradient within 10 min of exposure to the solute. The betaine uptake system consisted of a single, high-affinity transporter with an apparent K_s of 10 μM and an apparent maximum transport velocity of 1.15 nmol/min/mg of protein. The transporter appeared to be specific for betaine, since potential substrates, including glycine, sarcosine, dimethyl glycine, choline, and proline, did not significantly inhibit betaine uptake. *M. thermophila* TM-1 cells can also regulate the capacity for betaine accumulation, since the rate of betaine transport was reduced in cells pregrown in a high-osmolarity medium when 500 μM betaine was present. Betaine transport appears to be H^+ and/or Na^+ driven, since betaine transport was inhibited by several types of protonophores and sodium ionophores.

Methanogenic *Archaea* have been isolated from a variety of habitats, including lake sediments, hot springs, marine sediments, sewage sludge, and the intestinal tracts of animals (11). In all these environments, methanogens must cope with general osmotic stress due to fluctuating external solute concentrations. Like the *Bacteria* and *Eucarya*, the *Archaea* have adopted two general strategies for balancing intracellular osmotic pressure with external solute concentrations that include the accumulation of inorganic ions, such as potassium (14, 20, 34), and the accumulation of compatible organic solutes, such as glycine betaine (betaine) or amino acids, which can be synthesized (8, 32) or taken up from the environment (19, 25–27, 31).

Compatible solutes are often accumulated to high intracellular concentrations and do not usually serve as growth substrates. They aid in adjusting intracellular turgor pressure with external osmotic pressure to minimize water loss (36). Betaine is a common compatible solute alleviating osmotic stress in many organisms from all three domains (1, 3, 6, 13, 18, 25, 36). Betaine can be synthesized de novo in some organisms (19, 21) but is more commonly acquired from the environment by specific uptake systems. *Bacteria*, as demonstrated by the enteric bacteria *Escherichia coli* and *Salmonella typhimurium*, possess two betaine transporters (7, 21, 24). One transporter operates as a low-affinity transporter and appears to be constitutive (4). The other transporter has a high affinity for betaine and is inducible (5, 10, 22, 24). *Eucarya*, as demonstrated in MDCK (Madin-Darby canine kidney) cells, also possess both low- and high-affinity betaine transporters. However, the affinities of the eukaryotic betaine transporters appear to be much lower than observed for their counterparts among the *Bacteria*. Both of the MDCK eukaryotic betaine transporters appear to be inducible by hypertonicity (23, 35).

Betaine is a common compatible solute in many methanogenic *Archaea*, including many species of *Methanosarcina* (31), in the marine methanogens *Methanogenium cariaci*, *Methanogenium anulus* AN9, and *Methanococcus voltae*, and in the halophilic methanogens, including *Methanohalophilus zhilinae* and *Methanohalophilus mahii* (20, 25). However, little is known about betaine transport in any of these archaea. Previous physiological studies with *Methanosarcina thermophila* TM-1 demonstrated that this organism can adapt and grow at salt concentrations from 0.05 to 1.2 M (29, 31). It can differentially synthesize two compatible solutes, α-glutamate and N^{ε}-acetyl-β-lysine (Naβlys), depending on the osmolarity of the medium (29, 31, 32). It can also transport potassium ions (31). Finally, *M. thermophila* can alter its cell envelope by synthesis of a β-glucuronic acid- and galactosamine-containing sheath layer (30, 31). Although *M. thermophila* cannot synthesize betaine, it can accumulate this compatible solute when it is provided externally (31, 32): synthesis of Naβlys and α-glutamate is then suppressed. Since very little is known about solute transport in any of the methanogenic *Archaea*, we devised an anaerobic transport assay to measure betaine uptake in *M. thermophila* TM-1 cells. The substrate specificity and affinity of the betaine transporter were determined, along with the ability of cells to transport this compatible solute during growth at different concentrations of NaCl. These studies demonstrate that this methanogenic archaean possesses a single inducible betaine transporter with an energy requirement for either a sodium or a proton gradient.

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MATERIALS AND METHODS

Strains and culture media. *M. thermophila* TM-1, a representative of *Methanosarcina* spp., was used for all studies (29, 37). The basal carbonate-buffered (pH 6.8) anaerobic medium (29) was supplemented with 50 mM $MgCl_2$, 10 mM KCl, 1 mM $CaCl_2$, trace elements, and vitamins. Medium was prepared under a N_2-CO_2 (4:1) atmosphere and anaerobically dispensed into Wolin-Miller tubes that were sealed with butyl rubber stoppers and secured with aluminum crimp seals (29). After the tubes were autoclaved, 1 mM $Na_2S \cdot 9H_2O$ and 100 mM methanol were aseptically added with a syringe prior to cell inoculation. Osmolarity was varied by adding NaCl to a concentration between 0.1 and 0.8 M as indicated in the figures.

Growth rate experiments. Strain TM-1 was grown in the basal anaerobic medium at various osmolarities either with or without 500 μM betaine additions. Cultures were incubated at 35°C on a tube rotator, and the optical density at a 600-nm wavelength (OD_{600}) was measured every 2 to 4 h for 48 h. The cell growth rate was determined from the linear portion of the growth curve by computer-generated regression analysis (SlideWrite). Growth rates from triplicate growth experiments were within ± 12%.

Anaerobic procedures. For routine cell growth, a 10% inoculum of exponentially growing culture was aseptically inoculated into a 100-ml serum bottle containing 20 ml of fresh medium with a double-barreled 23-gauge Vacutainer needle (Belco Inc.). Cells were grown at 35°C for 18 to 24 h to an OD_{600} of 0.4 to 0.5 (mid-log phase of growth). Culture bottles were then introduced into a Coy anaerobic chamber, and the cells were transferred into centrifuge tubes with O-ring-sealed caps. Cells were centrifuged anaerobically at 3,000 × *g* for 15 min at 25°C (28, 29). To ensure that no oxygen was present, all syringes, centrifuge tubes, and needles were stored overnight in an anaerobic chamber (Coy Chamber Co.) and then rinsed with 100 mM $Na_2S \cdot 9H_2O$ prior to use. The cell pellets were resuspended in a 1/10 volume with fresh medium of the same osmolarity containing 1 mM $Na_2S \cdot 9H_2O$ and 100 mM methanol. Pooled cell suspensions were washed once and resuspended in the transport buffer to a final OD_{600} of between 3 and 4. This final cell suspension was preincubated for 30 min with gentle stirring at 25°C before transport assays were initiated. The 1-mm-diameter magnetic stir bars were scrubbed free of oxygen with N_2 gas prior to use. We determined that neither a HEPES buffer nor a phosphate buffer could be used in the betaine transport assay because no [^{14}C]betaine uptake was detected even though the cells were able to produce methane after resuspension in either buffer (data not shown). As a result, a carbonate-buffered cell medium was used as the buffer for all transport assays.

Preparation of [^{14}C]betaine. Synthesis of *methyl*-^{14}C-labeled betaine from *methyl*-^{14}C-labeled choline chloride (30 μCi, 0.2 mCi/mmol; Amersham) was performed according to the protocol of Perroud and LeRudulier (24). The [^{14}C]betaine product was separated from the reaction mixture on a Dowex 50W-X (H^+) column by elution with 2 M NH_4OH. To evaluate the purity of the product, samples (5 μl) were mixed with 1 M carrier standards of betaine and choline chloride and spotted onto Whatman 3MM paper. The electrophoretogram was run at 1,000 V for 30 min with 0.75 M formic acid as the buffer. The electrophoretogram was then dried and exposed to iodine fumes to localize the betaine (R_f = 0.22) and choline chloride (R_f = 0.66). The radioactivity was monitored by a radioactivity counter (Ambis Systems, San Diego, Calif.). The [^{14}C]betaine product yield was virtually 100% by this technique, and the typical radioactivity concentration of the product was 12 μCi/ml.

Transport assay with silicone oil centrifugation of cells. The protocol of Engel et al. (9) was adapted for *M. thermophila* TM-1 to allow the transport assays to be performed under anoxic conditions in stoppered 5-ml serum bottles. The appropriate mixture of [^{14}C]betaine and carrier betaine was added with a Hamilton gas-tight syringe through the septum into the serum bottle (Belco, Inc.). Betaine transport was initiated by adding 1.8 to 2.8 ml of cell suspension. A 300-μl volume was immediately drawn out from the reaction mixture, and triplicate 100-μl subsamples were pipetted onto 100 μl of silicone oil (Dow grade 550, density = 1.07 g/ml; Dow Chemical Co.) in each of three microcentrifuge tubes. Transport was terminated when the cell suspensions were separated from the betaine solution by centrifugation (17,000 × *g*) through the silicone oil underlay: this occurred in less than 1 min. After completion of the time course experiment, the microcentrifuge tube tips containing the visible cell pellets were cut, the cell pellets were dispersed in Ecoscint liquid scintillation cocktail, and the radioactivity was counted in a liquid scintillation counter. By this technique, the density difference between the reaction mixture and the silicone oil prevents carryover of the reaction mixture liquid. No droplets were associated with the cell pellet upon visual inspection of the tube. This was also confirmed by the lack of [^{14}C]betaine associated with the cell pellet when the methanogenic substrate methanol was omitted from the assay mixture.

In experiments, the total concentration of betaine in the transport reaction mixture was adjusted so that no more than 10% of the osmolyte was taken up; this condition was verified by comparing the total radioactivity in the cell pellet with the total radioactivity added in the reaction mixture. We evaluated whether the harvesting and washing steps resulted in the release of betaine into the surrounding fluid by preparing ethanol extracts of the TM-1 cells and of the fluid by quantitating the betaine with its periodide derivative (33). Transport rates were calculated from the amount of radioactive betaine taken up by the cells during a period of 10 to 45 min, depending on the osmolyte concentration. A line was fitted to the linear portion of the uptake curve by computer-generated regression analysis (SlideWrite). All data are mean values of triplicate replicates for each time point. Each experiment was conducted in duplicate unless otherwise noted. The means of the triplicate transport experiments agreed to within ±5%, while the means of duplicate transport experiments generally agreed to within ±10%.

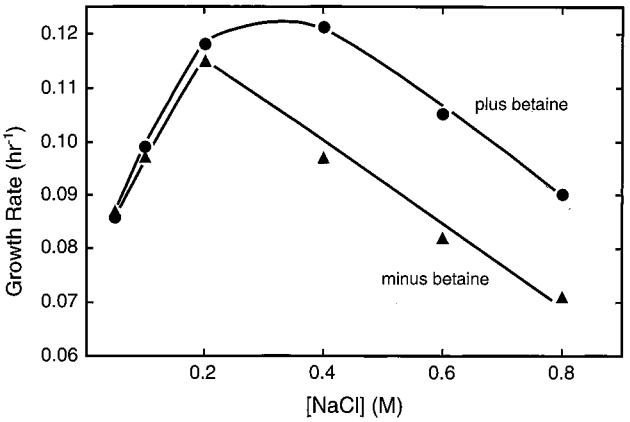

FIG. 1. Effect of exogenous betaine on growth of *M. thermophila* TM-1. Strain TM-1 was grown at 35°C without betaine or with 0.5 mM betaine in NaCl concentrations from 0.1 to 0.8 M; absorbance was measured at 600 nm, and the rate of cell growth is expressed in generations per hour.

Competition for betaine transport. Inhibition of betaine transport by the structural analogs glycine, sarcosine, dimethyl glycine, choline, and proline was tested in a competition assay. Each compound was evaluated at a molar ratio to betaine of 20:1 or 40:1; a control assay in which no competitor was included was also performed. In the competition assays, the cell suspensions were preincubated for 10 min with each potential competitor and transport was measured in the presence of the competitor. Transport was initiated by addition of the labeled betaine mixture (final concentration, 100 μM), and transport continued for 30 to 60 min. Inhibition was expressed as the percent inhibition of the rate of label uptake in the presence of each competitor compared with the rate of uptake of the control.

The properties of the betaine transporter were tested in an inhibition assay with the following compounds: the ATPase inhibitor *N*,*N*′-dicyclohexylcarbodiimide (DCCD) (2); the methyl coenzyme M reductase inhibitor bromoethane sulfonate (BES) (12); the sulfhydryl reagent *N*-ethylmaleimide (NEM) (8); the protonophores carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (15) and 3,3,4,5′-tetrachlorosalicylic acid (TCS) (2); the sodium ionophores monensin (2), ETH157, and ETH2120 (gift of M. Blaut); the potassium ionophore gramicidin D (15); and the Na^+/H^+ antiporter inhibitor harmaline (17). The inhibition assays were conducted in the same manner as the competition assays described above. The metabolic uncouplers were added at physiologically relevant concentrations based upon their ability to suppress methane production in strain TM-1.

Protein determination. Total cellular protein was determined by a modified Lowry method, since the intracellular compatible solutes present in strain TM-1 give a high background in the standard protein assay. Total cell protein was first precipitated by addition of 9% ice-cold trichloroacetic acid, and the mixture was allowed to sit on ice for 10 min. The supernate was removed, and the protein pellet was resuspended in distilled water by heating it at 70°C for 10 min. Bovine serum albumin (fraction V) was used as a standard and treated in the same fashion as the cell protein. When strain TM-1 was grown in 0.1 to 0.8 M NaCl, 1 OD_{600} unit of cells was equivalent to 346 μg of protein per ml.

Gas chromatographic analysis of methane production by TM-1. Methane formation was monitored by assaying the headspace gas of the 5-ml serum bottles or Wolin-Miller tubes. A Shimadzu model 8A gas chromatograph was equipped with a Hayes Sep-Q column and a flame ionization detector (31).

RESULTS

Effect of betaine on the growth rate of *M. thermophila* TM-1. In order to examine if the presence of betaine in the culture medium affects the rate of *M. thermophila* cell growth, cells were grown at different concentrations of NaCl ranging from 0.1 to 0.8 M in either the presence or absence of betaine (Fig. 1). Betaine additions had no effect on the cell growth rate at osmolarities of ≤0.2 M NaCl. However, above 0.2 M NaCl, cultures supplemented with betaine all grew noticeably faster

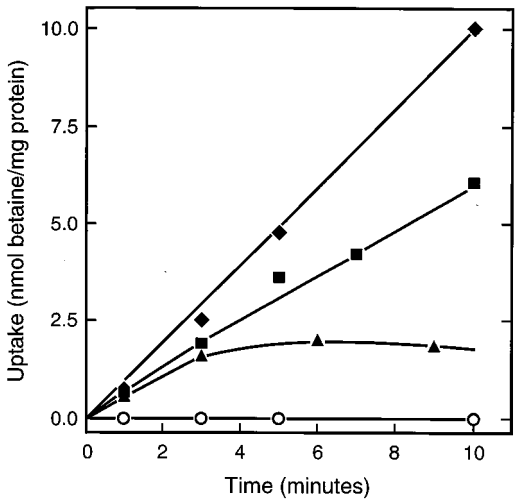

FIG. 2. Uptake of betaine by *M. thermophila* TM-1. Cells were incubated in 0.4 M NaCl transport buffer and with 100 mM methanol as the energy source (closed symbols); at 10 μM betaine, cells did not take up betaine without methanol (open symbols). The points shown are the mean values of triplicate samples per time point. Transport is expressed as nanomoles of betaine per milligram of protein. The concentrations of betaine were 4 μM (triangles), 8 μM (squares), and 24 μM (diamonds).

than did cultures that lacked betaine. The increased rate of cell growth above 0.2 M NaCl appears to correlate well with the accumulation of betaine by TM-1 cells (31) and the suppression of α-glutamate and Naβlys synthesis (31, 32).

Active transport of betaine. An anaerobic betaine uptake assay was developed for *M. thermophila* TM-1 (see Materials and Methods) (Fig. 2). Cells pregrown at 0.4 M NaCl and then incubated in 0.4 M NaCl with methanol present as a methanogenic substrate transported betaine at a concentration as low as 4 μM and at a rate of 0.33 nmol/min/mg of protein. The transport rate increased to a maximum rate of 1.0 nmol/min/mg of protein at 24 μM betaine (Fig. 2). The methanogenic substrate, methanol, was essential for this process: its omission resulted in the complete loss of betaine uptake by cells. Solute uptake also required the exclusion of oxygen from the transport assay: trace amounts of O_2 (ca. 10 to 25 μl of O_2) when added to the assay vial inhibited uptake by more than 99%.

Active transport of [^{14}C]glutamate. At NaCl concentrations ranging from 0.05 to 0.2 M NaCl, *M. thermophila* TM-1 accumulates α-glutamate as an osmolyte (31). As glutamate can be acquired by some organisms by either solute transport or de novo synthesis, strain TM-1 was examined for its ability to transport [^{14}C]glutamate under the same conditions we had observed for betaine transport. We were unable to observe α-glutamate transport when it was tested at either 10 or 50 μM in either 0.05 or 0.2 M NaCl transport buffer conditions.

Effect of NaCl on betaine transport rates. It is apparent that *M. thermophila* TM-1 can actively transport betaine over a wide range of salt concentrations and that it can regulate this capacity (Fig. 3). When cells were grown in the absence of added betaine at 0.1 M NaCl, they transported 10 μM betaine at a rate of 0.009 nmol/min/mg of protein. The uptake rate increased considerably as the NaCl level of the medium was raised to 0.4 M, where betaine was transported at a maximal rate of 0.70 nmol/min/mg of protein. Cells grown at NaCl concentrations above 0.4 M exhibited a somewhat reduced rate of betaine uptake.

We also examined the rate of betaine transport in cells pregrown at different concentrations of salt when 500 μM betaine was present (Fig. 3). At all osmolarities tested, cells exhibited a lower rate of betaine transport than was seen in cells grown without added betaine: the transport rates were lower by 2- to 40-fold. The reduced rates of betaine transport seen in cells pregrown with betaine present did not appear to be due to the leakage or export of betaine from the cells for several reasons. First, the TM-1 cells were always gently transferred from growth media to assay buffer of the same osmolarity to prevent osmotic shock. Under these conditions, we did not detect the release of betaine into the transport buffer of freshly washed cells by an assay for betaine (20, 33). Second, we noted that cells transferred from betaine-free medium to a medium containing 500 μM betaine exhibited intermediate betaine transport rates until several cell doublings had occurred.

Kinetics of betaine transport. To determine if strain TM-1 possesses betaine transporters with different substrate affinities, the kinetic parameters of betaine transport were determined with cells grown at 0.4 M NaCl with and without betaine. Initial linear rates of betaine transport were observed for osmolyte concentrations from 4 to 100 μM. In cells pregrown without betaine being present, double-reciprocal (Lineweaver-Burk) plots of the betaine transport rates versus substrate concentrations yielded a single line and resulted in an apparent K_s of 10 μM and a maximum transport velocity (V_{max}) of 1.15 nmol/min/mg protein (Fig. 4). With cells pregrown with 500 μM betaine, Lineweaver-Burk plot transformations of the betaine transport rates as a function of osmolyte concentrations yielded a second line with approximately the same apparent K_s (10 μM) but with an apparent V_{max} of 0.25 nmol/min/mg of protein (Fig. 4). The rate for betaine transport in cells preincubated with betaine was about fivefold lower than in cells pregrown without betaine.

Since increases in osmotic stress can induce or activate additional betaine transporters (5, 23), the kinetic parameters of betaine transport were also examined for cells pregrown and assayed at other NaCl concentrations. Cells grown in 0.2 M NaCl exhibited the same K_s (10 μM) and V_{max} (0.1 nmol/min/mg) whether they were grown with or without betaine. The same K_s (10 μM) but a higher V_{max} (0.5 nmol/min/mg) was derived for betaine transport in cells pregrown in 0.8 M NaCl

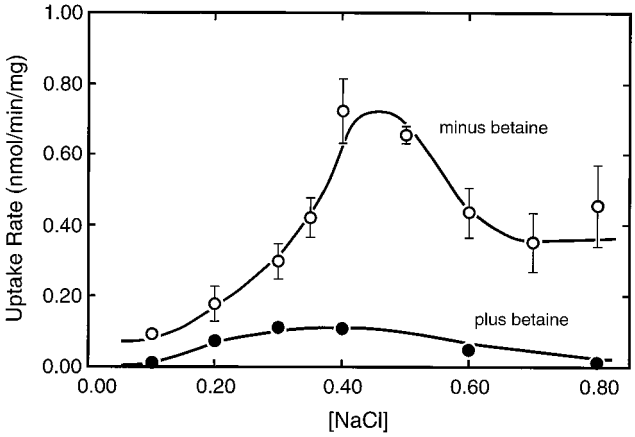

FIG. 3. Rates of betaine transport in *M. thermophila* TM-1 cells grown in media containing different concentrations of NaCl (in molar units). Betaine transport was assayed with 10 μM betaine. Cells were grown in the presence or absence of 500 μM added betaine. Methanol (100 mM) was present as an energy source in all assays. Error bars indicate the variation in values.

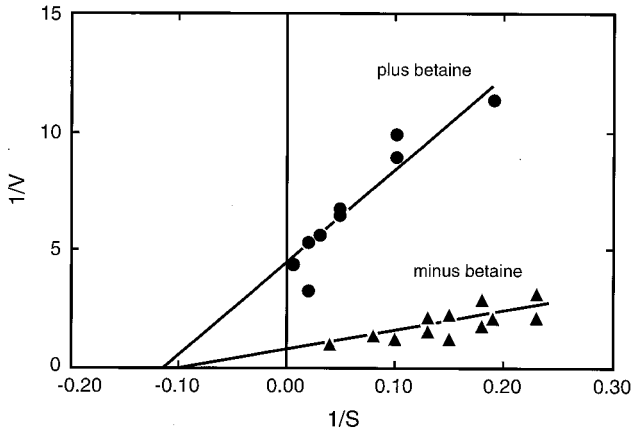

FIG. 4. Lineweaver-Burk plot transformations of betaine transport rates of *M. thermophila* TM-1. Cells were pregrown in medium containing 0.4 M NaCl with or without 0.5 mM betaine. Methanol (100 mM) was present as an energy source in all assays. Rates were based on the initial and linear portions of the uptake process. The rate of betaine uptake (V) is expressed in nanomoles per minute per milligram of protein. The betaine concentration (S) is in micromolar units. The lines fitted to the data were derived by regression analysis.

without betaine. The V_{max} for cells pregrown in 0.8 M NaCl with betaine was 0.05 nmol/min/mg (Table 1). These data suggest that *M. thermophila* TM-1 possesses only one betaine transporter and that it has a relatively high affinity for betaine.

Specificity of the betaine transporter. In order to determine if the transporter in strain TM-1 is specific for betaine or, alternatively, is a general transporter for betaine and other structurally related compounds, we tested if betaine transport was inhibited in a competition assay. When cell suspensions of strain TM-1 were incubated with glycine, sarcosine, dimethyl glycine, or choline, betaine transport rates were reduced by only 13 to 20%. Since studies with *E. coli* suggested that proline was also transported by the betaine transporter, and since many eukaryotic cells can accumulate proline as a compatible solute, we examined the ability of the TM-1 betaine transporter to take up proline. When betaine uptake was tested a 20-fold molar excess in the presence of proline, proline had little effect on the betaine transport rate (i.e., transport was inhibited by 30%). These experiments suggest that the betaine transporter in strain TM-1 is highly specific for betaine.

Effect of metabolic uncouplers on betaine transport. Since strain TM-1 requires the presence of the methanogenic substrate methanol for active transport of betaine, we tested the effect of a variety of metabolic uncouplers on betaine uptake to reveal information about the energy requirements of the transport system. When cells were incubated with methanol and either the methyl coenzyme M reductase inhibitor BES or the ATPase inhibitor DCCD, relatively little inhibition of betaine transport was observed (Table 2). Similar results were observed when NEM was used. However, certain protonophores and other ionophores were relatively effective in blocking betaine uptake. Betaine transport rates were inhibited by 85 to 95% when cell suspensions were incubated with 5 to 20 μM concentrations of either of the protonophores CCCP and TCS (Table 2). The sodium ionophores, including monensin, ETH157, and ETH2120, also inhibited betaine transport by 75 to 85% when they were tested at 20 μM (Table 2). Harmaline, an inhibitor of Na^+/H^+ antiporters, also inhibited betaine transport by about 50% when cells were incubated with 100 μM. Gramicidin D, an antibiotic which acts as a potassium ionophore, had no effect on betaine transport when it was tested at 10 μg/ml, but it completely inhibited transport at a concentration of 30 μg/ml. As noted previously, betaine uptake by cells required a methanogenic substrate (methanol) and was abolished by the presence of trace amounts of oxygen.

TABLE 1. Estimates of K_s and V_{max} values for betaine transport in *M. thermophila* TM-1 grown and assayed over a range of salt concentrations

NaCl concn (M)	Addition	Kinetic parameter[a]	
		K_s (μM)	V_{max} (nmol/min/mg)
0.2	Betaine	10	0.1
	None	10	0.1
0.4	Betaine	10	0.25
	None	10	1.15
0.8	Betaine	10	0.05
	None	10	0.5

[a] Substrate affinities and transport velocities derived from Lineweaver-Burk plot transformations of betaine uptake rates at the salt concentrations indicated.

TABLE 2. Effects of metabolic uncouplers on the uptake of 100 μM betaine in *M. thermophila* TM-1 grown and assayed in 0.4 M NaCl

Addition[a]	% Inhibition of betaine transport with indicated inhibitor at concn (μM)[b]:	
	20	100
None	0	0
BES	2	30
DCCD	22	50
NEM	30	65
CCCP	85	100
TCS	95	100
Monensin	75	100
ETH157	80	100
ETH2120	85	100
Gramicidin D	0	100
Harmaline	22	50

[a] The indicated compound was added at 20 or 100 μM 10 min prior to initiation of the assay. The compounds used were the ATPase inhibitor DCCD; the methyl coenzyme M reductase inhibitor BES; the sulfhydryl-reactive reagent NEM; the protonophores CCCP and TCS; the sodium ionophores monensin, ETH157, and ETH2120; the potassium ionophore gramicidin D; and the Na^+/H^+ antiporter inhibitor harmaline.

[b] Inhibition is expressed as the percent reduction in the rate of betaine transport in the presence of the inhibitor compared to the uninhibited rate of betaine uptake (in nanomoles per minute per milligram of protein).

DISCUSSION

Betaine is a compatible solute for the methanogenic archaeon *M. thermophila* TM-1. *M. thermophila* TM-1 cannot synthesize betaine, and it does not use exogenous betaine for growth (31). Instead, it responds to increasing osmotic stress by accumulating exogenous betaine. Betaine appears to be a truly compatible solute in *M. thermophila*, as growth rates were noticeably higher in cells grown at elevated levels of NaCl and when betaine was present than in cells that lacked betaine additions (Fig. 1).

Betaine accumulation appears to be a common response in methanogenic isolates from highly osmotic environments. For example, the marine methanogens *Methanococcus voltae*, grown in 0.22 M NaCl, *Methanogenium cariaci*, grown in 0.512 M NaCl (27), and *Methanogenium anulus* AN9, grown in 0.512

and 1.5 M NaCl (25), all accumulated betaine. The methanogen *Methanohalophilus mahii*, isolated from Great Salt Lake sediments, accumulated betaine when it was grown in 0.66 M NaCl, (27) and *Methanohalophilus zhilinae*, isolated from an alkaline lake sediment, accumulated betaine when it was grown in 0.66 and 1.5 M NaCl (25). Although eight strains of a halophilic methanogen, *Methanohalophilus* sp., accumulated intracellular betaine in response to 0.7 to 4.3 M NaCl by de novo synthesis (19), at least two of these strains also accumulated exogenous betaine when they were grown over a similar range of salt concentrations (0.7 to 3.1 M NaCl). Several *Methanosarcina* spp., isolated from osmotically diverse environments, also accumulated betaine in response to highly osmotic conditions (31). This appears to be a common trait among species within this genera, although the specificities and other properties of the uptake systems were uncharacterized prior to this study.

***M. thermophila* possesses one high-affinity transporter for betaine.** We developed an anaerobic transport assay for betaine that allowed us to evaluate the transport properties of *M. thermophila* TM-1. It appears to contain a single betaine transporter of relatively high affinity. This is in contrast to some eukaryotic (canine kidney cells [23]) and eubacterial (*S. typhimurium* [4, 5] and *E. coli* [10, 22]) cells.

The betaine transporter in TM-1 appears to have a high specificity for betaine, as structurally related compounds had little effect on betaine transport rates. These results are similar to those from competition assays performed with the betaine transporter in *E. coli*. Interestingly, the structural analog proline betaine significantly inhibits betaine transport in this enteric bacterium (21). We did not test proline betaine competition in our transport assays with *M. thermophila* TM-1. No data are available on the substrate specificities of eukaryotic betaine transporters.

Initial studies with both *E. coli* and *S. typhimurium* suggested that betaine transport was a secondary activity of the proline transporter. Subsequent investigations demonstrated that betaine is preferentially taken up and concentrated over proline to alleviate osmotic growth inhibition (7). However, proline was a poor competitor for the betaine transporter in *M. thermophila* TM-1. This suggests that the betaine transporter in TM-1 differs somewhat from the bacterial transporter. Furthermore, growth studies with TM-1 indicated that proline cannot replace betaine as an osmolyte since it is not accumulated by cells (31, 32) and that proline cannot stimulate the rate of cell growth at elevated osmolarity as betaine can (Fig. 1). This is in contrast to some enteric bacteria that possess a transporter specific for proline (7).

Osmotic stress, and not betaine, induces or activates the betaine transporter in strain TM-1. Osmotic stress appears to induce or activate the betaine transporter in TM-1, as cells pregrown and assayed in increasing amounts of NaCl exhibited an increasingly greater capacity for betaine transport, as evidenced by increases in the rate of betaine transport. Betaine transport in TM-1 was activated at 0.2 M NaCl and reached maximum capacity at 0.4 to 0.5 M NaCl. An increase in betaine transporter capacity with increasing osmotic stress was also observed in *Bacteria* and *Eucarya*. Both *E. coli* and *S. typhimurium* possess high-affinity betaine transporters which are activated by increasing osmotic stress. In *E. coli*, the high-affinity betaine transporter is activated at a relatively low (0.05 M) concentration of NaCl, with a plateau of activity from 0.1 to 0.3 M NaCl (22), while in *S. typhimurium*, betaine transport by the high-affinity transporter is activated at 0.15 M, with a maximum capacity at 0.5 M (5). In MDCK cells, both of the betaine transporters are activated by increasing osmolarity and over salt concentrations similar to those observed for *S. typhimurium* (23) and strain TM-1.

Strain TM-1 can regulate its capacity for betaine transport. Betaine transport rates were reduced in cells pregrown in media containing 500 μM betaine (Fig. 3). These results suggest that strain TM-1 can modulate the amount of the transporter protein in the cell. In *S. typhimurium*, no feedback control was evident for high-affinity betaine transport, as initial and steady-state rates of betaine transport appeared to be similar (5). Instead, regulation of the intracellular betaine concentration in response to fluctuating osmotic stress appears to be controlled by a betaine afflux system which is independent of the betaine transport system (16).

Although no attempt was made to specifically look for a betaine efflux system in eukaryotic cells, feedback control of intracellular betaine concentrations by regulating betaine transporter capacity appears to be the principal means of regulating cellular response to fluctuating osmotic stress in eukaryotes. Betaine transport rates in MDCK cells exposed to betaine over 2 days were approximately 50% lower than those in cells which had not been previously exposed to betaine (23). Direct evidence for feedback control of the betaine transporter by betaine or by protein inactivation in strain TM-1 awaits further study.

Betaine transport in strain TM-1 is linked to the proton and/or sodium transmembrane gradient. Our experiments with energy substrates and metabolic uncouplers suggest that betaine transport is an active process and is driven by a proton or sodium gradient. Metabolically active cells were required for betaine transport, since the absence of a methanogenic substrate (methanol) abolished betaine transport. However, inhibition of ATP synthesis and methyl reductase activity with DCCD or BES did not abolish betaine transport, which suggests that TM-1 did not specifically require active ATP synthesis or methanogenesis to drive betaine transport.

Although most of the inhibitors had a large effect when tested at a 100 μM concentration, compounds which collapse various proton or ion gradients in *M. thermophila* had the greatest effect at lower concentrations (20 μM). This finding suggests that metabolically active cells were required in order to maintain a transmembrane energy gradient in TM-1 cells. In particular, the protonophores CCCP and TCS strongly inhibited betaine transport, suggesting at least that betaine transport might be driven by a proton gradient. Although harmaline, an inhibitor of Na^+/H^+ antiporters, had a slight effect on betaine transport, this may have been the result of a secondary effect on proton or ion motive forces rather than an example of a primary effect on betaine transport.

Betaine transport systems in eukaryotes appear to be sodium dependent. In MDCK cell assays, Nakanishi et al. (23) were able to demonstrate that betaine transport was abolished by replacing the NaCl in the betaine transport medium with equimolar amounts of LiCl. On the other hand, betaine transport in the *Bacteria* appears to be driven by a proton gradient based upon evidence that betaine transport was inhibited by 2,4-dinitrophenol, a protonophore (*E. coli* [24]).

With regard to what is known about other transport systems in *Archaea*, amino acid transport also appears to be driven by either a proton or a sodium gradient (8, 15, 34). Protonophores and sodium ionophores had the greatest effect on isoleucine transport in *Methanococcus voltae*, while gramicidin D had little effect on isoleucine transport (8). Any direct evidence of a specific proton or of a sodium-driven betaine symporter in TM-1 will require the use of membrane vesicle systems.

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