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Influence of pH on Ammonia Accumulation and Toxicity in Halophilic, Methylotrophic Methanogens

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We studied the effects of pH and ammonia concentration on the growth of three methanogens. These three halophilic, methylotrophic methanogens, *Methanolobus bombayensis*, *Methanolobus taylorii*, and *Methanohalophilus zhilinaeae*, grew at environmental pH ranges that overlapped with each other and spanned the pH range from 7.0 to 9.5. During growth they had reversed membrane pH gradients (ΔpH) at all pH values tested. The ΔpH was in the range of −0.4 to −0.9 pH units, with the cytosol being more acidic than the environmental pH. *Methanohalophilus zhilinaeae* had the most negative ΔpH (−0.9 pH units). These negative pH gradients resulted in the accumulation of ammonium (NH_4^+), and when grown at the highest external ammonia concentrations that allowed good growth, cells had cytosolic NH_4^+ concentrations as high as 180 mM. The high concentrations of cytosolic NH_4^+ were accompanied by greater ΔpH and lower concentrations of the major cytosolic cation K^+ (compared with cells grown in medium with only 5 mM ammonia). *Methanolobus bombayensis* and *Methanolobus taylorii* were more sensitive to total external ammonia at higher external pH values, but the inhibitory concentration of un-ionized ammonia that resulted in a 50% reduction of the growth rate was about 2 to 5 mM, regardless of the pH. This is consistent with growth inhibition by ammonia in other bacteria. However, *Methanohalophilus zhilinaeae* was more resistant to un-ionized ammonia than any other known organism. It had a 50% inhibitory concentration for un-ionized ammonia of 13 mM at pH 8.5 and 45 mM at pH 9.5. We examined the effects of pH on three ammonia-assimilating activities (glutamine synthetase, glutamate dehydrogenase, and alanine dehydrogenase) in cell lysates and found that the pH ranges were consistent with the observed ranges of intracellular pH.

In most bacteria, proton-translocating ATPases equilibrate energy in ATP with a proton motive force ($\Delta_{\mu_{H^+}}$) across the cell membrane. The energy in the $\Delta_{\mu_{H^+}}$ is the sum of the energies in the transmembrane pH gradient (ΔpH) and the transmembrane electrical gradient (Δψ). Most of the energy in the $\Delta_{\mu_{H^+}}$ of *Methanolobus taylorii* is accounted for by its large Δψ (i.e., the outside of the cell is more positively charged); its ΔpH is therefore small or even negative (31). *Methanolobus taylorii* is a slight alkaliphile, and its small or negative ΔpH allows it to grow with a near-neutral cytosol even when the external pH is above 7. The active extrusion of potassium by this methanogen was suggested as a mechanism by which it increases its Δψ, thereby allowing it to grow with a low or negative ΔpH (31).

One of the consequences of a reversed ΔpH is the accumulation of ammonium in the cytosol. Ammonia can enter cells as the unprotonated form (NH_3), which diffuses readily across the cell membrane, equilibrating the intracellular and extracellular concentrations of NH_3 (22). On the other hand, ammonium (NH_4^+) does not readily diffuse through lipid membranes. Although the existence of NH_4^+ permeases has been postulated (22), these could not function in the presence of substantial concentrations of ammonia without establishing a futile cycle that would dissipate the $\Delta_{\mu_{H^+}}$ (13). The intracellular and extracellular concentrations of NH_4^+ are dependent on NH_3 concentration and the local pH (pK_a = 9.24) (11). Thus, cells whose intracellular pH is lower than the extracellular pH have an intracellular NH_4^+ concentration greater than that of their environment. In cells with a very negative ΔpH, cytosolic NH_4^+ may constitute a considerable fraction of the intracellular cations (48). For instance, *Methanohalophilus zhilinaeae* grows at pH 9.5 in medium with 20 mM total ammonia (NH_3 plus NH_4^+). If this methanogen maintained an internal pH of 7.5, it would have an NH_4^+ concentration of about 709 mM under these conditions.

At least two possible mechanisms of ammonia toxicity have been postulated: (i) un-ionized ammonia could directly inhibit the activity of cytosolic enzymes, or (ii) NH_4^+ accumulated inside cells might be toxic by its effect on intracellular pH (49) or the concentration of other cations such as K^+ (48). Either way, high pH and high total ammonia concentration could exert their toxicities synergistically. At higher pH values, a larger fraction of total ammonia is unprotonated (about 0.5% at pH 7 but almost 65% at pH 9.5). Also, if bacteria growing at a higher pH establish a more negative ΔpH to maintain a near-neutral cytosol, then the potential toxicity due to NH_4^+ accumulation would also be greater.

In the present study, we examined the adaptation of three members of the family *Methanosarcinaceae* to a range of external pH values and we tested the effect of this adaptation on the toxicity of ammonia.

(Portions of these results were presented previously [19].)

MATERIALS AND METHODS

Source of bacterial strains. The following methanogenic strains were obtained from the Oregon Collection of Methanogens (OCM) (Portland, Oreg.): *Methanolobus taylorii* GS-16 (OCM 58) (32), *Methanolobus bombayensis* B-1 (OCM 438) (20), and *Methanohalophilus zhilinaeae* WeN5 (OCM 62) (26) ("*Methanosalsus zhilinaeae*" [8]).

Media and culture conditions. Modifications (28) of the anaerobic techniques of Hungate (17) were used. Cells were grown statically at 40°C in MSH medium

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with 50 mM methanol as catabolic substrate. MSH medium is an anoxic medium of marine salinity, buffered with bicarbonate at pH 6.8 when equilibrated with pure CO_2, and containing minerals, yeast extract, and peptones (30). For growth of *Methanolobus bombayensis*, the concentration of $MgCl_2 \cdot 6H_2O$ was increased from 2.7 to 6.1 g/liter. For growth of *Methanolobus taylorii* and *Methanohalophilus zhilinaeae*, $CaCl_2 \cdot 2H_2O$ was omitted to avoid precipitates at high pH values. For media at pH 7.0 or 7.5, the pH was adjusted by diluting CO_2 in the gas phase with N_2 (the ratio of gases was determined empirically). For media at pH 8.5 and 9.5, the gas phase was 100% N_2 and pH was adjusted by adding sterile anoxic 4 M NaOH solution. MMSH was the same as MSH medium but with all organic constituents omitted.

Determination of specific growth. The specific growth rate (μ) was calculated from the rate of methane formation. We fit the Gompertz equation to the measured methane production in order to determine the most rapid growth rate of batch cultures (16, 50). The methane produced by the inoculum was also included in the calculations (34).

Ammonia inhibition of growth rate. The concentration of ammonia that inhibits the specific growth rate by 50% (the 50% inhibitory concentration [IC_{50}]) was estimated by measuring μ in medium with various concentrations of ammonia and extrapolating between the points by fitting these data to an equation. To find an equation that describes the relationship between concentration of an inhibitor and μ, we fitted over 35,000 equations (TableCurve; Jandel Scientific Co., San Rafael, Calif.) to several published data sets of growth rate in the presence of inhibitors. One simple equation [$y = a + b(x)^3$] fitted almost all the data sets well but was not satisfactory for some data sets. Observation of previously published data sets as well as our own indicated that μ was uninhibited at concentrations below some critical value and that the growth rate decreased linearly with the inhibitor concentration above that value. On the basis of these observations, we designed a linear equation that described this relationship, with three fitting parameters: a, the uninhibited μ c_1, the concentration below which no inhibition occurs; and c_2, the concentration above which growth is completely inhibited. The equation divides the growth range into two parts, uninhibited growth below c_1, and partially inhibited growth between c_1 and c_2: if $x < c_1$, $\mu = a$; if $c_1 < x < c_2$, $\mu = a/[(c_2 - c_1)(c_2 - x)]$; if $c_2 < x$, $\mu = 0$. On the basis of this equation, the IC_{50} is $0.5 \times (c_1 + c_2)$. The value of IC_{50} calculated by this equation was similar to the IC_{50} calculated by using the above cubic equation, but we used the linear equation for data analysis because it fitted all the data sets better than did the cubic equation.

Collection of cells. Cells in the late exponential phase were anoxically harvested by centrifugation at $10,000 \times g$ for 20 min at 4°C. Cultures were transferred to centrifuge bottles within an anaerobic chamber whose partial pressure of CO_2 matched approximately that of the culture medium. To remove O_2 from the centrifuge bottles, they were stored in an anaerobic chamber at least 48 h before use.

Determination of cell volume. Excess water was removed from cell pellets by inserting a piece of filter paper. The pellet was weighted, and its volume was determined picnometrically (27). Extracellular water in the pellet was measured by including 10 mM cellobiose (a cell-excluded solute) in the buffer for final washing of the cells; cellobiose was measured enzymatically (4) with cellobiose dehydrogenase after the cells were lysed by suspension in distilled water.

Preparation of aqueous cell extracts for intracellular ammonia, K^+, and Mg^{2+} analysis. Cell pellets were distributed in preweighed 1.5-ml centrifuge tubes, and the mass of each cell pellet was calculated. The pellet was lysed by adding approximately 5 ml of sodium dodecyl sulfate solution (1 g liter^{-1}) per g of pellet. The cell lysate was centrifuged at $14,000 \times g$ at 4°C for 4 min to remove cell debris, and the supernatant was analyzed for total ammonia, Na^+, K^+, and Mg^{2+}.

Preparation of cell extracts for enzyme assays. Cells were grown at their optimum pH values and growth conditions and were harvested anoxically. The cell pellets were washed twice with an anaerobic buffer containing 20 mM $NaHCO_3$, 30 mM $MgCl_2$ and 1 mM 2-mercaptoethanesulfonic acid. The pH of the buffer was set at 7 or 8.0 (for *Methanolobus bombayensis* and *Methanolobus taylorii*, respectively) by varying the ratio of N_2 and CO_2 in the gas phase, and the pH of the buffer for *Methanohalophilus zhilinaeae* (8.5) was equilibrated with N_2 and adjusted with 1 M NaOH. The cell pellets were suspended in the buffer to obtain a cell suspension of 40% (wt/vol). The cells were lysed by sonication (55 W for 20 min) (model SA520 sonicator with double-step microtip; Sonifier, Danbury, Conn.). The lysed-cell suspension was centrifuged at $10,000 \times g$ at 4°C for 20 min. The supernatant was frozen under anoxic conditions.

Enzyme assays. The activities of enzymes were estimated spectrophotometrically. The specific activity was expressed in of micromoles per minute per milligram of protein. Glutamate dehydrogenase was assayed by monitoring the oxidation of β-NADPH at 340 nm with α-ketoglutarate as the substrate ($\varepsilon_{340} = 6.22$ mM^{-1} cm^{-1}). The assay mixture contained Trizma (pH 7; Sigma) (84 mM), NH_4Cl (231 mM), sodium α-ketoglutarate (6.75 mM), and β-NADPH (0.225 mM) (21).

Glutamine synthetase activity was assayed by modifications (6) of the γ-glutamyltransferase method (42). The assay mixture contained imidazole-HCl (pH 7) (135 mM), hydroxylamine-HCl (18 mM), $MnCl_2$ (0.27 mM), sodium arsenate (25 mM), sodium ADP (0.36 mM), and L-glutamine (20 mM). The stop mixture (55 g of $FeCl_3 \cdot 6H_2O$, 20 g of trichloroacetic acid, and 21 ml of concentrated HCl per liter) was added to terminate the reaction. Under these conditions, 1 μmol of glutamyl hydroxamate gives an A_{540} of 0.532.

Alanine dehydrogenase activity was detected by the oxidation of NADH at 340 nm with sodium pyruvate as the substrate ($\varepsilon_{340} = 6.22$ mM^{-1} cm^{-1}). The assay mixture contained Trizma (pH 7) (87.5 mM), NH_4Cl (99 mM), sodium pyruvate (5 mM), and β-NADH (0.296 mM) (35).

Determination of optimum pH for enzyme activity. The crude extracts were assayed for the enzyme activities at pH values between 5 and 11. For the enzyme assays of glutamate dehydrogenase and alanine dehydrogenase, the following four buffers were used to obtain the indicated pH values: sodium acetate, pH 5; dibasic potassium phosphate, pH 5, 6, and 6.5; Trizma, pH 6.5 to 9 in increments of 0.5 pH units; and 2-*N*-cyclohexylaminoethanesulfonate, pH 9, 10, and 11. Enzyme activity was determined at a pH which was shared by two buffers to exclude the effect of the buffers on the enzyme activity, and activities with different buffers at the same pH were always similar. For the determination of the optimum pH for the glutamine synthetase activity, the assay mixture in the buffer imidazole-HCl was adjusted to various pH values by adding 1 M HCl or 2 M NaOH.

Analytical methods. Methane was analyzed by gas chromatography with flame ionization detection (25). Ammonia was quantified by estimating the ammonia nitrogen by flow injection and gas diffusion. This estimation was done with an FIA Star 5010 Analyzer (Tecator, Höganäs, Sweden). Na^+, K^+, and Mg^{2+} were quantified with an atomic absorption spectrophotometer with an acetylene-and-air flame. pH was measured by a combination electrode. The protein content of the crude extract was estimated by the bicinchoninic acid method (44) with bovine serum albumin as a standard. The A_{562} was measured.

Determination of intracellular pH. The intracellular pH was calculated from the following equation (40): $(pH)_{in} = (pH)_{out} + \log(NH_4{}^+{}_{out}/NH_4{}^+{}_{in})$, where subscripts in and out refer to intra- and extracellular conditions, respectively. We also tested cells' uptake of benzoate by incubating them in the presence of uniformly labeled [^{14}C]benzoate (1.7 μM; 0.2 mCi liter^{-1}) and measuring the radioactivity in the cell pellets.

RESULTS

Effect of ammonia on μ. We selected for study three methylotrophic, halophilic methanogens which had various pH optima for growth: *Methanolobus bombayensis*, *Methanolobus taylorii*, and *Methanohalophilus zhilinaeae*. These three methanogens grow at pH ranges that overlap with each other and span the pH range from 7 to 9.5. We studied the effects of ammonia on growth and intracellular cation concentrations of each of these three methanogens at two pH values each. *Methanolobus bombayensis* was least sensitive to ammonia, having an IC_{50} of 295 mM total ammonia during growth at pH 7.0 (Fig. 1A). *Methanolobus bombayensis* cells were only slightly more sensitive when grown at a higher pH (IC_{50} = 215 mM total ammonia during growth at pH 7.5). The slightly alkaliphilic *Methanolobus taylorii* was not very sensitive to ammonia during growth at the low pH (IC_{50} = 220 mM total ammonia at pH 7.5), but it was much more sensitive during growth at a higher pH (IC_{50} = 39 mM total ammonia at pH 8.5) (Fig. 1B). *Methanohalophilus zhilinaeae*, the most alkaliphilic of the three methanogens, was only moderately sensitive to ammonia, regardless of the pH of the medium (IC_{50} = 86 mM total ammonia at pH 8.5 and 81 mM total ammonia at pH 9.5) (Fig. 1C).

At very low initial ammonia concentrations (<5 mM), μ was sometimes lower than at slightly higher ammonia concentrations (Fig. 1). This was probably due to nitrogen limitations, and these datum points were omitted from our modeling of the inhibition of μ by ammonia.

The inhibitory effect of trimethylamine on the growth of these methanogens was additive with that of μ inhibition by ammonia (data not shown). For example, cells growing in the presence of 20 mM trimethylamine had an IC_{50} of total ammonia that was 20 mM lower than that of methanol-grown cells. The accumulation of trimethylamine during growth in low-ammonia environments may be an adaptive feature of reversed ΔpH, allowing the accumulation of the catabolic substrate for these methanogens.

Effects of external ammonia concentration on ΔpH and cytosolic $NH_4{}^+$. Under all conditions of growth tested in this

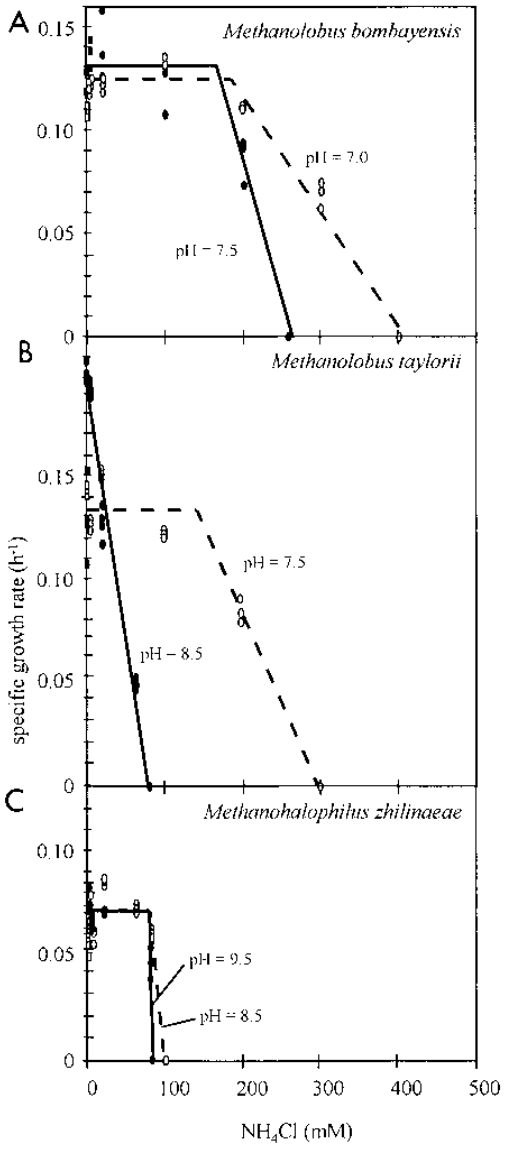

FIG. 1. Effects of external concentration of total ammonia on specific growth rates of three methanogens. Each methanogen was tested at two different pH values, *Methanolobus bombayensis* (A) at pH 7 and 7.5, *Methanolobus taylorii* (B) at pH 7.5 and 8.5, and *Methanohalophilus zhilinaeae* (C) at pH 8.5 and 9.5; in each case, the open symbols and dashed lines indicate data obtained at the lower pH and the filled symbols and solid lines indicate data obtained at the higher pH. The squares indicate data obtained at low ammonia concentrations that were not used in the fitting of the lines.

study, the cells maintained negative ΔpHs. Our attempts to measure the ΔpH by using ^{14}C-labeled benzoic acid were unsuccessful, because cytosolic benzoate concentrations were lower than the external benzoate concentrations. This is consistent with the possession of a negative ΔpH (10). On the other hand, methylammonium and ammonium are accumulated by cells with negative ΔpHs, and accumulation of these ions is often used to measure reversed ΔpHs (10, 14). Because methylamines are catabolic substrates of the methanogens in our study, they could be metabolized rapidly by these cells, resulting in the changing of their concentrations during cell collection. Therefore, we measured the ΔpH by monitoring ammonia accumulation. We grew the cells in the presence of various concentrations of ammonia and determined the cytosolic content of total ammonia. Each of the three methanogens accumulated ammonium, indicating that each had a negative ΔpH, with the cytosol being more acidic than the environmental pH (Fig. 2). In medium with a low ammonia concentration, the ΔpH for the three methanogens was −0.4 to −0.9 pH units, with the most alkaliphilic species (*Methanohalophilus zhilinaeae*) having the most negative ΔpH (Fig. 2). *Methanolobus bombayensis* accumulated cytosolic total ammonia that was proportional to the external total ammonia concentration at external ammonia concentrations up to about 60 to 80 mM (depending also on pH). This proportionality indicated that the ΔpH did not change appreciably. At external total ammo-

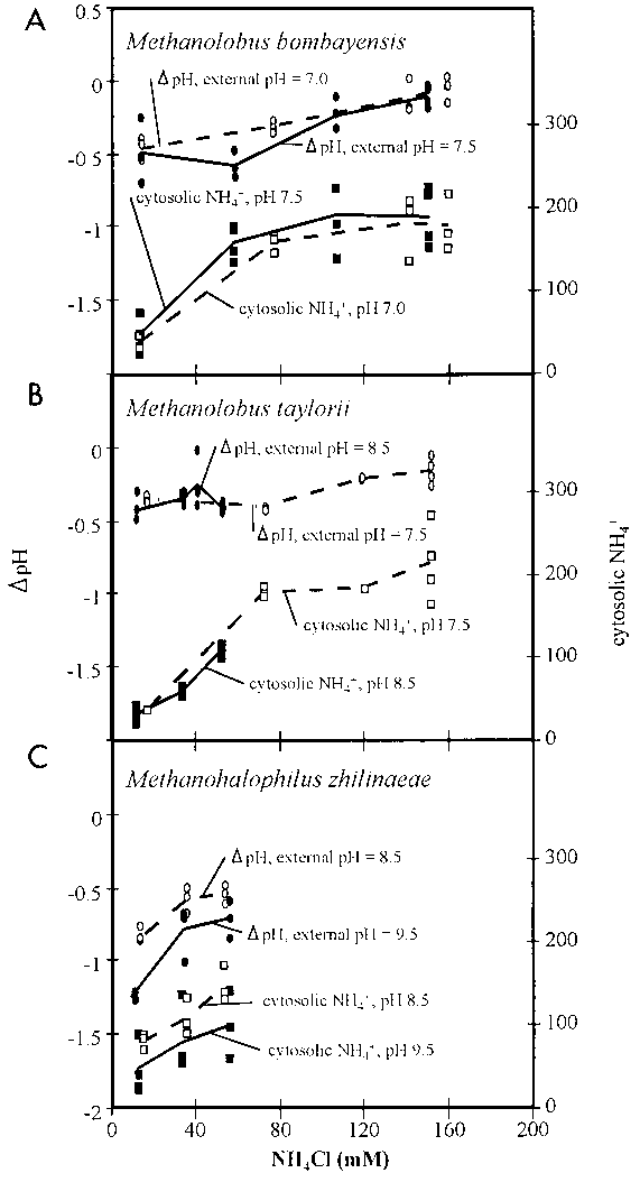

FIG. 2. Effects of external concentration of total ammonia on cytosolic NH_4^+ concentration (squares) and ΔpH (circles). Each methanogen was tested at two different pH values, *Methanolobus bombayensis* (A) at pH 7 and 7.5, *Methanolobus taylorii* (B) at pH 7.5 and 8.5, and *Methanohalophilus zhilinaeae* (C) at pH 8.5 and 9.5; in each case, the open symbols and dashed lines indicate data obtained at the lower pH and the filled symbols and solid lines indicate data obtained at the higher pH.

TABLE 1. Cytosolic ions and their dependence on extracellular pH and ammonia concentration

Organism and growth conditions[a]	External NH_4Cl concn (mM)	Intracellular concn (mM)					Internal osmotic strength	
		Total ammonia	NH_3	NH_4^+	Mg^{2+}	K^+	Calculated[b] (mOs)	% of medium[c]
Methanolobus bombayensis								
pH 7, low ammonia	13.4	39.5	0.07	39.5	65.9	275	827	61
pH 7, high ammonia	160	180	0.7	180	12.3	176	747	46
pH 7.5, low ammonia	13.8	46.3	0.2	46.1	87.6	294	942	70
pH 7.5, high ammonia	150	186	2.7	184	15.1	157	726	45
Methanolobus taylorii								
pH 7.5, low ammonia	17	36.9	0.3	36.6	19.7	195	521	40
pH 7.5, high ammonia	152	218	2.7	215	32.7	105	737	47
pH 8.5, low ammonia	12	31.3	1.8	29.5	35.4	255	675	53
pH 8.5, high ammonia	52.2	115	8.3	107	59.2	234	858	64
Methanohalophilus zhilinaeae								
pH 8.5, low ammonia	15.1	83.5	2.3	81.2	16.6	373	957	72
pH 8.5, high ammonia	53.5	157	8.2	149	24.9	138	647	47
pH 9.5, low ammonia	13.4	56.5	9.0	47.5	20.4	135	427	33
pH 9.5, high ammonia	55.4	139	36	103	35.7	56.1	424	32

[a] Low and high ammonia refer to ammonia concentrations during growth.
[b] Ionic strength of the cytosol was calculated from the measured concentrations of NH_4^+, K^+, and Mg^{2+} and from the assumed presence of monovalent anions.
[c] The calculated ionic strength of the cytosol expressed as a percentage of the calculated ionic strength of the medium, the latter being calculated from the inorganic medium constituents.

nia concentrations above 60 to 80 mM, ammonium accumulation was less than proportional to external ammonium, accompanied by a ΔpH that was higher (less negative) at higher external ammonia concentrations. Likewise, for *Methanolobus taylorii* growing at pH 7.5, internal ammonium was proportional to external ammonium at external total ammonia concentrations up to about 75 mM and was accompanied by little change in ΔpH, but at higher external total ammonia concentrations, a lower proportion of ammonium was accumulated and the ΔpH was higher (Fig. 2B). For *Methanolobus taylorii* growing at pH 8.5, the internal ammonium concentration was always proportional to the external total ammonia concentration, so the ΔpH did not change through the range of external ammonia concentrations at which this species was able to grow (Fig. 2B). For *Methanohalophilus zhilinaeae*, the proportion of cytosolic ammonium relative to external total ammonia decreased slightly throughout the range of concentrations tested, indicating a slight increase in ΔpH at higher external ammonia concentrations.

Intracellular concentrations of K^+, Mg^{2+}, and Na^+. One suggested mechanism by which ammonia may exert its toxicity is its influence on the concentrations of other cytosolic cations (49). We attempted to measure the cytosolic concentrations of Na^+, K^+, and Mg^{2+} in cells grown at various concentrations of ammonia. The cytosolic Na^+ concentration was always lower than the concentration in the culture medium (600 mM), so it was impossible to measure the cytosolic Na^+ concentration accurately. The concentration of cytosolic Na^+ in other halophilic methanogens in the family *Methanosarcinaceae* is also very low (27).

K^+ is often a major cytosolic cation, and it is reported to play a role in pH homeostasis and osmoregulation of cells (3), including *Methanolobus taylorii* (31). We found that all three methanogens accumulated K^+, but *Methanolobus bombayensis* and *Methanolobus taylorii* showed decreased cytosolic K^+ concentrations when grown at high concentrations of external total ammonia (Table 1). In *Methanohalophilus zhilinaeae* grown at a high pH, the cytosolic K^+ was not lower even at the highest external ammonia concentrations tolerated (Fig. 3).

Mg^{2+} is an important ion in the action of many enzymes that catalyze ATP-dependent reactions (43), and it also may function to stabilize proteins in the presence of denaturing concentrations of ammonia. We found that cells of all three of these methanogens accumulated Mg^{2+} (Fig. 3). Figure 3 shows that the cytosolic Mg^{2+} content of *Methanolobus bombayensis* decreased when the external ammonia concentration was high. The requirements of this organism for a high external Mg^{2+} concentration may therefore be related to its sensitivity to ammonia. In contrast, the Mg^{2+} contents of *Methanolobus taylorii* and *Methanohalophilus zhilinaeae* were elevated when the external ammonia concentrations were high (Fig. 3B and C).

The cations Mg^{2+}, K^+, and NH_4^+ were the major cytosolic cations and were present at concentrations sufficient to affect cytosolic osmolarity. These methanogens are halophiles with weak cell walls (26, 33, 47) that do not allow the development of significant turgor pressure (27), so the osmolarity of the cytosol must approximately match that of the methanogen's environment. The osmolarity of the measured cations of these cells (plus their presumed counter ions) was a substantial fraction of the osmolarity of the culture medium in which the cells were grown (Table 1). The osmolarity of detected cytosolic ions in *Methanolobus bombayensis* (grown in medium at pH 7.5 with 13.8 mM total ammonia) was about 69% that of its medium concentration. The remaining 31% of cytosolic osmolarity may have been provided by undetected inorganic cations or by organic molecules such as compatible solutes, as reported previously for halophilic methanogens (24, 38, 46). The concentration of cytosolic ions in *Methanolobus taylorii* (pH 8.5 and 12 mM total ammonia) accounted for only about 53% of its osmolarity, and those of *Methanohalophilus zhilinaeae* (pH 9.5 and 13.4 mM total ammonia) accounted for only 33% of its osmolarity, possibly indicating higher concentrations of organic compatible solutes in these species.

Activities of ammonia-assimilating enzymes. Each of the three methanogens tested contained detectable activities of each of the three ammonia-assimilating enzymes previously found in methanogens (15): glutamate dehydrogenase, glutamine synthetase, and alanine dehydrogenase. The activity levels of glutamine synthetase and alanine dehydrogenase were

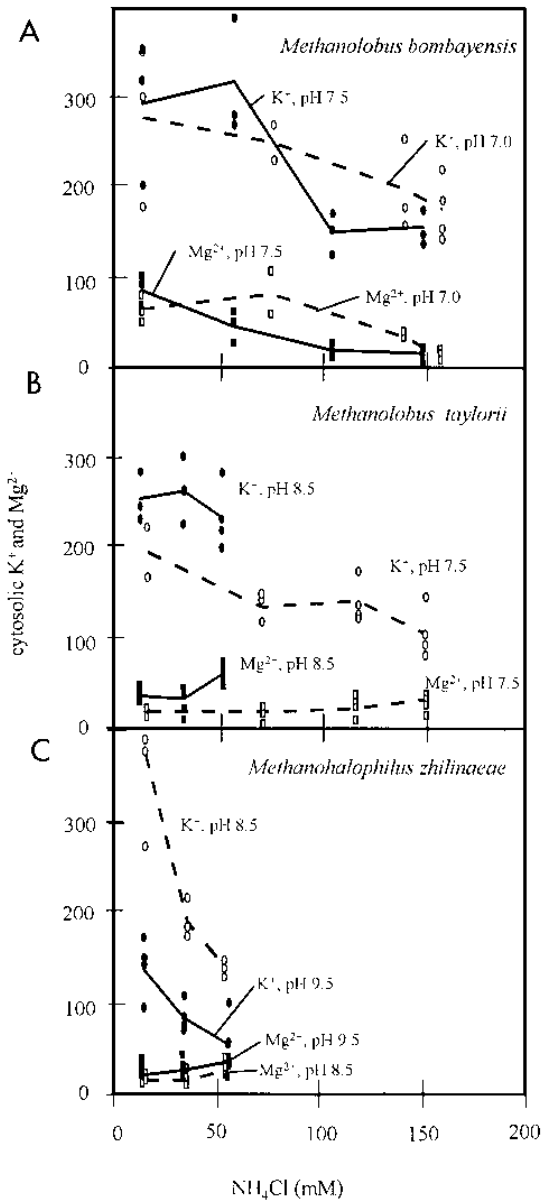

FIG. 3. Effects of external concentration of total ammonia on cytosolic concentrations of K^+ (circles) and Mg^{2+} (squares). Each methanogen was tested at two different pH values, *Methanolobus bombayensis* (A) at pH 7 and 7.5, *Methanolobus taylorii* (B) at pH 7.5 and 8.5, and *Methanohalophilus zhilinaeae* (C) at pH 8.5 and 9.5; in each case, the open symbols and dashed lines indicate data obtained at the lower pH and the filled symbols and solid lines indicate data obtained at the higher pH.

detectable in each strain but were low, except that the alanine dehydrogenase activity level was somewhat elevated in *Methanolobus taylorii* (Table 2). The glutamate dehydrogenase activity level in *Methanolobus bombayensis* was also low, but those in *Methanolobus taylorii* (150 mU) and *Methanohalophilus zhilinaeae* (400 mU) were much higher.

DISCUSSION

To understand the possible inhibitory mechanisms of ammonia, it is important to consider the chemical interaction of ammonia and cells. When a cell is exposed to an increased extracellular ammonia concentration, un-ionized ammonia equilibrates across the cell membrane, thereby increasing the cytosolic concentration of un-ionized ammonia. If the cytosolic pH remains constant, there is a proportional increase in the cytosolic NH_4^+ concentration as well. The protons absorbed by NH_3 molecules to become NH_4^+ can lead to the temporary alkalization of the cytosol, as occurs when *Vibrio alginolyticus* is exposed to 20 mM diethanolamine at pH 9.6 (29). In a well-buffered cytosol with small changes in external ammonia concentration, this reequilibration of intracellular NH_3 and NH_4^+ may not result in a significant change in internal pH (and thus in ΔpH). We found that the ΔpH of *Methanolobus taylorii* was the same at all concentrations of external total ammonia tested up to about 75 mM (Fig. 2B). Under these conditions, the cytosolic NH_4^+ concentration of this organism was as great as 180 mM, yet there was no significant change in ΔpH.

This lack of significant change in ΔpH can be explained by considering the effects of an increasing ΔpH on the activities of proton extrusion and H^+-dependent ATPase. Increased ΔpH would tend to increase the $\Delta_{\mu_{H^+}}$ (of which ΔpH is a component), and an increased $\Delta_{\mu_{H^+}}$ would energetically inhibit proton extrusion, resulting in a temporary net influx of protons. This net influx of protons would diminish the change in ΔpH and impose a corresponding decrease in $\Delta\psi$ until the energy in the $\Delta_{\mu_{H^+}}$ was reequilibrated. The $\Delta\psi$ may effect a redistribution of other cations such as K^+ across the cell membrane. Such a redistribution occurs in *V. alginolyticus*, which appears to contain an H^+/K^+ antiporter (29). Similarly, the methanogens at pH 7 to 7.5 accommodated increased environmental ammonia concentrations up to a given point by maintaining a relatively constant ΔpH (Fig. 2) and decreasing the cytosolic K^+ content (Fig. 3). At higher external ammonia concentrations, the ΔpH increased and concentrations of cytosolic K^+ decreased. Methanogens grown at higher external pHs allowed their ΔpHs as well as their intracellular ammonium concentrations to increase in response to growth at higher extracellular concentrations of total ammonia (Fig. 2). This response could alleviate sensitivity to ammonia, because if cells had not responded by increasing their ΔpHs they would have accumulated very high cytosolic NH_4^+ concentrations. For instance, *Methanolobus taylorii* growing at pH 7.5 would have a cytosolic NH_4^+ concentration of about 683 mM if it maintained its ΔpH at -0.5 during growth at its IC_{50} of total ammonia.

TABLE 2. Activities and pH ranges of ammonia-assimilating enzymes

Organism and activity detected	Sp act (mU/mg of protein)	pH optimum	pH range for activity[a]
Methanolobus bombayensis			
Glutamine synthetase	2	7	6.5–8.0
Glutamate dehydrogenase	2	7	6.5–7.5
Alanine dehydrogenase	2	6	5.5–7.5
Methanolobus taylorii			
Glutamine synthetase	2	7	6.5–8.0
Glutamate dehydrogenase	128	7	6.5–8.5
Alanine dehydrogenase	37	8	7.0–8.5
Methanohalophilus zhilinaeae			
Glutamine synthetase	1	7	6.0–8.5
Glutamate dehydrogenase	386	8	7.0–8.5
Alanine dehydrogenase	3	8.5	7.5–9.0

[a] The range of pH values at which the enzyme activity was at least one-third of the activity measured at the optimum pH value.

Other studies indicating that un-ionized ammonia is the toxic component of total ammonia were supported by our results for *Methanolobus bombayensis* and *Methanolobus taylorii*. These two species tolerated 2 to 5 mM un-ionized NH_3 regardless of external pH or NH_4^+ concentration (Table 1). These results suggest that growth inhibition by ammonia during anaerobic digestion can be controlled by adjustment of the pH. Similarly, un-ionized ammonia (NH_3) in the range of 2 to 5 mM inhibits by 50% the growth of other methanogenic isolates (18, 48) and ruminal bacteria (37), methanogenesis in anaerobic digestors (2, 7, 12, 23, 45), and oxygenic photosynthesis by pure cultures (5) and in oxidation ponds (1). However, photosynthesis by the alkaliphile *Spirulina platensis* is more tolerant of ammonia (50% inhibition at 8.5 mM NH_3) (5), and likewise the alkaliphile *Methanohalophilus zhilinaeae* tolerated even higher concentrations of un-ionized ammonia (IC_{50} of 13 mM at pH 8.5 and 45 mM at pH 9.5 [Fig. 1C]). *Methanohalophilus zhilinaeae* is the only methanogen tested whose IC_{50} of un-ionized ammonia was dependent on pH and was greater than 5 mM. That *Methanohalophilus zhilinaeae* was able to tolerate a much higher concentration of un-ionized ammonia during growth at pH 9.5 than during growth at pH 8.5 suggests that something other than un-ionized ammonia was toxic at pH 8.5. However, pH 8.5 is near the lower end of the range of pHs at which *Methanohalophilus zhilinaeae* grows (μ at pH 8.5 is 60% as high as that at 9.5 [9]), and it is possible that the inhibition of growth was due to the additive effects of independent toxicity of un-ionized ammonia and low pH. Another possible explanation for the toxic effects of total ammonia on *Methanohalophilus zhilinaeae* is the effects of the accumulated cytosolic NH_4^+. NH_4^+ accumulation was correlated with decreased cytosolic concentrations of potassium even though the high internal pH of this methanogen minimizes the accumulation of NH_4^+. Regardless of the growth pH, this methanogen had an extraordinary resistance to ammonia.

Some bacteria such as *Escherichia coli* (10) adjust their ΔpH depending on external pH, as a method for homeostatic control of cytosolic pH. Other organisms, such as various ruminal bacteria (41), maintain a constant ΔpH, allowing their cytosolic pH to change with external pH. Each of the three methanogens in this study, when grown in medium with a low ammonia concentration, behaved as the latter, maintaining a fixed ΔpH regardless of the external pH.

Three mechanisms of ammonia assimilation in methanogens have been demonstrated (15). At low ammonia concentrations (<1 mM), cells use glutamine synthetase to catalyze ATP-dependent synthesis of glutamine from ammonia and glutamate (36). Each of the three methanogens in this study had glutamine synthetase activity, but the level of activity was low, as expected, because the cells were all grown at high ammonia concentrations. *Methanolobus bombayensis* had low levels of each of the three ammonia-assimilating enzymes. *Methanolobus taylorii* had a moderate level of alanine dehydrogenase activity (40 mU) and a high level of glutamate dehydrogenase activity (150 mU), and *Methanohalophilus zhilinaeae* had a very high glutamate dehydrogenase activity (400 mU). The high levels of ammonia-assimilating activities of the latter two methanogens could be useful to the cells for the synthesis of nitrogen-containing compatible solutes such as glycine betaine (39) and α- and β-glutamate (24), which these two species accumulate in response to elevated external osmolarities. These two methanogens also had much larger deficits of cytosolic ions relative to the environmental concentrations of ions, and *Methanolobus bombayensis* may rely on the synthesis of compatible solutes less than these two strains do.

Cytosolic enzymes must be able to function at the pH of the cytosol. The pH optima for the three examined ammonia-assimilating enzymes were consistent with the observed ranges of intracellular pH: 6.0 to 7.0 for *Methanolobus bombayensis*, 7.0 to 8.0 for *Methanolobus taylorii*, and 7.0 to 8.5 for *Methanohalophilus zhilinaeae.*

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