Physiology of Halophilic, Methylotrophic Methanogens

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To my dear mother

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ABSTRACT

Physiology of Halophilic, Methylotrophic Methanogens.

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Several methanogens of the family *Methanosarcinaceae* were characterized phenotypically and phylogenetically. *Methanolobus bombayensis* and *Methanolobus vulcani* were characterized physiologically and taxonomically. The influence of ammonia on cytosolic pH and ammonia toxicity was studied for *Methanolobus bombayensis*, *Methanolobus taylorii* and *Methanohalophilus zhilinaeae*.

The first of these three methanogens, *Methanolobus bombayensis* B-1 (= OCM 438), was isolated from sediments of Arabian Sea near Bombay, India. This strain grew on methylamines, methanol, and dimethyl sulfide, but it did not catabolize H_2 + CO_2, acetate or formate. The cells were non motile, irregular coccoids (diameter, 1.0 to 1.5 μm) that occurred singly. Electron micrographs showed a cell membrane and a protein cell-wall. The cells grew fastest at mesophilic temperatures, neutral pH, and salinity near that of the ocean, but they required higher (30 mM) concentrations of divalent cations (Mg^{2+} and Ca^{2+}). The cells grew in mineral medium, but they were greatly stimulated by yeast extract and peptones. The guanine-plus-cytosine content of the DNA was 39.2 ± 0.1%. The comparison of 16S rRNA sequences showed that the strain B-1 was

phylogenetically related to *Methanolobus vulcani*, but the sequences of these organisms differed by 2%, indicating that they belong in separate species.

Methanolobus vulcani PL-12/M, was isolated by König, Stetter and Thomm. The characterization of this strain was rudimentary and thus it was further studied. It grew on methylamines and methanol but not on dimethyl sulfide, formate, acetate or $H_2 + CO_2$. The cells grew rapidly at mesophilic temperatures, at neutral pH (6 to 7.5), and in medium supplemented with 0.1 to 1.2 M NaCl and 13 mM Mg^{2+}. The cells grew in mineral medium plus biotin and catabolic substrate, but their growth was stimulated by yeast extract and peptones. *Methanolobus vulcani* was physiologically similar to *Methanolobus tindarius* and had a similar 16S rRNA sequence, although the results of DNA-DNA hybridization experiments indicate that these organisms should be considered separate species.

Three halophilic, methylotrophic, methanogens, *Methanolobus bombayensis*, *Methanolobus taylorii* and *Methanohalophilus zhilinaeae*, which grew at environmental pH ranges that overlapped with each other and spanned the pH range from 7.0-9.5, had reversed membrane pH gradients (ΔpH) at all pH values tested. The ΔpH of these three methanogens was in the range of -0.4 to -0.9 pH units, with cytosol more acidic than environment. *Methanohalophilus zhilinaeae* had the most negative ΔpH (-0.9 pH units). Consequence of these negative pH gradients was the accumulation of ammonium (NH_4^+), with cytosolic NH_4^+ concentration as high as 180 mM during growth. The high concentrations of cytosolic NH_4^+ were accompanied by higher ΔpH and lower concentrations of the major cytosolic cation K^+. *Methanolobus bombayensis* and *Methanolobus taylorii* were more sensitive to total external ammonia at higher external pH, but the inhibitory concentration of un-ionized ammonia that resulted in 50% reduction of growth rate (IC_{50}) was about 2-6 mM regardless of pH. This is consistent with ammonia inhibition of other bacteria. *Methanohalophilus zhilinaeae* on the contrary, was

more resistant to un-ionized ammonia than any other known organism. It had an IC_{50} for un-ionized ammonia of 13 mM at pH 8.5 and 45 mM at pH 9.5. We examined the effect 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.

1. BACKGROUND

The concept proposed by Woese and associates reclassified all living organisms in to three "domains" to include: eukaryotes, bacteria and archaeobacteria (110). The prokaryotic organisms are now considered to comprise two phylogenetically distinct groups: eubacteria and archaeobacteria. The eubacteria consist of the traditional bacterial groups including photosynthetic bacteria, blue-green bacteria, endospore-forming bacteria, actinomycetes and spirochetes, and on the other hand archaeobacteria consist of, methanogens, extreme halophiles and thermoacidophiles (Fig.1) (41). One group of the archaeobacteria, methanogenic bacteria, are considered a critical group of bacteria, because of their phylogenetic diversity and as the only producers of a hydrocarbon, methane.

Methanogens require anoxic conditions and highly reduced conditions for growth (97). These bacteria use carbon dioxide and or a methyl group as the terminal electron acceptor and produce methane as their catabolic end product. In spite of fastidious requirements, these bacteria are found in diverse habitats which are associated with decomposition of organic matter. They are found in bogs, anaerobic digestors, aquatic sediments, hydrothermal submarine vents and geothermal springs (14, 41, 105). In animals they are found in rumens of herbivores, mammalian intestines, human oral cavity, guts of insects (97). Over the years many methanogens have been isolated from various habitats. These methanogens have been identified and classified depending on their phenotypic and phylogenetic characteristics.

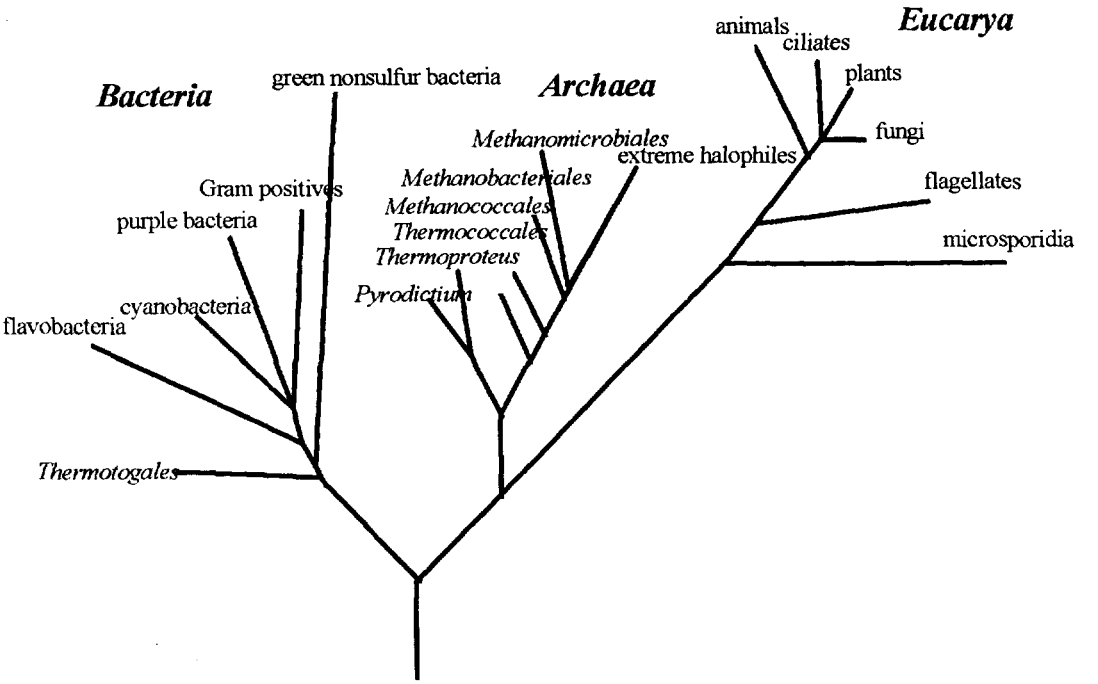

FIG. 1 : The universal phylogenetic tree (57).

Taxonomy of methanogens

Methanogens use only limited catabolic substrates such as: H_2 + CO_2, formate, acetate, methanol, methylamines, secondary and cyclic alcohols with CO_2, ethanol, methanol with H_2, dimethyl sulfide, methane thiol and pyruvate (97). Catabolic substrates and products are one of the important tools used for classification of other bacteria, but the limited catabolic range of methanogens leaves few easily determined phenotypic characteristics, requiring the use of other tools for classification; one such tool is genomic relatedness. Thus, the taxonomy of methanogens is strongly based on sequencing and cataloging of 16S rRNA. In addition, other phylogenetic methods have been applied such as: DNA/DNA hybridization (12), protein sequencing (70), sequencing gene for the methyl-coenzyme M reductase (*mcr*A) (98).

Table 1. Taxonomy of methylotrophic methanogens

Order / Family / Genus / Species
Methanobacteriales
Methanococcales
Methanomicrobiales
Methanosarcinales
Methanosarcinaceae
Methanosarcina
M. barkeri, M. mazeii, M. thermophila, M. acetivorans, M. vacuolata, M. siciliae
Methanolobus
M. tindarius, M. vulcani, M. taylorii, M. bombayensis, M. oregonensis
Methanococcoides
M. methylutens, M. burtonii
Methanohalophilus
M. mahii, M. halophilus
Methanosalsus
M. zhilinaeae
Methanohalobium
M. evestigatum
Methanosaetaceae
Methanopyrales

Based on 16S rRNA sequence data, methanogens are classified into five orders within the kingdom Archaeobacteria: *Methanobacteriales*, *Methanococcales*, *Methano–microbiales*, *Methanosarcinales* and *Methanopyrales* (Table 1) (14). The family *Methanosarcinaceae*, the subject of this dissertation, together with *Methanosaetaceae* make up the order *Methanosarcinales*. Members of *Methanosarcinaceae* are all methylotrophic, growing exclusively on methyl compounds such as methanol and methylamines. Some can grow on dimethyl sulfide or methane thiol and few others can also grow on acetate. Methanogens of this family are mesophilic or thermophilic, and habitats range from fresh water to saturated brines (14). Cells of *Methanosarcinaceae* are coccoid, occurring singly, in clusters, or in pseudosarcinal aggregates; their cell walls are often only protein (13). Based on phylogeny, the methanogens of *Methanosarcinaceae* form a coherent group. This phylogenetic classification was based on 16S rRNA sequences (Fig. 2) and confirmed by partial sequences of the genes encoding methyl-coenzyme M reductase (*mcr* I). The trees generated using these sequences were similar (98).

This family has six genera: *Methanosarcina*, *Methanolobus*, *Methanococcoides*, *Methanohalophilus*, *Methanosalsus* and *Methanohalobium* (14). The genus *Methanosarcina* contains organisms which grow on methylotrophic substrates and sometimes acetate or $H_2 + CO_2$. Except for *Methanosarcina siciliae* all the other methanogens in this genus are nonhalophilic. Members of the other genera are all halophilic and exclusively methylotrophic. *Methanohalophilus* is a genus of moderate halophiles and *Methanohalobium* has one extremely halophilic species (*Methanohalobium evestigatum*). The other three genera, *Methanococcoides*, *Methanolobus* and *Methanosalsus*, are slightly halophilic.

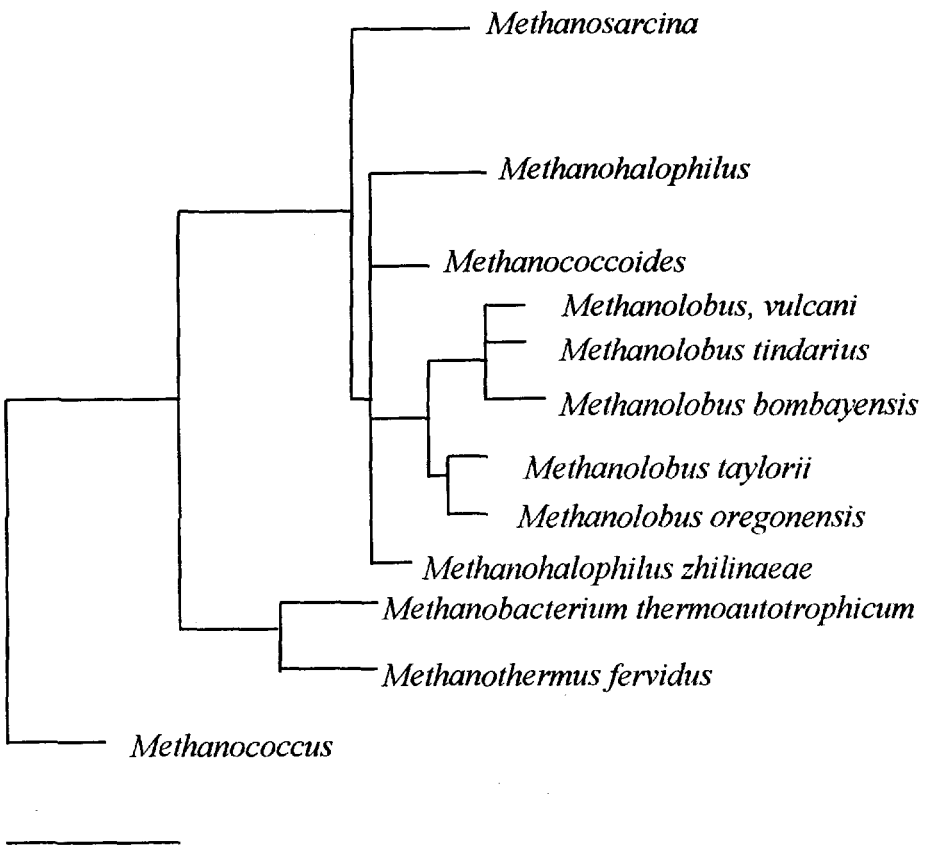

FIG.2 Fitch trees based on 16S rRNA sequences (98).

Methanogens belonging to genera *Methanolobus* and *Methanosalsus* were included in the present studies. Prior to this study *Methanolobus* had four species: *Methanolobus tindarius*, *Methanolobus vulcani*, *Methanolobus taylorii* and *Methanolobus oregonensis*. The strain B-1 which was isolated from the Arabian sea near Bombay, India was further characterized during this work and identified to be a new species and named as *Methanolobus bombayensis*. Of the species of *Methanolobus*, the

characterization of *Methanolobus vulcani* was most rudimentary, further characterization of this species was made a part of this study.

The second section of this dissertation examines the influence of pH on ammonia accumulation and toxicity, three methanogens with three different pH optima for growth were selected; *Methanolobus bombayensis*, *Methanolobus taylorii* and an alkaliphile *Methanohalophilus zhilinaeae* (Based on phylogeny, *Methanohalophilus zhilinaeae* belongs to a new genus, *Methanosalsus zhilinaeae*, but has not been formally transferred (14)).

Ecological range of methanogens

Methanogens occupy many diverse habitats. One of the purpose of this dissertation was to discover how methanogens can physiologically adapt to these environmental extremes. Various ecological factors such as availability of substrates, pH, temperature, salinity, and oxygen tolerance can influence the physiology of these bacteria. For instance, methanogens from marine habitat are mostly halophilic or halotolerant, likewise methanogens from hot springs are thermophilic (41).

Catabolic substrates

The most widespread catabolic pathways carried out by the methanogens include the reduction of CO_2. H_2 is the most common reductant, with H_2 being oxidized to H^+. Other substrates used to reduce CO_2 include, formate, primary and secondary alcohols. One genus, *Methanosphaera*, grows only by reducing methanol with H_2. These catabolic pathways are the only ones available to methanogens other than methanogens in the order *Methanosarcinales*. Methanogens in order *Methanosarcinales*, grow by catabolizing

methanol, methylamines and methylsulfides (41). Some species in this order are also able to grow with environmentally important acetate as a catabolic substrate (14).

Methanogens classified in the family *Methanosarcinaceae* utilize a broader range of catabolic substrates; many confined to methyl group containing compounds. These methanogens use methylated compounds such as methanol, methylamines (mono, di and tri methylamines) and methylated sulfides. In natural habitats methanol is probably not a major substrate and is derived in small amounts from compounds such as pectin (86). Methylamines are derived from compounds such as: choline (constituent of lipid lecithin), betaine (glycine betaine) and trimethylamine-*N*-oxide (25, 114). Betaine, which acts as a osmoprotectant, is often accumulated by cells of organisms growing in saline habitats. Trimethylamine-*N*-oxide, an osmolyte of fish, is reduced to trimethylamine by anaerobes in decaying fish and is a major volatile compound responsible for the characteristic odor of rotting fish. Thus, considerable amounts of these osmoprotectants can be expected to occur in saline and hypersaline environments with decomposing biomass. On the other hand, methylated sulfides are only minor methane precursors, and can be derived from thiomethyl group of methionine (115) or sulfonium osmoprotectants such as dimethylpropiothetin (47). Many halophilic methanogens found in saline environments use methylated compounds as their catabolic substrates (14).

Salinity

Methanogens are found in range of salinity from fresh water to hypersaline (14). All methanogens including those from fresh water require at least 1 mM Na^+, which is required for bioenergetics (44, 67). On the other hand, most of the marine methanogens require high levels of sodium chloride (about 0.5 M) for most rapid growth (14). The extreme halophile *Methanohalobium evestigatum* requires 4.3 M sodium chloride for optimum growth (113). Halophilic and extremely halophilic methanogens growing in such

high sodium chloride concentrations are known to accumulate cytosolic solutes to equalize the external and internal osmolarity. These cytosolic solutes (compatible solutes) may be either salts such as K^+ or organic compounds (55, 56, 95).

pH

Most methanogens grow at neutral pH (41). Methanogenic activity occurs at pH values as low as 4 and as high as 9.7. For instance, methanogenesis occurs in peat bogs having pH values of 4.0 (109), yet acidophilic methanogens (growing optimally below pH 5.0) have not been isolated. However, some species in the genus *Methanosarcina* grow fairly well at pH 4.5 although they also grow well at neutral pH (59). At the alkaline end of the pH spectrum, alkaliphilic methanogens occur in alkaline, saline lakes as well as other habitats with alkaline pH. For instance, *Methanohalophilus zhilinaeae* grows fastest at pH 9.2, and is isolated from a hypersaline lake in Egypt which has pH around 9.7 (61).

Temperature

Most of the methanogens are mesophilic, but there are methanogens growing at extremes of temperatures. Some methanogens grow at very high temperature such as *Methanopyrus kandler*, isolated from shallow marine hydrothermal vent grows at 100 °C (54). At the other end of temperature scale *Methanococcoides burtonii*, isolated from a lake in Antarctica grows fastest at 10 °C (32).

Autecology

Halophily

Bacteria growing in saline and hypersaline conditions respond to changes in extracellular solute concentration by the accumulation of low-molecular weight compounds or ions (compatible solutes). The main function of these solutes is to reduce intracellular water activity without perturbing protein function. These solutes are either ions, such as K^+, or organic compounds (20). Halophilic methanogens accumulate compatible solutes such as: K^+, α-glutamate, β-glutamate, β-glutamine, glycine-betaine, *N,N*-dimethylglycine and N^{ε}-acetyl-β-lysine (56, 82, 95, 96). The four methanogens studied in this dissertation (*Methanolobus bombayensis*, *Methanolobus vulcani*, *Methanolobus taylorii* and *Methanohalophilus zhilinaeae*) are halophiles and isolated from saline environments. In addition to being halophilic these methanogens have weak, protein cell envelopes (42, 43, 61, 73), which do not allow them to develop significant turgor pressure (62). These halophilic methanogens accumulate compatible solutes in order to match the osmolality of the cytosol with that of their environment. *Methanolobus taylorii* is known to accumulate α-glutamate, β-glutamine, N^{ε}-acetyl-β-lysine whereas *Methanohalophilus zhilinaeae* accumulates α-glutamate and glycine-betaine (62).

pH

Bacterial survival requires that the cytosolic pH is maintained within range that allows the activity of all necessary cytosolic enzymes. This limits the range of pH which some bacteria grow. Some bacteria are able to regulate their cytosolic pH within narrow limits while external pH varies over a wider range (16). This regulation of cytosolic pH implies control over the maintenance of a gradient of pH across the cell membrane (ΔpH).

This concept is complicated by the integral role ΔpH plays in the bioenergetics of organisms.

Bioenergetics

The proton translocating ATP synthase utilizes the proton motive force ($\Delta\mu_{H+}$) to drive the phosphorylation of ATP. The energy in $\Delta\mu_{H+}$ is the sum of the energies in two membrane gradients: the transmembrane chemical gradient of H^+ (ΔpH) and transmembrane electrical gradient ($\Delta\Psi$). This relation is expressed as (66):

$$\Delta\mu_{H+} = \Delta\Psi - 2.303\ RT/F \cdot \Delta pH$$

Where R = gas constant T = absolute temperature F = Faraday's constant.

pH homeostasis

There is substantial evidence that many bacteria maintain pH gradients with cytosolic pH more neutral than environmental pH. For instance, acidophiles have a cytosolic pH around 6.0 to 7.0 (positive ΔpH), neutrophiles have a cytosolic pH (pH 7.0 to 8.0, positive ΔpH) which is slightly alkaline than environmental pH. Some alkaliphiles maintain cytosolic pH of 8.5 to 9.0 (negative or 'reversed ΔpH) when growing at external pH which is high (5).

Many bacteria regulate their cytosolic pH within limited range while external pH varies over a wider range. For instance, when *Escherichia coli* grows at pH 7.8 it maintains cytosolic pH close to 7.8 and has ΔpH of 0. However, when it grows above or below environmental pH 7.8, the ΔpH changes to minimize the change in cytosolic pH (6, 75). Similarly, some methanogens such as, *Methanobacterium thermoautotrophicum* and

Methanospirillum hungateii maintain their cytosolic pH within narrow limits by adjusting their ΔpH (40).

Some bacteria do not adjust ΔpH. They maintain a constant ΔpH and allow their cytosolic pH to change (16). For example, the external pH of *Acetobacterium wieringae* (an acetogenic bacterium) decreased from 7.0 to 5.0 during growth, accordingly, the cytosolic pH changed from 7.1 to 5.5 and thus maintained fairly constant ΔpH (63). Likewise, in the present studies three methanogens (*Methanolobus bombayensis*, *Methanolobus taylorii* and *Methanohalophilus zhilinaeae*) maintained a constant ΔpH when grown at various external pH.

Temperature

Temperature is one of the most important environmental factors influencing the growth and survival of organisms. Bacteria growing at high temperatures must maintain structure and function of their macromolecules (proteins, nucleic acids, lipids, and other cellular components) (19). Many bacteria growing at high temperatures have thermostable proteins. (104). Similarly, methanogens growing at extreme temperatures have to adapt to their environment. Some methanogens produce 'heat shock' proteins (37) and others accumulate high concentrations of metabolites such as cyclic 2,3-diphosphoglycerate (36). Cyclic 2,3-diphosphoglycerate may play a role in thermostabilty, but this has not yet established (27).

Fatty acid and Ammonia toxicity

Anaerobic waste treatment comprises of three major stages: hydrolysis, acetogenesis, and methanogensis. Generally anaerobic biodegradation proceeds at its

optimum only in a narrow pH range (6.4 - 7.5). This is mainly because the methanogens flourish at this pH range and their growth is very sensitive to any change in medium pH. Under balanced digestion conditions the volatile fatty acids are used during methane formation and this helps to maintain pH range, 6.4 - 7.5. However, if imbalance develops then the acid former outpace the methanogens and volatile organic acids build up in the digestors. This usually leads to 'sour' digestors. Many methanogens are sensitive to fatty acids. The probable reason for fatty acid toxicity is the accumulation of fatty acids in cytosol.

The hydrophobic nature of cell membranes inhibits the distribution of ionized fatty acid (45). However, protonated form of fatty acids are unionized and equilibrate readily across the membrane. Usually, cells which maintain alkaline cytosol (positive ΔpH) will tend to accumulate fatty acids and this accumulation will be proportional to ΔpH. Thus acidophiles which maintain a strong ΔpH might be more susceptible to fatty acid toxicity.

Another frequent cause of digestor failure is ammonia toxicity. Ammonia is produced during degradation of proteinaceous material. Ammonia behaves analogously to fatty acids, except that the unprotonated form (NH_3) can diffuse through the membrane and the protonated form (NH_4^+) cannot. Cells growing at high pH usually maintain acidic cytosol (negative ΔpH) and consequence of 'reversed' ΔpH is the accumulation of NH_4^+. At very negative ΔpH more amount of NH_4^+ accumulates in the cytosol and may constitute the major fraction of the cytosolic cations. Thus at very high external pH the effect of ammonia accumulation on intracellular pH increases.

Most bacteria are sensitive to NH_3 and this sensitivity increases with external pH (107). Likewise, methanogens are sensitive to ammonia and this inhibitory effect of ammonia increases with pH (51). Similarly, in the present studies *Methanolobus bombayensis* and *Methanolobus taylorii* were sensitive to ammonia and tolerated about 2-

6 mM of NH_3. However, *Methanohalophilus zhilinaeae* was extraordinarily resistant to NH_3 and tolerated 45 mM of NH_3 at pH 9.5.

2. ISOLATION AND CHARACTERIZATION OF *Methanolobus bombayensis*, A METHYLOTROPHIC METHANOGEN THAT REQUIRES HIGH CONCENTRATIONS OF DIVALENT CATIONS

Results in this part have been presented in the following publication:

Kadam, P. C., D. R. Ranade, L.Mandelco, and D. R. Boone. Int. J. Syst. Bacteriol. (1994) 44:603-607.

INTRODUCTION

One of the salient physiological feature of the methanogens is their catabolic specialization. Methanogens use substrates such as H_2 + CO_2, acetate, methylamines. Some marine methanogens use either H_2 + CO_2 (14, 21, 24, 31, 41, 108) or acetate (93), but many marine isolates and nearly all isolates from hypersaline sources are obligately methylotrophic (12, 58, 61, 76, 112, 113), which grow exclusively on methyl compounds (such as methyl amines and methanol). Some of these obligate methylotrophs can also catabolize dimethyl sulfide and methane thiol (21, 30, 70, 74). *Methanosphaera* species, which grow by using H_2 to reduce methanol to methane, are not considered as obligate methylotrophs, nor are *Methanosarcina* species that catabolize acetate or H_2 + CO_2 as well as methylotrophic substrates.

The family *Methanosarcinaceae* contains all known halophilic, methylotrophic methanogens. These include extreme halophiles (in the genus *Methanohalobium*), moderate halophiles (in the genus *Methanohalophilus*), and slight halophiles (in the genera *Methanolobus*, *Methanococcoides* and one species of *Methanosarcina*, *Methanosarcina siciliae*). *Methanosarcina siciliae* is synonymous with *Methanolobus siciliae* (72). One more species was included in the genus *Methanolobus*, which was a slightly halophilic, methylotrophic methanogen that required high concentrations of divalent (Ca^{2+} or Mg^{2+}); *Methanolobus bombayensis* sp. nov. strain B-1.

MATERIALS AND METHODS

Source of inoculum.

Strain B-1 was isolated from the sediments of the Arabian Sea near Bombay, India. The sample was transported to the laboratory under anoxic conditions within 24 h.

Media and culture techniques.

Modifications (65) of the anaerobic techniques of Hungate (38) were used. The composition of SWM (Sea water medium) was (per liter): 1.0 g of Na_2SO_4, 30 g of NaCl, 0.7 g of KCl, 10 g of $MgCl_2 \cdot 6H_2O$, 1.0 g of $CaCl_2$, 0.2 g of $NaHCO_3$, 0.1 g of K_2HPO_4, 0.25 g of NH_4Cl, 2 mg of $Fe(NH_4)_2(SO_4)_2$, 2 g of yeast extract, 2 g of peptones, 1.0 mg of resazurin, and 10 ml of trace element solution (106). All the constituents except sulfide were dissolved in water, boiled and cooled under an O_2-free mixture of N_2 and CO_2 (4:1). The medium was distributed to serum bottles under the same gas mixture, then sealed and autoclaved (121°C, for 20 min). Sulfide from a sterile anoxic stock solution was added before inoculation. The pH of the medium was 7.2. For solid medium, 18 g of purified agar per liter was added.

For other experiments a modification of MSH medium (70), a bicarbonate-buffered medium containing yeast extract and peptones and with 29.2 g of NaCl per liter was used. The medium was modified by increasing its concentration of $MgCl_2 \cdot 6H_2O$ from 2.7 g to 6.1 g per liter. Methanol (50 mM) was added as catabolic substrate. To measure the effect of pH on growth, the pH of the medium was modified by adjusting the ratio of N_2 and CO_2 in the gas. Adjusting the fraction of CO_2 provided media with pH values between 6.8 (with 100% CO_2) and 8.0 (100% N_2). Media with pH values lower

than 6.8 were obtained from medium with 100% CO_2 by further adjusting the pH with HCl. Media with pH values higher than 8.0 were obtained from medium with 100% N_2 by adding NaOH. The pH drop during growth (due to CO_2 production) was small; the greatest pH decreases (0.25 pH units) occurred during growth in medium with an initial pH of 8.0.

Determination of catabolic substrates.

The use of catabolic substrates was tested by inoculating cultures into media with various catabolic substrates (20 mM of soluble substrates or 0.7 atm partial pressure of gaseous substrates) and monitoring methanogenesis. Methane formation was compared to controls lacking catabolic substrate, and cultures producing significant amounts of methane were transferred to fresh medium. The methanogenic rates of these cultures were evaluated to determine whether methanogenesis was accompanied by growth. Those not producing significantly more methane than controls lacking substrate were checked for methanogenesis after at least 4 weeks of incubation.

Determination of requirement for growth factors

Cultures were inoculated in mineral MSH medium which was MSH medium without the organic constituents (yeast extract, peptones and 2-mercaptoethanesulfonate). The growth was monitored on the basis of methanogenesis. In order to check the requirement of the organic constituents for growth, each of the following constituents were added to the mineral MSH medium and methanogenesis was monitored: 3 mM 2-mercaptoethanesulfonate, 4 mM sodium acetate, 5 mM glycine betaine, a vitamin mixture (106), 2 g per liter of peptone and 2 g per liter each of yeast extract and peptones.

Determination of growth rates.

The specific growth rates (μ) were calculated from the amount of methane produced during growth (13). The specific growth rate during exponential growth was analyzed by linear regression of the logarithm of total methane accumulated (including the methane produced by the inoculum (76) and time. Inocula were grown under conditions similar to experimental conditions.

Analytical technique.

Methane was measured by gas chromatography with a flame-ionization detector (58). The DNA was isolated by the method of Marmur (60) and its mole percent guanine-plus-cytosine was determined by high-performance liquid chromatography after enzymatic hydrolysis (64). Cells were examined microscopically with an epifluorescence microscope equipped with a type O2 filter set (Carl Zeiss Inc., Thornwood, N.Y.).

The reverse transcriptase method was used to sequence the 16S rRNA. Samples for electron microscopy were fixed in a cacodylate-buffered solution containing osmium tetroxide (10 g/liter) and glutaraldehyde (25 g/liter) (2); Mg^{2+} (30 mM) was included in all of the buffers.

RESULTS AND DISCUSSION

Isolation.

Sediment samples were inoculated into four bottles of SWM medium (2 ml of inoculum into 18 ml of medium). Two bottles had 50 mM acetate as catabolic substrate and two bottles had 50 mM methanol. After 30 days incubation at 35°C, the methanol cultures but not acetate cultures had produced methane. One methanol enrichment culture

was grown in medium with vancomycin ·HCl (0.1 g/liter) to decrease the proportion of non methanogenic contaminants, and this culture was inoculated into roll-tube medium for further purification. A well-isolated colony appearing after 2 weeks of incubation was picked and aseptically transferred to liquid medium under a stream of O_2-free N_2. This culture was diluted and reinoculated into roll-tube medium. The procedure was repeated until single type of colony was obtained. The resulting culture was axenic, based on: microscopic examination of wet mounts; the presence of a single colony type in roll tube medium; and the absence of growth in either SWM medium devoid of substrate or in nutrient broth with 10 g of glucose per liter. The strain B-1 was named and deposited in the Oregon Collection of Methanogens as OCM 438.

Colonial and cell morphology.

Surface colonies were circular, convex with entire margins, colorless, translucent, and 2 to 3 mm in diameter after incubation for 7-10 days. The cells were very irregular coccoids with a diameter of 1.0 to 1.5 μm. The cells were nonmotile, and thin section electron micrographs showed no flagella. The Gram-stain reaction was negative.

The cells lysed immediately when 0.1 g of sodium dodecylsulfate per liter was added, indicating a proteinaceous cell wall (14). Electron microscopy also showed an S-layer (Fig. 3). When suspended in a buffer of the same osmolality but without any $MgCl_2$ or $CaCl_2$ the cells became turgid and spherical. In *Methanococcus voltaei*, Ca^{2+} and Mg^{2+} stabilize the cell envelope and are tightly bound to the wall protein (41); whether they serve the same function in strain B-1 is unknown.

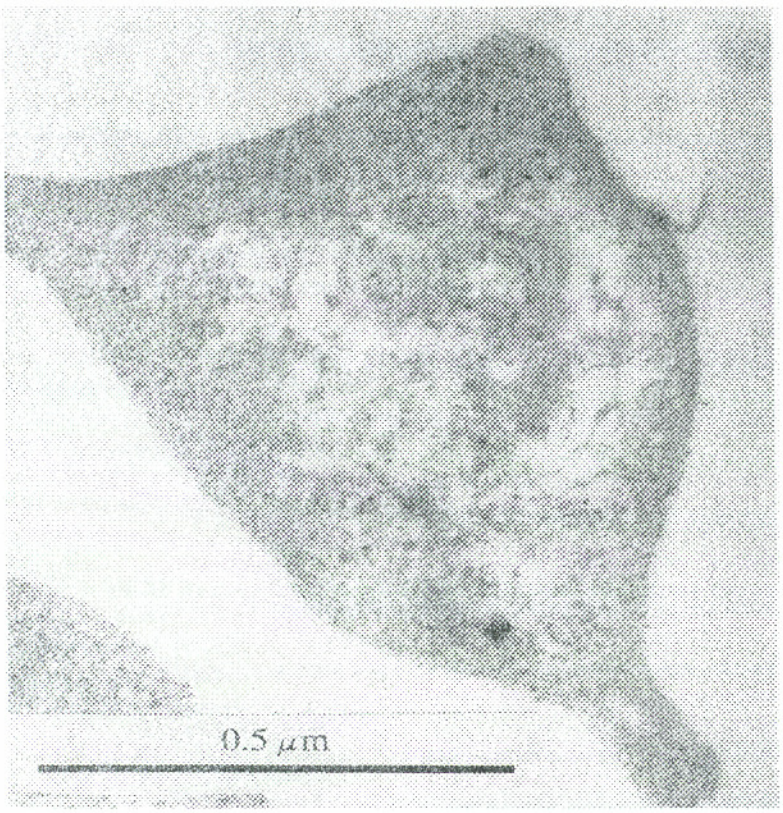

FIG. 3. Thin-section electron micrograph showing a coccoid cell grown in liquid medium containing 30 mM Mg^{2+}.

Methylotrophy and environmental factors for growth.

Physiological studies conducted with modified MSH medium and with SWM medium gave similar results. Strain B-1 catabolized methanol, trimethylamine, and dimethyl sulfide. Cultures with H_2 + 20 mM methanol produced the same amount of methane as those with 20 mM methanol only. Cultures did not use formate, acetate, or $H_2 + CO_2$.

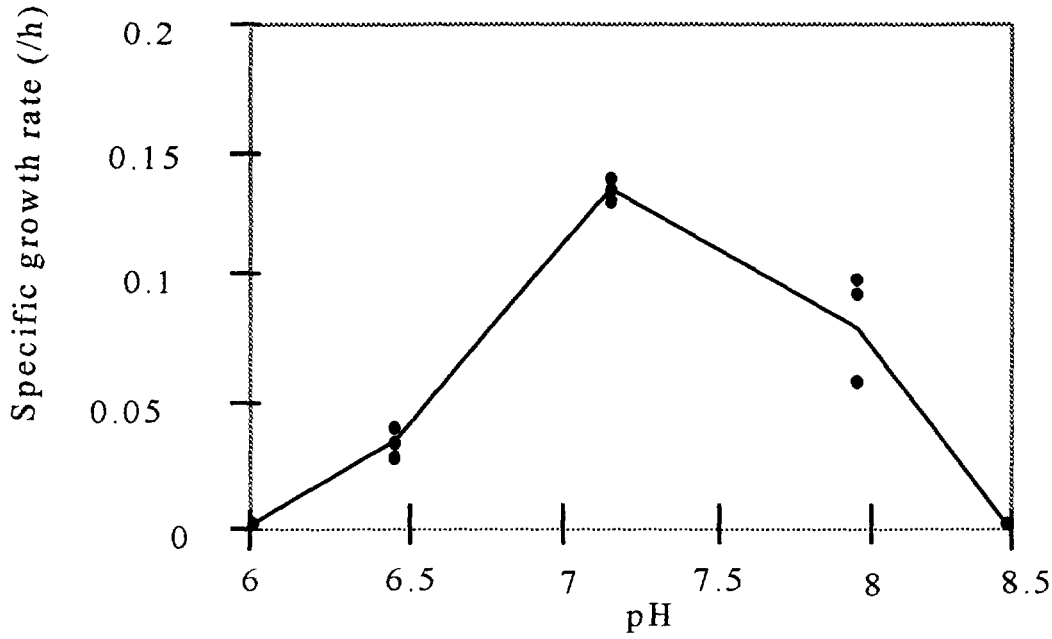

FIG. 4. Effect of initial pH on the specific growth rate of strain B-1. The results of three replicate measurements at each of five pH values are shown (some of the symbols overlap). The solid line connects the average.

Cells grew fastest near neutral pH values (Fig. 4) and growth was not detected below pH 6.5 and above 8.0. The range of salinity for strain B-1, was 0.1 M to 2.0 M and cell grew fastest at about 0.5 M NaCl (Fig. 5). As compared to other strains of *Methanolobus*, strain B-1 tolerated higher salt concentration (2 M NaCl).

The strain was mesophilic (Fig. 6). Analysis with the square-root model (49, 79) of the effect of temperature on specific growth rate indicated that cells could not grow at or below 13.2°C nor at or above 45.7°C, and that they could grow fastest at 36.6°C (Fig. 6). However, growth was not detected at 45°C.

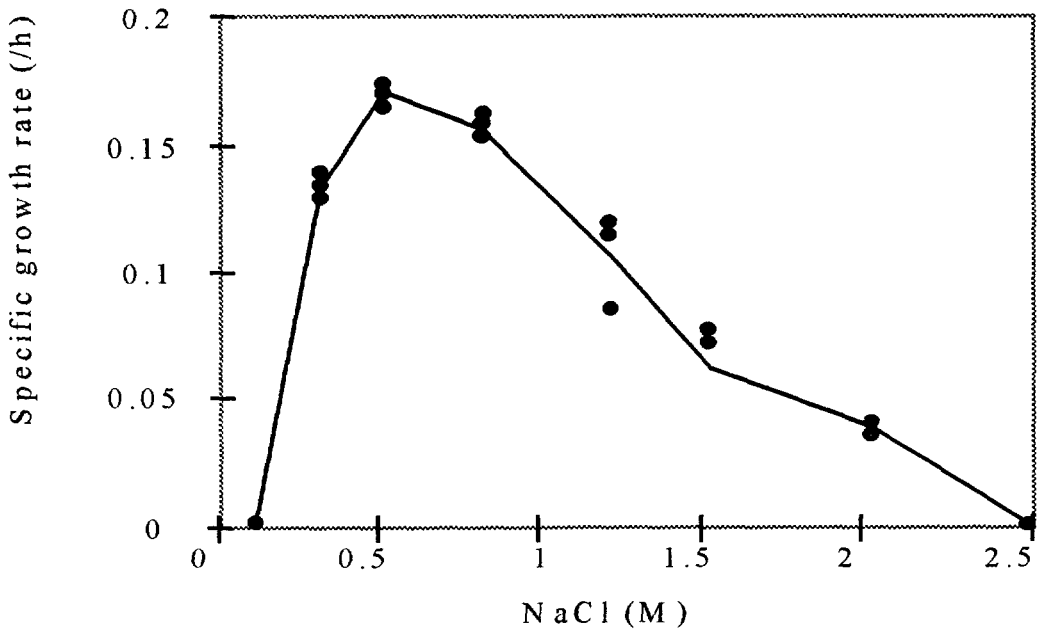

FIG. 5. Effect of NaCl on the specific growth rate of strain B-1. The results of three replicate measurements at each of eight levels of salinity are shown (some of the symbols overlap). The solid line connects the average growth rates at the levels of salinity tested.

Requirement for high concentration of Mg^{2+}.

Strain B-1 did not grow in MSH medium (with 13 mM Mg^{2+} and 3 mM Ca^{2+}) unless the concentration of Mg^{2+} or Ca^{2+} was increased. When Ca^{2+} in the medium was 3 mM, cells grew well with 30 mM to 85 mM Mg^{2+} but did not grow with 20 mM Mg^{2+}. Cells also grew in MSH medium when Mg^{2+} was kept at 13 mM if Ca^{2+} was increased to 20 mM. Although, species of the genera *Methanococcoides* and *Methanolobus* generally grow fastest in medium with 40 to 60 mM Mg^{2+} (50, 58, 70, 94), they differed from strain B-1 because they were able to grow in MSH medium with 13 mM Mg^{2+} and 3 mM Ca^{2+}.

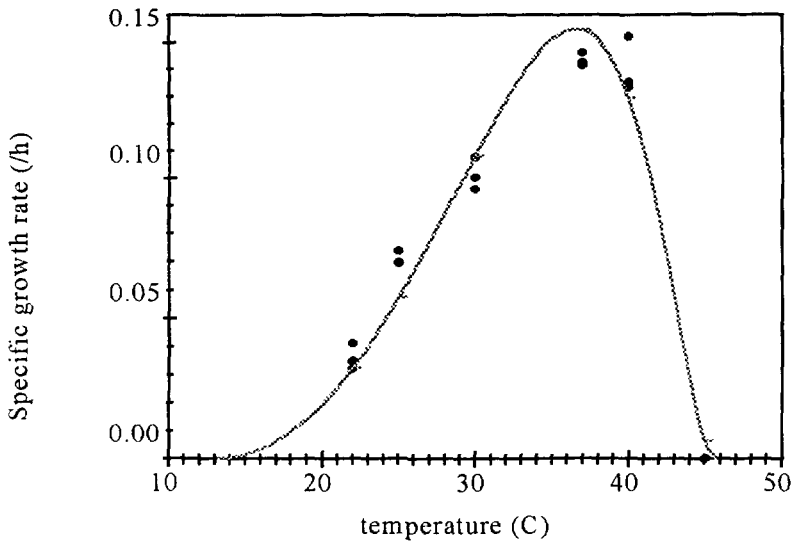

FIG. 6. Effect of temperature on the specific growth rate of strain B-1. The results of three replicate measurements at each of six temperatures are shown (some of the symbols overlap). The solid line is the best fit (49) as determined by the square root model (79).

Divalent cations have a significant role in biological systems and are known to maintain ultrastructure of prokaryotes. Elevated Mg^{2+} and Ca^{2+} concentrations cause the disaggregation of *Methanosarcina* species and *Methanolobus taylorii* GS-16 (=*Methanolobus* strain GS-16 (11, 73, 74). Also, these cations may increase the ability of sludge to settle in anaerobic waste treatment: packets of coccoidal cells similar to *Methanosarcina thermophila* increase in presence of Mg^{2+} in thermophilic upflow anaerobic sludge-blanket reactors (87). *Methanospirillum* respond to medium with low concentration of Ca^{2+} by growing as long non-flagellated filaments (29).

Requirement for growth factors.

Strain B-1 grew in mineral medium (μ = 0.050 h^{-1}) with methanol added as the catabolic substrate. However, it grew faster when other organic compounds were added, including 2-mercaptoethanesulfonate (μ = 0.094 h^{-1}), sodium acetate (μ = 0.106 h^{-1}), glycine betaine (μ = 0.108 h^{-1}), a vitamin mixture (2) (μ = 0.107 h^{-1}), or peptone (μ = 0.129 h^{-1}). Fastest growth occurred with each of yeast extract and peptones, (μ = 0.156 h^{-1}).

DNA analysis.

The guanine-plus-cytosine content of the DNA was 39.2 ± 0.1 mol%. The 16S rRNA sequence of strain B-1 was compared with the sequences of other methanogens, and found to be most similar to sequences of *Methanolobus* strains, especially to that of *Methanolobus vulcani*, to which it had a 2% difference.

Taxonomy

The physiology, morphology and 16S rRNA comparisons indicate that the strain B-1 belongs in the genus *Methanolobus* (Fig. 2). According to a previous classification, this genus included three species, *viz.*, *Methanolobus tindarius, Methanolobus vulcani* and *Methanolobus siciliae* (103). However, based on 16S rRNA sequences comparison and phenotypic characterization, *Methanolobus siciliae* was transferred to genus *Methanosarcina* as *Methanosarcina siciliae* (72). Those studies also indicated that strain GS-16 (74) belongs in the genus *Methanolobus*, and the name *Methanolobus taylorii* was later proposed (73). *Methanohalophilus oregonensis* also belongs in *Methanolobus*, and its transfer as *Methanolobus oregonensis* was suggested but not formally proposed (14).

Comparisons of 16S rRNA sequences to these and other methanogens indicated that strain B-1 was most similar to *Methanolobus vulcani* PL-12/M, but the sequences

were 2% different. A difference of 2% in 16S rRNA sequences suggests that strains belong in separate species (14). Strain B-1 differed from *Methanolobus vulcani* in some phenotypic characteristics. Unlike *Methanolobus vulcani* (69), strain B-1 required a high concentration of divalent ions. Strain B-1 was also slightly more alkaliphilic than *Methanolobus vulcani* PL-12/M strain B-1 grew well at pH 8 but did not grow at pH 6 (Fig. 4), whereas *Methanolobus vulcani* PL-12/M grew well at pH 6 but did not grow at pH 8 (43).

Strain B-1 also differed phenotypically from other species that belong in *Methanolobus*. Strain B-1 differed from *Methanohalophilus oregonensis* WAL1 (= "*Methanolobus oregonensis*") and *Methanolobus taylorii* GS-16 in that it could not grow at pH above 8. Strain B-1 grew in mineral medium with its catabolic substrate as the only organic addition, (as does *Methanolobus tindarius* (50), whereas *Methanohalophilus oregonensis* requires vitamins for its growth (58). These phylogenetic and phenotypic distinctions indicate that strain B-1 represents a new species of *Methanolobus*. The description of the new species, *Methanolobus bombayensis*, has been proposed (42)

***Methanolobus bombayensis* sp. nov.** *Methanolobus bombayensis* (bom.bay.enNsis. N. L. adj. bombayensis, from Bombay, indicating the source of the type strain). Cells are irregular, nonmotile, coccoids (diameter 1 to 1.5 μm) occurring singly. Stains gram negative. Lysed by 0.1 g of sodium dodecyl sulfate per liter. Surface colonies are circular, convex, translucent, colorless, with entire margins, and have a diameter of 2 to 3 mm after 7-10 days of incubation.

Catabolic substrates are methanol, trimethylamine, and dimethyl sulfide, but not formate, acetate, or $H_2 + CO_2$. Grows in mineral medium plus catabolic substrate; yeast extract and peptones are highly stimulatory.

Sodium and magnesium are required for growth. Fastest growth with 0.5 M Na^+, $\geq$ 0.03 M Mg^{2+}, pH 7.2, and 37°C.

The mole percent guanine-plus-cytosine of the DNA is 39.2 $\pm$ 0.1 (determined by chromatographic analysis of bases). The habitat is marine sediments. The type strain is strain B-1 (= OCM 438), which was isolated from sediments in the Arabian Sea, near Bombay, India.

3. PHYSIOLOGICAL CHARACTERIZATION AND EMENDED DESCRIPTION OF *Methanolobus vulcani*

Results in this part have been presented in the following publication:

Kadam, P. C., and D. R. Boone. Int. J. Syst. Bacteriol. (1995) **45:**400-402.

INTRODUCTION

Methanolobus is a genus of slightly halophilic, methylotrophic methanogens and in the earlier section a discussion on the characterization of new strain, *Methanolobus bombayensis* was discussed. In 1989 (103), this genus was recognized to contain three species, *Methanolobus tindarius* (50), *Methanolobus vulcani*, and *Methanolobus siciliae*, but since that time *Methanolobus siciliae* was transferred to the genus *Methanosarcina* as *Methanosarcina siciliae* (72). In addition to *Methanolobus vulcani* and *Methanolobus tindarius*, three other species should be classified as *Methanolobus*, based on their phylogeny as determined by 16S rRNA sequences (Fig. 2). These are *Methanolobus taylorii* [Oremland, 1994 #7489; *Methanolobus bombayensis* (42), and *Methanohalophilus oregonensis* (14, 58). Thus the genus *Methanolobus* has total five species and all five species have been phenotypically characterized, (14, 42, 50, 72-74). However, the characterization of *Methanolobus vulcani* (103) is rudimentary and no formal proposal has been made to transfer this latter species to the genus *Methanolobus* (14). Comparisons of partial sequences of methyl-coenzyme M-methylreductase genes confirm this group of five species as a phylogenetically coherent group (98). This section discusses the physiological characterization of *Methanolobus vulcani* and its emended description.

MATERIALS AND METHODS

Source of organism.

Methanolobus vulcani PL-12/M was isolated from sea sediments near Vulcano Island, Italy (103). For the present study *Methanolobus vulcani* PL-12/M was obtained from the Oregon Collection of Methanogens (OCM 157).

Media and culture techniques

Modifications (65) of the anaerobic techniques of Hungate (38) were used. $MSHCO_2$ medium (70), a bicarbonate-buffered medium containing yeast extract and peptones and with 29.2 g of NaCl per liter was used. Methanol (50 mM) was added as catabolic substrate. To measure the effect of pH on growth, the pH of the medium was modified by adjusting the ratio of N_2 and CO_2 in the gas. Adjusting the fraction of CO_2 provided media with pH values between 6.8 (with 100% CO_2) and 8.0 (100% N_2). Media with pH values lower than 6.8 were obtained from medium with 100% CO_2 by further adjusting the pH with HCl. Media with pH values higher than 8.0 were obtained from medium with 100% N_2 by adding NaOH.

Determination of catabolic substrates.

The use of catabolic substrates was tested by inoculating cultures into media with various catabolic substrates (20 mM of soluble substrates or 0.7 atm partial pressure of gaseous substrates) and monitoring methanogenesis. Methane formation was compared to controls lacking catabolic substrate, and cultures producing significant amounts of methane were transferred to fresh medium. The methanogenic rates of these cultures were evaluated to determine whether methanogenesis was accompanied by growth. Those not

producing significantly more methane than controls lacking substrate were checked for methanogenesis after at least 4 weeks of incubation.

Determination of growth rates.

The specific growth rate (μ) was determined by measuring the amount of methane formed during growth (13). The specific growth rate during exponential growth was analyzed by using the software package TableCurve (Jandel Scientific, San Rafael, Calif.) to fit to the Gompertz equation (33, 116) the total methane accumulated *versus* time. The modified Gompertz equation was as follows:

$$y = A \times \exp\{- \exp [(\mu_m \times e/A) \times (\lambda - t) + 1]\}$$

where y = logarithm of the number of organism as a function of time; μ_m = the maximum specific growth rate; λ = the lag time; t = time; the asymptote A = the maximal value of the number of organism (it is the asymptote value for the number of organisms reached as t approaches infinity) and e = the base unit for natural logarithms. Inoculum-produced methane was included in this calculation (77).

Determination of optimum temperature for growth

Specific growth rates at various temperatures were determined (Fig. 9) and were fitted (by using the TableCurve software) to the square-root equation (79):

$$\mu = \{b(T-T_{min}) \bullet c[1+\exp(T_{max}-T)]\}^2,$$

where μ is the specific growth rate, T is the temperature, T_{min} is the minimum temperature for growth, T_{max} is the maximum temperature for growth, and b and c are fitting parameters. The fitted curve obtained for *Methanolobus vulcani* is shown in Fig. 9, and the fitting parameters (T_{min}, T_{max}, b and c) are shown in Table 2.

Analytical technique.

Methane was measured by gas chromatography with flame-ionization detection (58). Cells were examined microscopically with an epifluorescence microscope equipped with a type O2 filter set (Carl Zeiss Inc., Thornwood, N.Y.). The reverse transcriptase method was used to sequence the 16S rRNA.

RESULTS AND DISCUSSION

Cell morphology

The cells were irregular coccoids with diameter of 1.0 to 1.25 μm when grown in $MSHCO_2$ medium plus 50 mM methanol. The cells lysed when 0.1 g of sodium dodecylsulfate per liter was added, indicating the presence of proteinaceous cell wall.

Environmental factors for growth

Methanolobus vulcani catabolized methanol and trimethylamine. The cultures did not catabolize H_2 either in the presence or absence of 50 mM methanol. Cultures also did not grow on formate, acetate, or dimethyl sulfide.

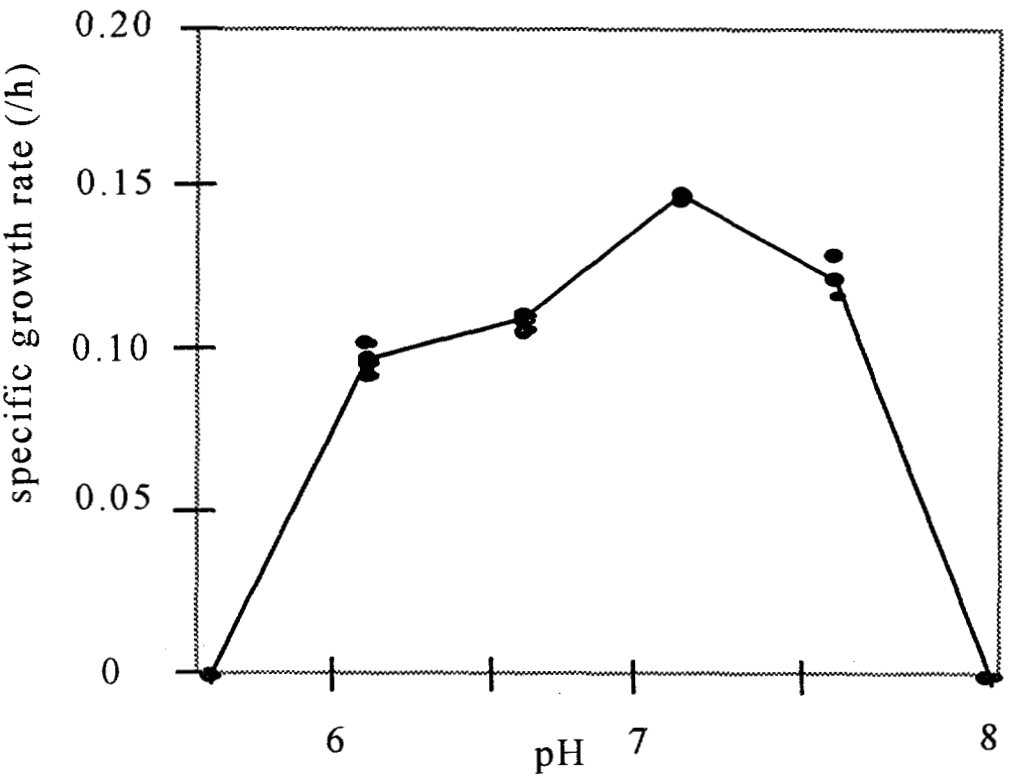

FIG. 7. Effect of pH on the specific growth rate of *Methanolobus vulcani*.

Cells grew fastest at neutral pH values (Fig.7), and did not grow below pH 6.0 and above pH 7.5. In contrast, most other species in this genus are alkaliphilic or alkalitolerant, growing well at pH values above 7.5 (Table 2). Cultures were slightly halophilic, growing fastest in the presence of 0.1 to 1.2 M NaCl (Fig. 8).

Although the T_{min} of *Methanolobus vulcani* was 13°C, the growth was not detected at temperatures even as high as 20°C (Fig. 9). However, cells grew fastest at 37 °C. Cells grew well in the presence of 13 mM of Mg^{2+} but grew poorly at Mg^{2+} concentrations below 0.5 mM or above 80 mM.

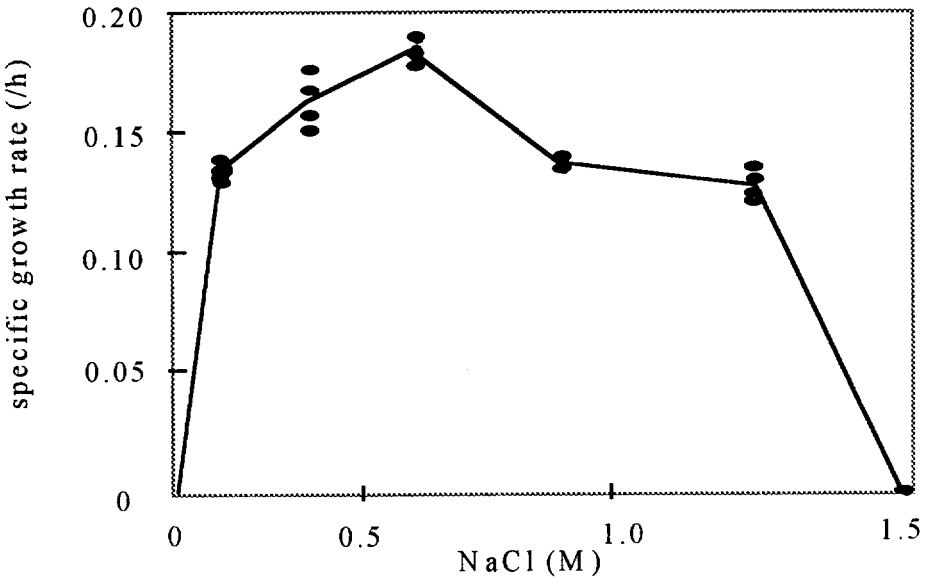

FIG. 8. Effect of salinity on the specific growth rate of *Methanolobus vulcani*.

Methanolobus vulcani did not grow in $MSHCO_2$ mineral medium ($MSHCO_2$ medium with organic constituents omitted) plus methanol, but it grew in this medium when biotin was added (specific growth rate = 0.09 h^{-1}). Growth was fastest (0.13 h^{-1}) with 2 g of yeast extract and 2 g of peptone per liter.

Taxonomy

The placement of *Methanolobus vulcani* in the genus *Methanolobus* together with *Methanolobus tindarius*, *Methanolobus taylorii*, *Methanolobus bombayensis*, and *Methanohalophilus oregonensis* is consistent with phylogenetic relationships and phenotypic characteristics (Table 2). Among these species, *Methanolobus vulcani* is most closely related to *Methanolobus tindarius*.

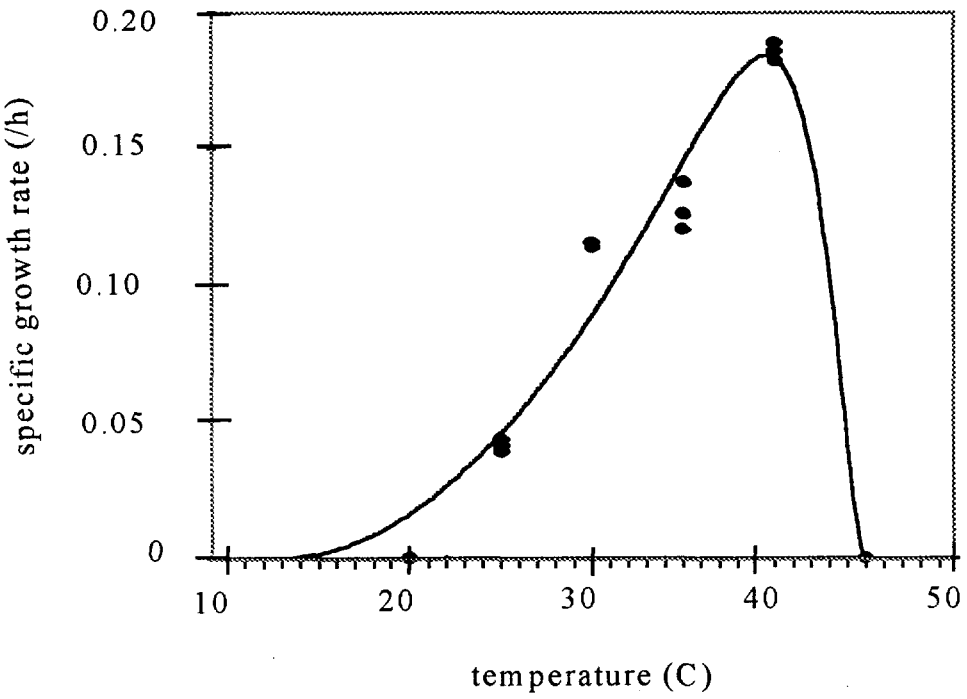

FIG. 9. Effect of temperature on the specific growth rate of *Methanolobus vulcani*.

The most significant phenotypic differences were that *Methanolobus vulcani* required biotin and had slightly higher temperature and pH optima. The percent difference of the 16S rRNA sequences of these two species is 1.6% (Fig. 2), a magnitude of difference that is found between pairs of closely related species or between strains classified together within a single species (14, 28). Based on these physiological and phylogenetic similarities, some might consider these two species to be synonymous. However, stability of taxonomy should be preserved unless changes are well supported, and we continue to consider these as separate species. The low DNA-DNA reassociation value of 29% between these two species (103) also indicates these species should remain separate.

Methanolobus vulcani has greater phylogenetic and phenotypic differences from the other organisms listed in Table 2. Differences in 16S rRNA sequences between *Methanolobus vulcani* and these species are 2% or greater. *Methanolobus vulcani* differs from *Methanohalophilus oregonensis* and *Methanolobus taylorii* by lacking alkalitolerance and by not catabolizing dimethyl sulfide. *Methanolobus vulcani* differs from *Methanolobus bombayensis* in its ability to grow without high concentrations of divalent cations.

Emended description of *Methanolobus vulcani.*

Methanolobus vulcani Stetter, König and Thomm 1989. (vul.ca'ni. L. gen. n. vulcani from Vulcan, the god after whom Vulcano Island was named (insula Vulcani). Irregulary coccoid, Gram negative, nonmotile. Lysed by 0.1 g of sodium dodecylsulfate per liter. Grows on methanol, or methylamines, but not dimethyl sulfide, formate, acetate, or $H_2 + CO_2$. Biotin and catabolic substrate are the only organic compounds required for growth; yeast extract and peptone are stimulatory. Grows well at 37 °C, pH 7.2, and with 0.5 M NaCl and 13 mM Mg^{2+}. The guanine-plus-cytosine content of the DNA is 39 mol%. The type strain PL-12/M (OCM=157). Habitat: Sea sediments near Vulcano Island, Italy.

Table 2. Characteristics of species of *Methanolobus* and *Methanohalophilus oregonensis*.

Species	Type strain	Other designations[a]	G+C content (mol%)	Use of dimethyl sulfide	Growth factor(s)	T_{min} (°C)[b]	T_{opt} (°C)[b]	T_{max} (°C)[b]	b[b]	c[b]	Optimum pH	pH range[c]	Optimum Na^+ concn (mM)	Na^+ concn range (mM)[c]	Optimum Mg^{2+} concn (mM)	Mg^{2+} concn range (mM)[c]	% 16S rRNA difference[d]
Methanolobus tindarius	Tindari 3	OCM 150, DSM 2278	46	–	None	7	28	50	0.264	0.0039	6.5	5.5–8.0	490	60–1,270	ND	ND	1.61
Methanolobus vulcani	PL-12/M	OCM 157, DSM 3029	39	–	Biotin	13	40	45	0.0167	0.487	7.2	6.0–7.5	500	100–1,200	13	0.5–85	
Methanolobus taylorii	GS-16	OCM 58, DSM 9005	41	+	Biotin	11	39	40	0.0157	3.01	8	7.0–8.6	600	200–1,200	40	13–40	2.28
Methanolobus bombayensis	B-1	OCM 438, DSM 7082	39	+	None	13	37	46	0.0208	0.182	7.2	6.5–8.0	500	300–2,000	33	30–80	2.13
Methanohalophilus oregonensis	WALI	OCM 99, DSM 5435	41	+	Biotin, thiamine	7	34	44	−0.0120	0.182	8.6	7.4–9.5	500	100–1,600	40	13–220	3.13

[a] OCM, Oregon Collection of Methanogens, Oregon Graduate Institute of Science & Technology, Portland; DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany.

[b] T_{min}, T_{opt}, and T_{max} are the minimum, optimum, and maximum growth temperatures, respectively, and b and c are the two other fitting parameters used in the square-root equation (when the units growth rate are $hour^{-1}$).

[c] Range at which the specific growth rate was at least 10% of the growth rate under optimal conditions.

[d] Calculated by comparing 16S rRNA sequences with the *Methanolobus vulcani* 16S rRNA sequence. Sequence data were obtained from the Ribosomal Database Project

4. INFLUENCE OF pH ON AMMONIA ACCUMULATION AND TOXICITY IN HALOPHILIC, METHYLOTROPHIC METHANOGENS

Portions of these results were submitted in an abstract to the 96th General Meeting of the American Society for Microbiology, New Orleans, Louisiana , 1996.

Results in this part have been submitted for publication:

Kadam, P. C., and D. R. Boone. Appl. Environ. Microbiol. (1996).

Introduction

Ammonia toxicity in methanogens

Ammonia can inhibit microbes in anaerobic digestors and other anaerobic water-treatment processes (3, 53). Often in anaerobic digestion methanogens are considered to be more sensitive to ammonia than other microbes (51, 52). This sensitivity to ammonia is attributed to the unprotonated form of ammonia (NH_3) which diffuses readily across cell membranes, equilibrating the intracellular and extracellular concentrations of NH_3 (48). On the other hand, ammonium (NH_4^+) ion does not readily diffuse through lipid membranes (Fig. 10). Although NH_4^+ permeases have been postulated (48), these could not function in the presence of substantial concentrations of ammonia without establishing a futile cycle that would dissipate the proton motive force ($\Delta\mu_{H+}$) (22). The intracellular and extracellular concentrations of NH_4^+ are dependent on NH_3 concentration and pH (pK_a = 9.24) (17). Thus, cells whose intracellular pH is lower than the extracellular pH ('reversed' ΔpH) have an intracellular NH_4^+ concentration greater than that of their environment. In cells with very negative ΔpH, cytosolic NH_4^+ may constitute a considerable fraction of the intracellular cations (100). 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.

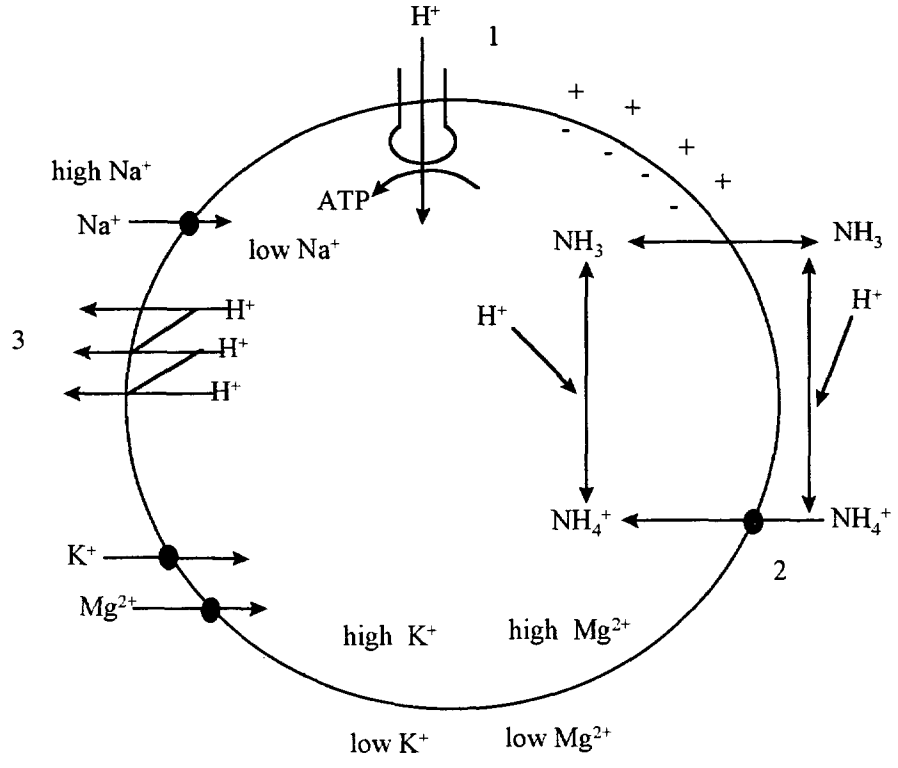

FIG. 10. Diffusion of NH_3 and distribution of cations such as H^+, Na^+, NH_4^+ K^+ and Mg^{2+} across the cell membrane. 1. ATP synthase; 2. Carrier mediated transport; 3. Electron transport chain.

At least two possible mechanisms of ammonia toxicity have been postulated: (i) un-ionized ammonia may directly inhibit the activity of cytosolic enzymes, or (ii) accumulated NH_4^+ inside cells might be toxic by its effect on intracellular pH (101) or the concentration of other cations such as K^+ (100). Either way, high pH and high total ammonia concentration may exert their toxicity 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 higher pH establish a more negative ΔpH to maintain

near-neutral cytosol, then the potential toxicity due to NH_4^+ accumulation would also be greater.

So far the work on inhibitory effect of ammonia is limited to either hydrogen utilizing or acetate utilizing methanogens (10, 26, 51, 92, 100-102). In the following section three halophilic, methylotrophic methanogens of the family *Methanosarcinaceae* were adapted to a range of external pH values, and the effect of this adaptation on the toxicity of ammonia was studied.

Materials and Methods

Source of bacterial strains.

The following methanogenic strains were obtained from the Oregon Collection of Methanogens (OCM; P.O. Box 91000, Portland, OR 97291-1000): *Methanolobus taylorii* GS-16 (OCM 58) (73), *Methanolobus bombayensis* B-1 (OCM 438) (42) and *Methanohalophilus zhilinaeae* WeN5 (OCM 62) (61) (= "*Methanosalsus zhilinaeae*" (14)).

Media and culture conditions.

Modifications (65) of the anaerobic techniques of Hungate (38) were used. Cells were grown statically at 40°C in MSH medium 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 (70). For growth of *Methanolobus bombayensis*, the concentration of $MgCl_2 \cdot 6H_2O$ was increased from 2.7 g to 6.1 g per liter. For growth of *Methanolobus taylorii* and *Methanohalophilus zhilinaeae*, $CaCl_2 \cdot 2H_2O$ was omitted to avoid

precipitates at high pH. 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 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. The data of measured methane production was fit to the Gompertz equation in order to determine the most rapid growth rate of batch cultures (33, 116). The methane produced by the inoculum was also included in the calculations (77).

Ammonia inhibition of growth rate.

The concentration of ammonia that inhibits the specific growth rate by 50% (IC_{50}) was estimated by measuring the specific growth rate 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, over 35,000 equations (TableCurve, Jandel Scientific Co., San Rafael, Calif.) were fit 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 growth rate decreased linearly with the inhibitor concentration above that value. Based on these observations, a linear equation was designed that described this relationship, with three fitting parameters: a, the uninhibited specific growth rate; 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:

$$\text{If } x < c_1,\ \mu = a;\ \text{if } c_1 < x < c_2,\ \mu = a/((c_2-c_1)(c_2-x));\ \text{if } c_2 < x,\ \mu = 0$$

Based on this equation, the $IC_{50} = 0.5 \times (c_1 + c_2)$. The value of IC_{50} calculated by this equation was similar to the IC_{50} calculated by the above cubic equation but the linear equation for data analysis was used because it fitted all the data sets better than the cubic equation.

Collection of cells.

Cells in the late exponential phase were anoxically harvested by centrifugation at 10,000 × *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 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 weighed and its volume was determined picnometrically (62). 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 (7) with cellobiose dehydrogenase after cells were lysed by suspension in distilled water.

Preparation of the 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 dodecylsulfate solution (1 g $liter^{-1}$) per gram of pellet. The cell lysate was centrifuged at 14,000 × *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 free extracts for enzyme assays.

Cells were grown at their optimum pH and growth conditions and harvested anoxically. 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. 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) (Sonifier model SA520 with double-step microtip, Danbury, Conn.). The lysed cell suspension was centrifuged at 10,000 × 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 Units 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 (ε_{340n} = 6.22 mM^{-1} cm^{-1}). The assay mixture

contained: Trizma, pH 7, 84 mM; NH_4Cl, 231 mM; sodium α-ketoglutarate, 6.75 mM; and β-NADPH, 0.225 mM (46).

Glutamine synthetase activity was assayed by modifications (9) of the γ-glutamyltransferase method (89). 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 0.532 units of absorbance at 540 nm.

Alanine dehydrogenase activity was detected by the oxidation of NADH at 340 nm with sodium pyruvate as the substrate ($\varepsilon_{340n} = 6.22\ mM^{-1}\ cm^{-1}$). The assay mixture contained: Trizma, pH 7, 87.5 mM; NH_4Cl, 99 mM; sodium pyruvate, 5 mM; and β-NADH, 0.296 mM (78).

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 four buffers were used to obtain various pH values: sodium acetate at pH 5; dibasic potassium phosphate at pH 5, 6, and 6.5; Trizma at pH of 6.5 to 9 in increments of 0.5 pH units; and 2-N-cyclohexylaminoethanesulfonate at pH 9, 10, and 11. Enzyme activity was determined at a pH value 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 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 (58). Ammonia was quantified by estimating the ammonia nitrogen by flow injection and gas diffusion. This estimation was done using 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 (91) with bovine serum albumen as a standard. The absorbance was measured at 562 nm.

Determination of intracellular pH.

The intracellular pH was calculated from the following equation (84): $(pH)_{in} = \log[(\text{total ammonia})_{in}/(\text{total ammonia})_{out}\ (10^{pK}+10^{pHout})-10^{pK}]$. The uptake of benzoate by cells was also tested this was done by incubating them in the presence of uniformly labeled ^{14}C-benzoate (1.7 μM, 2.2 mCi liter^{-1}) and measuring the radioactivity in cell pellets.

Results

Effect of ammonia on specific growth rate.

Three methylotrophic, halophilic methanogens which had various pH optima for growth were selected: *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 7-9.5. The effect of ammonia on growth and intracellular cation concentrations of each of these three methanogens each at two pH values was studied.

Table 3. The IC_{50} value for ammonia.

	IC_{50} (total ammonia) (mM)	NH_3 concentration at IC_{50} (mM)
Methanolobus bombayensis		
pH 7.0	295	1.69
pH 7.5	215	3.84
Methanolobus taylorii		
pH 7.5	220	3.93
pH 8.5	39	6.01
Methanohalophilus zhilinaeae		
pH 8.5	86	13.2
pH 9.5	81	45.1

Methanolobus bombayensis was least sensitive to ammonia, having an IC_{50} of 295 mM total ammonia during growth at pH 7.0 (Fig. 11a). Cells were only slightly more sensitive when grown at a higher pH value (IC_{50} = 215 mM total ammonia during growth at pH 7.5) (Table. 3). 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. 11b). *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. 11c) (Table 3).

At very low initial ammonia concentrations (< 5 mM) μ was sometimes lower than at slightly higher ammonia concentrations (Fig. 11). This was probably due to nitrogen limitations, and these data 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 ammonia inhibition. For example, cells growing in the presence of 20 mM trimethylamine had an IC_{50} for 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.

Effect of external ammonia concentration on ΔpH and cytosolic NH_4^+.

Under all conditions of growth tested in this study, the cells maintained negative ΔpH. The ΔpH couldn't be accurately measured by using ^{14}C-labeled benzoic acid because cytosolic benzoate concentrations were lower than external benzoate concentration. This is consistent with the possession of a negative ΔpH (16). On the other hand, methylammonium and ammonium are accumulated by cells with a negative ΔpH, and accumulation of these ions is often used to measure reversed pH gradients (16, 23).

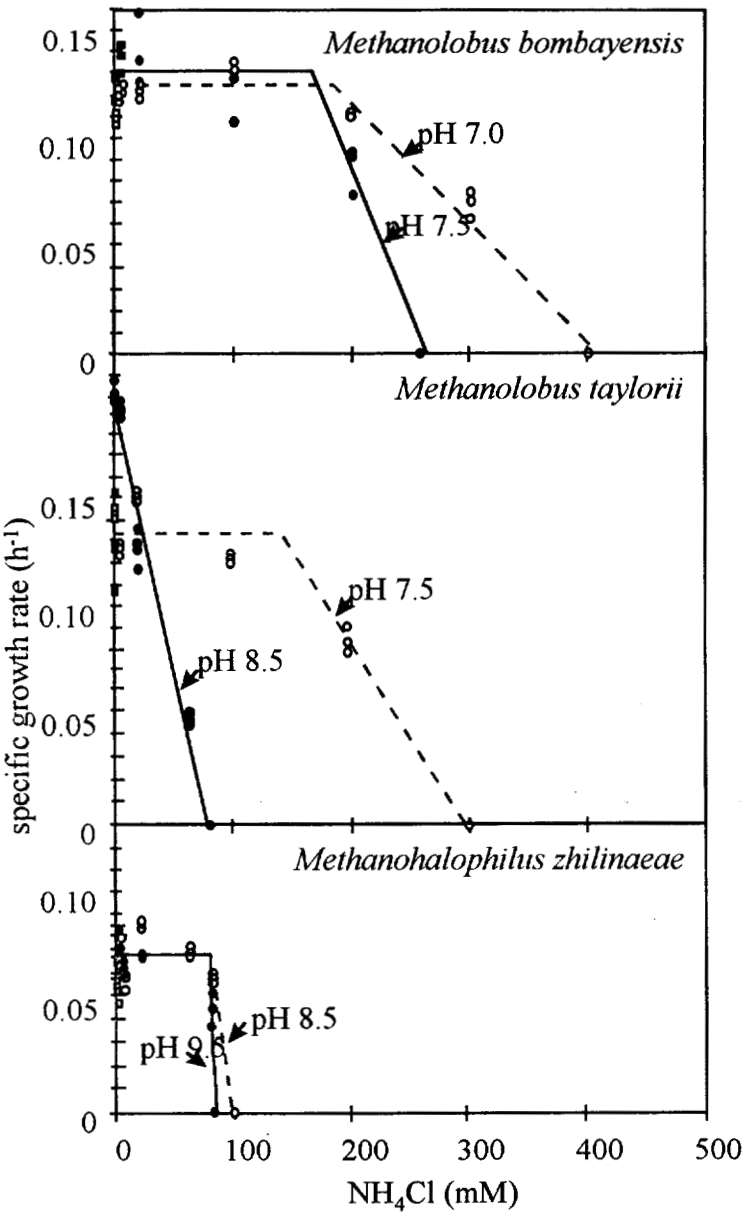

FIG. 11. Effect of external concentration of total ammonia on specific growth rates of three methanogens. Each methanogen was tested at two different pH values, *Methanolobus bombayensis* at pH 7 and 7.5, *Methanolobus taylorii* at pH 7.5 and 8.5, and *Methanohalophilus zhilinaeae* at pH 8.5 and 9.5; in each case the open symbols and dashed lines indicate data at the lower pH and the filled symbols and solid lines indicate data at the higher pH value. The squares indicate data at low ammonia concentrations that were not used in the fitting of the lines.

The cells were grown in the presence of various concentrations of ammonia and the cytosolic content of total ammonia was determined. Each of the three methanogens accumulated ammonium, indicating that they had a negative ΔpH, with cytosol more acidic than their environment (Fig. 12).

In medium with 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. 12).

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 ammonia 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, 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. 12b). 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. 12b). 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

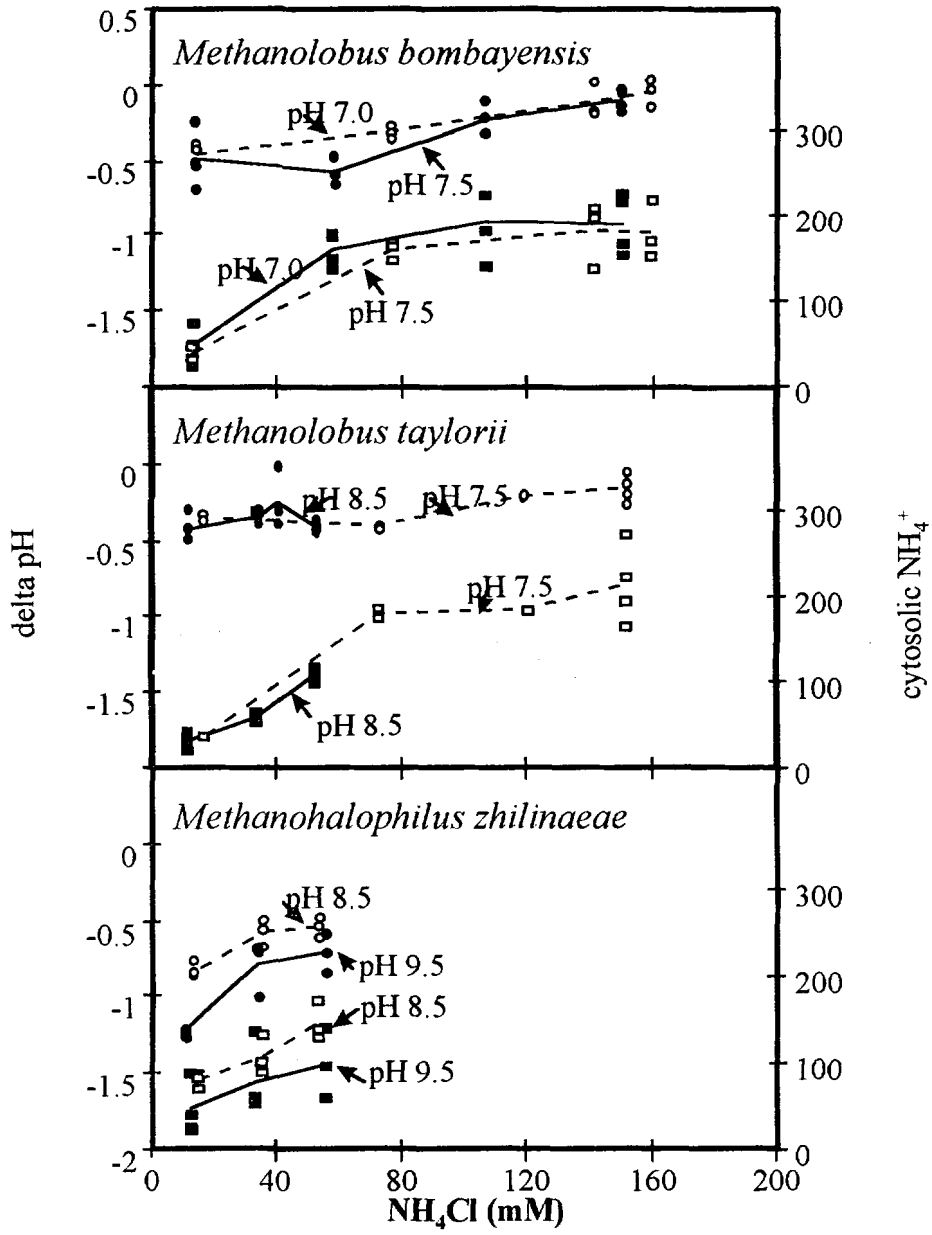

FIG. 12. Effect of external concentration of total ammonia on cytosolic NH_4^+concentration (squares) and ΔpH (circles). Each methanogen was tested at two different pH values, *Methanolobus bombayensis* at pH 7 and 7.5, *Methanolobus taylorii* at pH 7.5 and 8.5, and *Methanohalophilus zhilinaeae* at pH 8.5 and 9.5; in each case the open symbols and dashed lines indicate data at the lower pH and the filled symbols and solid lines indicate data at the higher pH value.

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 (101). The cytosolic concentrations of Na^+, K^+, and Mg^{2+} in cells grown at various concentrations of ammonia was determined. 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. Cytosolic Na^+ in other halophilic methanogens in the family *Methanosarcinaceae* is also very low (62).

K^+ is often a major cytosolic cation, and it is reported to play a role in pH homeostasis and osmoregulation of cells (4), including *Methanolobus taylorii* (71). All three methanogens accumulated K^+, but they decreased their cytosolic K^+ concentration when grown at high concentrations of external total ammonia (Fig. 13).

Mg^{2+} is an important ion in the action of many enzymes that catalyze ATP-dependent reactions (90) and it also may function to stabilize proteins in the presence of denaturing concentrations of ammonia. The cells of all these three methanogens accumulated Mg^{2+} (Fig. 13). Figure 13 shows that the cytosolic Mg^{2+} content of *Methanolobus bombayensis* was decreased when external ammonia concentration was high. The requirements of this organism for high external Mg^{2+} concentration may therefore be related to its sensitivity to ammonia. In contrast, the Mg^{2+} content of *Methanolobus taylorii* and *Methanohalophilus zhilinaeae* was elevated when external ammonia concentration were high (Fig. 13b and 13c).

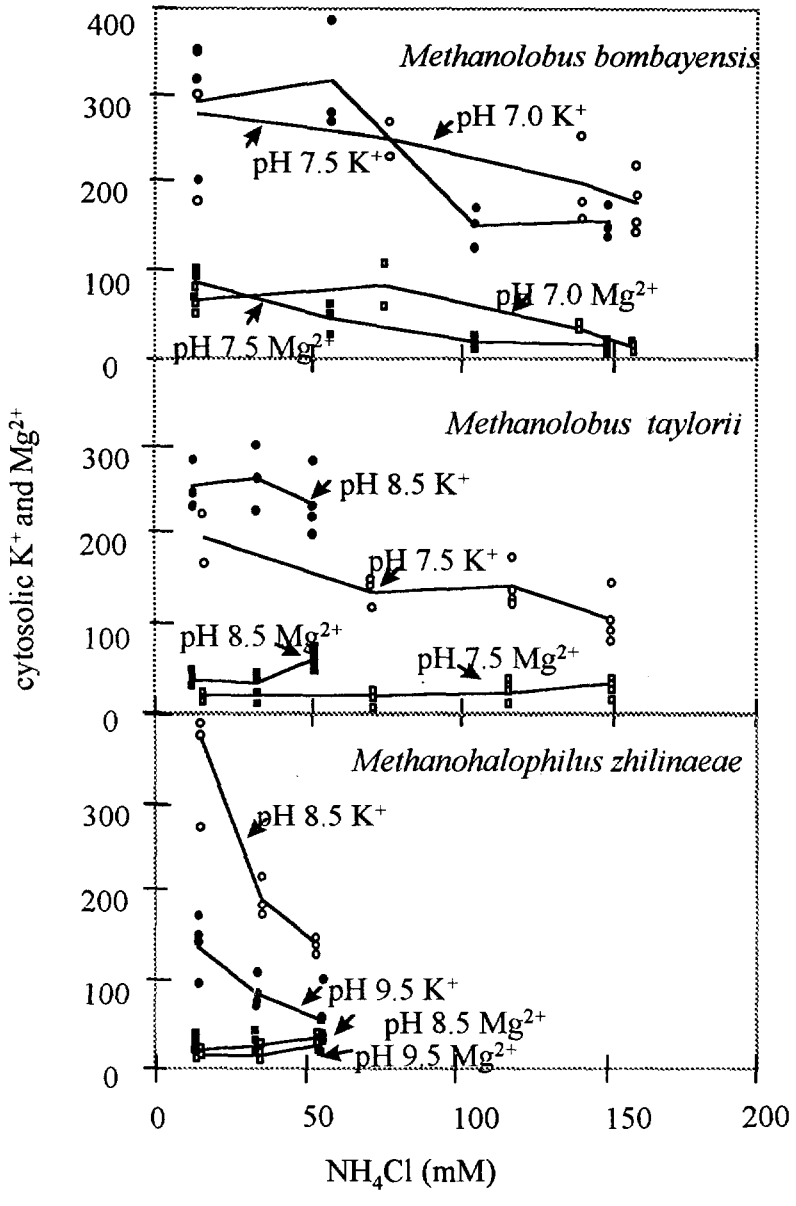

Figure 13. Effect of external concentration of total ammonia on cytosolic concentrations of K^+ (circles) and Mg^{2+} (squares). Each methanogen was tested at two different pH values, *Methanolobus bombayensis* at pH 7 and 7.5, *Methanolobus taylorii* at pH 7.5 and 8.5, and *Methanohalophilus zhilinaeae* at pH 8.5 and 9.5; in each case the open symbols and dashed lines indicate data at the lower pH and the filled symbols and solid lines indicate data at the higher pH value.

Table 4. Cytosolic ions and their dependence on extracellular pH and ammonia concentration.

Organism	external NH_4Cl (mM)	Intracellular concentrations (mM) total ammonia	NH_3	NH_4^+	Mg^{2+}	K^+	Internal osmotic strength calculated[a] (mOs)	% of medium[b]
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]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.
[b]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.

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 a weak cell walls (61, 74, 99) that do not allow the development of significant turgor pressure (62), so the osmolarity of the cytosol must approximately match that of their environment. The osmolarity of these cultures exerted by the detected cations and their counter ions was a substantial fraction of the osmolarity of their culture medium (Table 4). The osmolarity of detected cytosolic ions in *Methanolobus bombayensis* (grown in medium at pH 7.5 with 13.8 mM total ammonia) was about 70% that of its medium concentration, whereas the concentration of cytosolic ions in *Methanolobus taylorii* (grown at pH 8.5 and 12 mM total ammonia) was only about 53% of its medium concentration. *Methanohalophilus zhilinaeae* (pH 9.5 and 13.4 mM total ammonia concentration) had the least cytosolic ion concentration (33% of its medium concentration). The deficit in calculated cytosolic osmotic pressure (relative to the culture medium) may be made up by undetected inorganic cations or by organic molecules such as compatible solutes, as reported for halophilic methanogens (56, 82, 95).

Activities of ammonia-assimilating enzymes.

There was detectable activity of each of the three ammonia-assimilating enzymes previously found in methanogens (27): glutamate dehydrogenase, glutamine synthetase, and alanine dehydrogenase. The activities of glutamine synthetase and alanine dehydrogenase were detectable in each strain, but the activities were low except that alanine dehydrogenase activity was somewhat elevated in *Methanolobus taylorii* (Table 5). Glutamate dehydrogenase activity in *Methanolobus bombayensis* was also low, but glutamate dehydrogenase activity in *Methanolobus taylorii* (150 mU) and *Methanohalophilus zhilinaeae* (400 mU) was much higher.

Table 5. Activities and pH ranges of ammonia assimilating enzymes.

	Specific activity (mU/mg 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 at which enzyme activity was at least one-third of the activity measured at the optimum pH value.

Discussion

To understand the possible inhibitory mechanisms of ammonia, it is important to consider the chemical interaction of ammonia and cells. When a cell at constant pH is exposed to an increased extracellular ammonia concentration, un-ionized ammonia would equilibrate across the cell membrane, thereby increasing the cytosolic concentration of un-ionized ammonia. If the cytosolic pH remained constant, this would cause a proportional increase in the cytosolic NH_4^+ concentration as well. Because of the protons absorbed by NH_3 molecules to become NH_4^+, this may lead to temporary alkalization of cytosol as occurs when *Vibrio alginolyticus* is exposed to 20 mM diethanolamine at pH 9.6 (68). In a well-buffered cytosol with small changes in external ammonia concentration this re-equilibration of intracellular NH_3/NH_4^+ may not result in a significant change in internal pH

(and thus in ΔpH). However, the ΔpH of *Methanolobus taylorii* was the same at all concentrations of external total ammonia tested up to about 75 mM (Fig. 12b). 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 (Fig. 12b). This lack of significant change in ΔpH can be explained by considering 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 $\Delta\mu_{H^+}$ re-equilibrated. The $\Delta\Psi$ may effect a redistribution of other cations such as K^+ across the cell membrane. Such a redistribution occurs in *Vibrio alginolyticus*, which appears to contain an H^+/K^+ antiporter (68). Similarly, the methanogens at pH 7 to 7.5 accommodated increased environmental ammonia concentrations up to a given point by maintaining relatively constant ΔpH (Fig. 12) and decreasing their cytosolic content of K^+ (Fig. 13). At higher external ammonia concentrations the ΔpH increased and concentrations of cytosolic K^+ decreased. Methanogens grown at higher external pH allowed their ΔpH as well as intracellular ammonium to increase in response to growth at higher extracellular total ammonia (Fig. 12). If cells had not responded by increasing their ΔpH, they would have accumulated very high cytosolic NH_4^+ concentration. For instance, *Methanolobus taylorii* growing at pH 7.5 would have a cytosolic NH_4^+ concentration of about 683 mM if had maintained its ΔpH at -0.5 during growth at its IC_{50} for total ammonia.

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 2-6 mM un-ionized NH_3 regardless of external pH or NH_4^+ concentration (Table 3). Similarly un-ionized ammonia (NH_3) in the

range of 2-5 mM inhibits by 50% the growth of other methanogenic isolates (39, 100), rumen bacteria (81), methanogenesis in anaerobic digestors (3, 10, 18, 51, 92), and oxygenic photosynthesis by pure cultures (8) 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) (8), 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, Table. 3). *Methanohalophilus zhilinaeae* is the only methanogen tested whose IC_{50} for un-ionized ammonia was dependent on pH, and was greater than 5 mM un-ionized ammonia. If un-ionized NH_3 concentration was the toxic factor, its concentration should have been the same at the IC_{50} for total ammonia at different pH values. 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. Another possible explanation for the toxic effects of total ammonia on *Methanohalophilus zhilinaeae* is 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. This methanogen was isolated in enrichment medium at pH 9.5 and with approximately 20 mM each of NH_4Cl and trimethylamine (15, 61), which would have supported the growth only of methanogens with such a tolerance to ammonia.

Some bacteria such as *Escherichia coli* (16) adjust their ΔpH depending on external pH, as a method for homeostatic control of cytosolic pH. Other organisms, such as various ruminal bacteria (85), maintain a constant ΔpH, allowing their cytosolic pH to change with external pH. Each of the three methanogens grown in medium with low ammonia behaved as the latter, maintaining a fixed ΔpH regardless of the external pH.

Three mechanisms of ammonia assimilation have been demonstrated in methanogens (27). At low ammonia concentrations (<1 mM) cells use glutamine synthetase to catalyze ATP-dependent synthesis of glutamine from ammonia and glutamate (80). Each of the three methanogens had glutamine synthetase activity, but the activity was low, as expected because cells were all grown at high ammonia concentrations. *Methanolobus bombayensis* had low levels of each of the three ammonia assimilating enzymes. *Methanolobus taylorii* had moderate alanine dehydrogenase activity (40 mU) and high glutamate dehydrogenase activity (150 mU), and *Methanohalophilus zhilinaeae* had very high glutamate dehydrogenase activity (400 mU). The high levels of ammonia-assimilating activities of these latter two methanogens may be useful to the cells for the synthesis of nitrogen-containing compatible solutes such as glycine betaine (83) and α- and β-glutamate (56), which these two species accumulate in response to elevated external osmolarities. These two methanogens also had a much larger deficit of cytosolic ions relative to the concentrations of ions in their environment, and *Methanolobus bombayensis* may rely less on the synthesis of compatible solutes than these two strains.

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: 6.0-7.0 for *Methanolobus bombayensis*, 7.0-8.0 for *Methanolobus taylorii* and 7.0-8.5 for *Methanohalophilus zhilinaeae*.

CONCLUSIONS

1. Environments and enrichment media that are chemically or physically unusual such as those high Mg^{2+} concentration, can recover new species of microbes such as *Methanolobus bombayensis*, a halophilic, methylotrophic methanogen isolated from Arabian Sea sediment near Bombay, India.

2. *Methanolobus bombayensis* and *Methanolobus taylorii* tolerated 2-6 mM of un-ionized ammonia (NH_3) regardless of external pH. However, *Methanohalophilus zhilinaeae*, an alkaliphile, had an extraordinarily high resistance to NH_3, and this tolerance was dependent on external pH. NH_3 concentration of 13 mM at pH 8.5 and 45 mM at pH 9.5 inhibited by 50% the growth of *Methanohalophilus zhilinaeae*. Thus at pH 8.5, in addition to NH_3 other factors might have caused inhibition of growth.

3. *Methanolobus bombayensis*, *Methanolobus taylorii* and *Methanohalophilus zhilinaeae* maintained an acidic cytosol ('reversed' ΔpH). *Methanohalophilus zhilinaeae* the most alkaliphilic species had the most negative ΔpH (-0.9 pH units). All these three methanogens did not change their ΔpH with external pH.

4. *Methanohalophilus zhilinaeae*, an alkaliphile, grew well with high cytosolic pH (cytosolic pH was near 8.5 at external pH of 9.5). This was supported by measuring the pH range of activities of three enzymes; glutamine synthetase, glutamate dehydrogenase and alanine dehydrogenase, these were all active at high pH (8.5-9.0).

OTHER FINDINGS

1. Based on phylogeny (16S rRNA sequencing and cataloging) *Methanolobus vulcani* was most similar to *Methanolobus tindarius*. However, *Methanolobus vulcani* was

phenotypically different; it required biotin and had slightly high temperature and pH optima.

2. *Methanolobus bombayensis*, *Methanolobus taylorii* and *Methanohalophilus zhilinaeae* accumulated K^+, and their cytosolic K^+ concentration decreased when grown at high external ammonia concentration. The accumulation of NH_4^+ along with slight change in ΔpH (ΔpH increased slightly at high external ammonia concentration) might have influenced the cytosolic K^+ content.

3. *Methanohalophilus zhilinaeae* when grown in medium at pH 9.5 and with 13.4 mM total ammonia had the least cytosolic ionic concentration (33% of that of its medium) as compared to *Methanolobus bombayensis* and *Methanolobus taylorii*. This deficit in calculated osmolytes (relative to the culture medium) may be made up other compatible solutes. *Methanohalophilus zhilinaeae* is known to accumulate α-glutamate and glycine betaine.

4. Activity of three ammonia-assimilating enzymes, (glutamine synthetase, glutamate dehydrogenase and alanine dehydrogenase) was detected in *Methanolobus bombayensis*, *Methanolobus taylorii* and *Methanohalophilus zhilinaeae*.

5. Activity of glutamate dehydrogenase was high in *Methanolobus taylorii* (150 mU) and highest in *Methanohalophilus zhilinaeae* (400 mU). The high activity in *Methanohalophilus zhilinaeae* may be necessary for the cells for the synthesis of nitrogen-containing compatible solutes.

FUTURE DIRECTIONS

Further studies on role of Mg^{2+} in the physiology of *Methanolobus bombayensis*

Mg^{2+} is one of the major cytosolic divalent cations in living cells. It is important in the action of many enzymes that catalyze ATP-dependent reactions (90). In addition, Mg^{2+} ions are also known to maintain ultrastructure of prokaryotes. *Methanolobus bombayensis* required high concentration of Mg^{2+} (minimum 30 mM) for growth. Cells lost their morphology and became turgid and spherical when Mg^{2+} was omitted from the buffer suspension. *Methanolobus bombayensis* cells accumulated Mg^{2+} and when cells were grown in higher concentration of ammonia, the cytosolic Mg^{2+} content decreased and ΔpH increased (Fig. 13 and Fig. 12). The future efforts should focus on the role of Mg^{2+} in osmotic and pH adaptation by this strain. It will be interesting to understand if Mg^{2+} plays role in stabilizing the cell envelope proteins and in bioenergetics of this organism.

Further studies on determining the transmembrane membrane potential (ΔΨ) of *Methanolobus taylorii, Methanolobus bombayensis* and *Methanohalophilus zhilinaeae*

In most bacteria proton-translocating ATPase equilibrate energy in ATP with $\Delta\mu_{H^+}$, across the cell membrane. This energy in $\Delta\mu_{H^+}$ is the sum of the energies in the ΔpH and ΔΨ (66). The measurement of $\Delta\mu_{H^+}$ is usually in the range of −150 to −200 mV in accord with theoretical requirements for ATP synthesis. Most bacteria maintain a ΔpH (alkaline inside) some others maintain a 'reversed' ΔpH (acidic inside). Thus those cells

which maintain 'reversed' ΔpH mostly compensate change in $\Delta\mu_{H^+}$ by maintaining a strong $\Delta\Psi$ (34).

The present studies indicated that *Methanolobus bombayensis*, *Methanolobus taylorii* and *Methanohalophilus zhilinaeae* maintained a reversed ΔpH (acidic inside) (Fig. 12). It might be possible that these cells maintain a strong $\Delta\Psi$. To prove this hypothesis, further study should involve the determination of $\Delta\Psi$ for these organism and also calculate $\Delta\mu_{H^+}$. The present study also showed that at high ammonia concentration, ΔpH increased (became less negative). Thus further studies should also include the determination of $\Delta\Psi$ at high ammonia concentration. This will elucidate the effect of ammonia on $\Delta\Psi$ and on $\Delta\mu_{H^+}$.

Future studies on determination of ΔpH and $\Delta\Psi$ of acidotolerant methanogens

The work on determination of ΔpH and $\Delta\Psi$ has included methanogens growing at neutral or alkaline pH (40, 45, 71, 88, 101). Many bacteria growing at very low pH usually maintain a strong ΔpH and thus have a low $\Delta\Psi$ (cytosol less negative). This adaptation allows these bacteria to maintain their cytosolic pH more neutral than their environment. Future studies should focus on the ΔpH and $\Delta\Psi$ of acidotolerant methanogens such as *Methanosarcina barkeri* and *Methanosarcina vacuolata* which grow well at pH 4.5 (59).

Further work on alkaliphiles

In this work ammonia toxicity was studied in three methanogens: *Methanolobus bombayensis*, *Methanolobus taylorii* and *Methanohalophilus zhilinaeae*. *Methanohalophilus zhilinaeae* differed from the other two methanogens in that it had an extraordinary tolerance to NH_3 (IC_{50} at pH 9.5 was 45 mM NH_3). In addition, *Methanohalophilus zhilinaeae* is phylogenetically different from the other two

methanogens and belongs to another genus (14). Thus, a methanogen belonging to same genus as *Methanolobus* may not show the same characteristics as *Methanohalophilus zhilinaeae*. Another alkaliphilic species of the genus *Methanolobus, Methanolobus oregonensis* should be examined for ammonia toxicity.

In addition to *Methanolobus oregonensis* and *Methanohalophilus zhilinaeae* there are two more alkaliphilic methanogens: *Methanobacterium alcaliphilum* (111) and *Methanobacterium thermoalcaliphilum* (14). There is no information in the literature on the cytosolic pH of any of these alkaliphilic methanogens. We found that *Methanohalophilus zhilinaeae* had a cytosolic pH near 8.5, and it will be interesting to examine the cytosolic pH of the other three methanogens. This knowledge on cytosolic pH for the alkaliphiles will help to detect alkaliphilic or alkalitolerant enzymes which can be of industrial importance, such as protease (that used in detergents (35)).

In present study we found that *Methanohalophilus zhilinaeae* which had alkaline cytosol had also alkalitolerant enzymes such as glutamate dehydrogenase, glutamine synthetase, and alanine dehydrogenase, and activity for these enzymes was detected at pH range 8.5-9.0. Future studies should be focused on exploring various other alkaliphilic or alkalitolerant enzymes and also checking the stability of these enzymes at high pH. The stability of the enzymes can be checked by exposing the enzyme to the alkaline pH, which enzyme tolerates and then determining the activity at its optimum pH.

Methanohalophilus zhilinaeae has an extraordinary tolerance to NH_3 (IC_{50} at pH 9.5 was 45 mM of NH_3). Such a high concentration of NH_3 might influence the various cell constituents especially; proteins. Probably in *Methanohalophilus zhilinaeae* most of these cell constituents must be moderately tolerant to ammonia. Further studies should elucidate the ammonia tolerance of various proteins and enzymes.

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Publications

1. Kadam, P. C., D. R. Ranade, L. Mandelco, and D. R. Boone. 1994. Isolation and characterization of *Methanolobus bombayensis* sp. nov., a methylotrophic methanogen that requires high concentrations of divalent cations. Int. J. Syst. Bacteriol. **44:** 603-607.

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Poster Presentations

1. Kadam, P. C., and D. R. Boone. 1996. Influence of pH on ammonia accumulation and toxicity in halophilic, methylotrophic methanogens. 96th ASM General Meeting, New Orleans, Louisiana.

2. Ranade, D. R., and P. C. Kadam. 1989. Methanogens from aquatic sediments in India. 5th International Symposium on Microbial Ecology, Kyoto, Japan.