Study on Two Methylotrophic and Halophilic Methanogens, *Methanosarcina siciliae* HI350 and *Methanolobus taylorii* GS-16

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To Professor Zhe-shu Qian,
Who introduced me to the wonderful world of science

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Study on Two Methylotrophic and Halophilic Methanogens, *Methanosarcina siciliae* HI350 and *Methanolobus taylorii* GS-16

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Strain HI350 was isolated from an oil well in the Gulf of Mexico. This strain was similar to *Methanolobus siciliae* T4/M^T (T = type strain) morphologically and physiologically. Cells of both strains were irregular, nonmotile, coccoid, and 1.5 to 3.0 μm in diameter. Cells occurred singly, in pairs or sometimes in massive clumps consisting of pseudosarcinae. Their catabolic substrates included methanol, trimethylamine, dimethyl sulfide, and methane thiol, but not H_2-CO_2, formate, or acetate. Growth was fastest in the presence of 0.4 to 0.6 M Na^+, in the presence of 60 to 200 mM Mg^{2+}, at pH 6.5 to 6.8, and at 40°C. Growth was stimulated by yeast extract. Sequence analysis of 16S rRNA showed that *Methanolobus siciliae* T4/M^T was closely related to *Methanosarcina*, especially to *Methanosarcina acetivorans* C2A^T. Thus, we proposed to transfer *Methanolobus siciliae* T4/M^T to the genus *Methanosarcina* as *Methanosarcina*

siciliae with strain HI350 as its reference strain.

Degradation of dimethyl sulfide or methane thiol by strain HI350 was complete, and stoichiometric quantities of methane and hydrogen sulfide were formed. Studies of cell-free extracts suggested that enzymes for the degradation of dimethyl sulfide and methane thiol were inducible, whereas those for the degradation of methanol or trimethylamine were constitutive.

Methanolobus taylorii GS-16, a moderately halophilic and alkaliphilic methanogen, grows over a wide pH range from 6.8 to 9.0. Cells suspended in medium with pH above 8.2 reversed their ΔpH making their cytosol more acidic than the medium. The decreased energy in the proton-motive force due to the reverse ΔpH was partly compensated by an increased $\Delta\psi$. The cytosolic acidification by strain GS-16 at alkaline pH was accompanied by K^+ extrusion. The cytosolic K^+ concentration was 110 mM in cells suspended at pH 8.7, but it was 320 mM K^+ in cells suspended at neutral pH. High external K^+ concentration (210 mM or higher) inhibited strain GS-16 at alkaline pH, perhaps by preventing K^+ extrusion. Cells suspended at pH 8.5 and 300 mM external K^+ failed to acidify their cytosol. The key observation indicative of the involvement of K^+ transport in cytosolic acidification was that valinomycin (0.8 μM), a K^+ uniporter, inhibited the growth of strain GS-16 only at alkaline pH. Experiments with resting cells indicated that, at alkaline pH, valinomycin uncoupled catabolic reactions from ATP synthesis. Thus, a K^+/H^+ antiporter was proposed to account for the K^+ extrusion and the uncoupling effect of valinomycin at alkaline pH.

INTRODUCTION

Methanogens represent a diverse group of strictly anaerobic *Archaeobacteria* which obligately produce methane as an end product of their energy metabolism. One of the most salient physiological feature of the methanogens is their extreme specialization of catabolic substrates. As a group, methanogens are limited to a few small molecules, including H_2-CO_2, formate, acetate, methanol, methylated amines and methylated sulfur compounds such as dimethyl sulfide and methane thiol. Many methanogens can grow only on one or two of these substrates. The most versatile *Methanosarcina* species are able to use H_2-CO_2, acetate, methanol, and methylated amines.

Dimethyl sulfide was recognized as a growth substrate for only a few methanogens: *Methanolobus taylorii* (16, 54, 97), *Methanolobus oregonensis* (= *Methanohalophilus oregonensis*) (16, 68), and *Methanohalophilus zhilinaeae* (77). A number of *Methanosarcina* species tested were incapable of using this compound (97). Although methane thiol was detected in methanogenic enrichment culture with dimethyl sulfide as the sole substrate (51, 52) and in dimethyl-sulfide-grown pure culture of *Methanolobus taylorii* (54, 97), methanogens growing on methane thiol as a catabolic substrate had not been reported at the time when this study began. Part I of this

dissertation describes the characterization of a halophilic methanogen, strain HI350, which belongs in the genus *Methanosarcina*. Strain HI350 grows on dimethyl sulfide and methane thiol, as well as methanol and methylamines. Degradation of dimethyl sulfide and methane thiol by strain HI350 is complete. Enzymes involved in the catabolism of those sulfur compounds are inducible.

Tolerance of extreme environmental conditions is perhaps another physiological feature of methanogens (29). Interestingly, all three dimethyl-sulfide-using methanogens reported were isolated from alkaline environments, and they are all alkaliphilic (16). Results presented in Part II of this dissertation show that *Methanolobus taylorii* GS-16 was sensitive to valinomycin only during growth at alkaline pH. Upon resuspension in alkaline medium, cells reversed ΔpH and released cytosolic K^+. A possible role of K^+ extrusion in pH homeostasis by strain GS-16 is proposed.

PART I

CATABOLISM OF METHYLATED SULFUR COMPOUNDS BY *Methanosarcina siciliae* HI350 and OTHER METHYLOTROPHIC METHANOGENS

Results in this part have been presented in the following publications: 1) **Ni, S., and D. R. Boone.** Int. J. Syst. Bacteriol. (1991) **41:**410-416; 2) **Ni, S., and D. R. Boone.** p. 796-810, *In* R. S. Oremland (ed.), Biogeochemistry of global change. Chapman & Hall, Inc., New York (1993); 3) **Ni, S., C. R. Woese, H. C. Aldrich, and D. R. Boone.** Int. J. Syst. Bacteriol. (submitted)

BACKGROUND

Occurrence of dimethyl sulfide and methane thiol in environment.

Dimethyl sulfide and methane thiol are important compounds of the sulfur cycle in various environments (4, 20, 22, 32, 35, 51, 52, 53, 54, 112, 128, 149, 151). Dimethyl sulfide is more abundant in nature than methane thiol. It has been estimated that this compound accounts for one-half of the biogenic input of volatile sulfur into the atmosphere, with nearly 75% of it being generated in marine environments (4). Dimethyl sulfide and methane thiol are formed during the biodegradation of organic sulfur compounds and the biological methylation of inorganic sulfide. Dimethylsulfoniopropionate and methionine are the major organic precusors, but 3-methiolpropionate, S-methylcysteine,and S-methymethionine ma also participate (126). Principal routes for microbial formation of dimethylsulfide and methane tiol are summarized in Fig. 1.

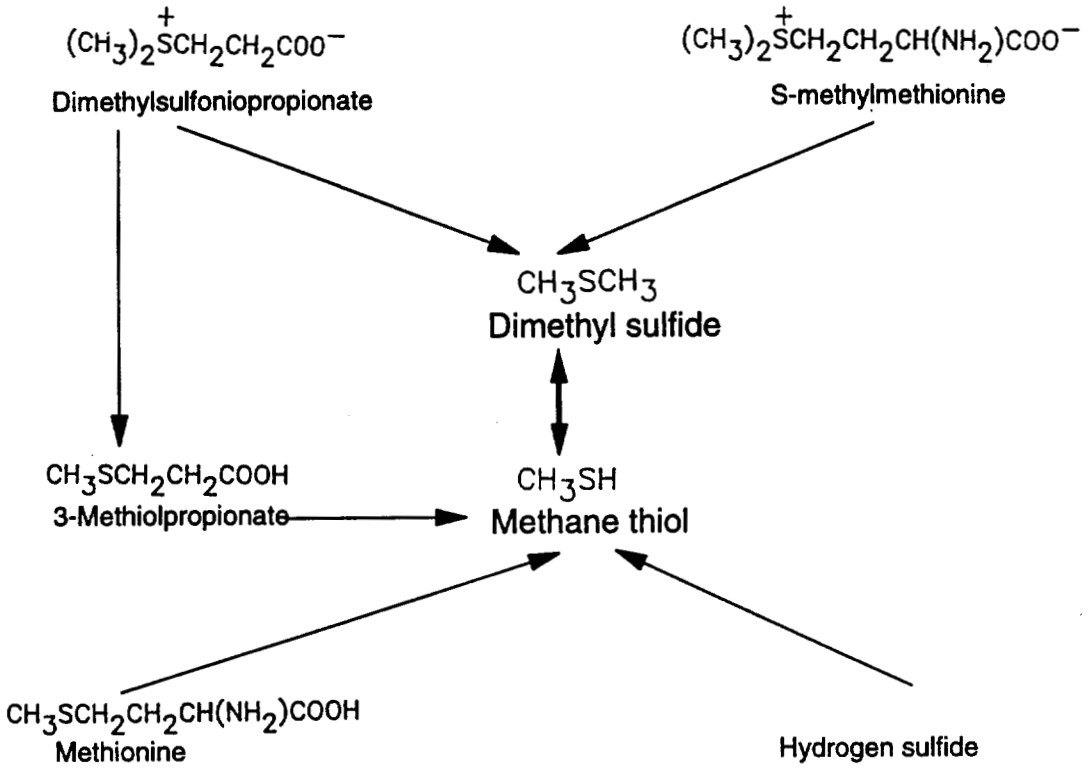


Figure 1. Major routes for microbial formation of dimethyl sulfide and methane thiol.

Dimethylsulfoniopropionate is the major precursor of dimethyl sulfide. Dimethylsulfoniopropionate is present some species of marine phytoplankton, macroalgae, and salt marsh plants. (46, 108, 137). In these organisms dimethylsulfoniopropionate probably acts as a compatible solute for osmoregulation (54, 60, 128, 151). Cytosolic concentrations of dimethylsulfoniopropionate may reach molar levels (126). It is released upon cell lysis and further degraded to dimethyl sulfide and acrylate by various organisms such as *Clostridium* sp., *Pheaocystis pouchetti*, *Polysiphonia lanosa*, and *Gyrodinium cohnii*. Dimethylsulfoniopropionate can also be abiotically degraded to dimethyl sulfide and acrylate at high pH (> 12). Zooplankton grazing on dimethylsulfoniopropionate-containing phytoplankton cells also contribute to the generation of dimethyl sulfide in the marine environments (139).

Alternatively, dimethylsulfoniopropionate is degraded to form methane thiol (127). Dimethylsulfoniopropionate is initially demethylated to 3-methiolpropionate which is then demthiolated to produce methane thiol. Bacteria that use this pathway were recently isolated from a variety of marine environments and from cultures of marine phytoplankton (138). Methionine is the other major precursor of methane thiol (150). Microorganisms produce methane thiol from methionine under either oxic or anoxic conditions (128). Bacteria capable of this transformation include *Proteus vulgaris*, *Enterobacter aerogenes*, *Escherichia coli*, and species of *Pseudomonas* and *Clostridium*. Among fungi, species of *Penicillium*, *Candida*, *Aspergillus* and *Scopulariopsis* are capable of forming methane thiol from methionine.

Biological methylation of inorganic sulfide is another pathway for the formation

of dimethyl sulfide and methane thiol. Various heterotrophic bacteria contain thiol S-methyltransferases, which carry out the methylation reaction of H_2S and S-adenosylmethionine (27). Methoxylated aromatic compounds such as syringate (the lignin monomers) also serve as the donors of methyl group for the methylation of H_2S (32). Because of the abundance of both H_2S and methylated compounds in nature, generation of dimethyl sulfide and methane thiol by this methylation process is probably more significant than by the degradation pathway from methionine and dimethylsulfoniopropionate. The emission of those volatile sulfur compounds into the atmosphere has been linked to acid rain and even to the global climate regulation (20, 128). However, microbial degradation of dimethyl sulfide may decrease its flux into the atmosphere.

Microbial degradation of dimethyl sulfide and methane thiol

Aerobic degradation. Dimethyl sulfide and methane thiol are most common in oxic regions in the environment. Microbes which grow aerobically on dimethyl sulfide are either species of *Hyphomicrobium* or *Thiobacillus* (128). *Hyphomicrobium* species are strictly methylotrophic. *Hyphomicrobium* strain S can use dimethyl sulfide and dimethyl sulfoxide. In addition to dimethyl sulfide and dimethyl sulfoxide, *Hyphomicrobium* strain GE can grow well on methylamine, dimethylamine, and trimethylamine N-oxide and poorly on formate or trimethylamine. In these organisms, dimethyl sulfoxide and dimethyl sulfide are completely oxidized to H_2SO_4 and CO_2.

Dimethyl sulfide is oxidized to formaldehyde and methane thiol by dimethyl sulfide monoxygenase, and then a methane thiol oxidase catalyzes the methane thiol to sulfide plus formaldehyde. Sulfide is eventually oxidized to sulfate by this enzyme. NAD-dependent formaldehyde and formate dehydrogenases catalyze the oxidation of formaldehyde to CO_2. The methane thiol oxidase of *Hyphomicrobium* strain GE has been purified (125).

Anaerobic degradation. Under anaerobic conditions where light is present, dimethyl sulfide is oxidized to dimethyl sulfoxide by the phototrophic bacterium *Thiocystis* sp. (147). In sediments, dimethyl sulfide is metabolized by both sulfate-reducing and methanogenic bacteria, yielding hydrogen sulfide and methane (51, 53, 128, 149, 150). When dimethyl sulfide is present at low concentrations (<2 μM), it is used mainly by sulfate reducers, but at higher concentrations it is used by methanogens (53).

Since the concentration of dimethyl sulfide in natural anoxic environments seldom exceeds 2 μM (21), sulfate-reducing bacteria are likely the major agents of degradation. However, pure cultures of dimethyl-sulfide-using sulfate-reducing have not been reported. Instead, several strains of obligately methylotrophic methanogens were shown to use dimethyl sulfide as their substrate: *Methanolobus taylorii* GS-16 (16, 95, 97), *Methanohalophilus zhilinaeae* WeN5 (77), *Methanolobus oregonesis* WAL1 (= *Methanohalophilus oregonensis*) (68). Other methylated compounds such as methanol, mono-, di-, and trimethylamine can also serve as their catabolic substrates. These halophilic bacteria were isolated from marine sediments and salt lakes. The pathway of dimethyl sulfide degradation in those obligate, methylotrophic methanogens is unknown. Probably, it

resembles the pathway of methanol degradation.

Pathway of Methanol Degradation by Methanogens

Studies of *Methanosarcina barkeri* have provided most of our knowledge of the biochemistry of methanogenic methanol degradation. In this organism, methanol conversion involves two different routes: the reduction of the substrate to methane and concomitant methanol oxidation to CO_2 (Fig. 2) according to the following overall reactions:

$$CH_3OH + H_2O \rightarrow CO_2 + 6H^+ + 6e^- \quad (1)$$

$$3CH_3OH + 6H^+ + 6e^- \rightarrow 3CH_4 + 3H_2O \quad (2)$$

$$\text{net: } 4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O \quad (3)$$

Methanol reduction to methane. Methanol reduction to methane is initiated by the transfer of the methyl group to coenzyme M (HS-CoM). CH_3S-CoM synthesis proceeds in two enzymatic reactions. These reactions are catalyzed by a methyltransferase complex which consists of two methyltransferases, MT1 and MT2 (133). MT1 comprises about 15% of the soluble protein in methanol-grown *Methanosarcina barkeri*, and has been partially purified. Purification required strictly anoxic conditions, and even then, major loss in activity occurs. Native MT1 has an apparent molecular mass of 122 kDa and contains two different subunits of 34 kDa and 53 kDa in an $\alpha_2\beta$ configuration. Corrinoid (3.4 mol) per 122 kDa trimer is present, conferring an orange-brown color to the enzyme. The corrinoid is characteristics of B_{12}

derivatives and is identified as a cobamide with the nitrogenous base 5-hydroxybenzimidazole as the (α) ligand (106). This corrinoid prosthetic group, known as B_{12}HBI, is tightly, but not covalently bound to the holoenzyme.

MT1 transfers the methyl group from methanol to B_{12}HBI to form methylcobalamin (135). The central cobalt atom must be maintained at its reduced form of Co(I) for the MT1 to be active. In the presence of mild oxidants (traces of O_2, oxidized flavins or viologen dyes) Co(I) is oxidized to pentacoordinate (Co(II)-corrin and hexacoordinate Co(III)-corrin, and MT1 becomes catalytically incompetent. This at least partly explains inactivation of the enzyme during its purification. MT1, however, may be reductively reactivated in vitro by an enzyme system from *M. barkeri* (134, 132). MT1 reactivation requires H_2, ATP, hydrogenase, ferredoxin and a heat-labile, oxygen-sensitive enzyme (methyltransferase-activating protein) (48). MT1 in the presence of hydrogen displays a sharp absorption peak at 390 nm only when ATP is added. This peak is characteristic of Co(I) cobamide. With small amounts of methanol, spectral changes that occur during reaction of MT1 with methanol indicate that the central cobalt atom undergoes changes from its highly-reduced Co(I) state to Co(III). The corrin is trapped as methyl-B_{12}-HBI. When extracted under the appropriate conditions, up to 80% of the total cobamide in *Methanosarcina barkeri* consists of methyl-B_{12}HBI (37). The reactivation mechanism is unknown. At pH 7, the reduction potential of the Co(I)/Co(II) couple in free cobamide is -640 mV (48), although the redox potential of enzyme-bound B_{12}HBI may be more positive. The reduction potential of H_2/H^+ couple is -414 mV. So the redox potential of the electrons derived from hydrogen must be lowered perhaps at

the expense of ATP hydrolysis, to a level which permits Co(II) reduction to Co(I).

Next, MT2 transfers the methyl group from methylcobalamin to HS-CoM to form CH_3-CoM. MT2 has been purified to apparent homogeneity (136). It is composed of a single polypeptide of about 40 kDa.

CH_3-CoM is reduced to CH_4 by methyl-coenzyme M reductase complex. Methyl-coenzyme M reductase constitutes about 10% of the cell protein of methanol-grown *Methanosarcina barkeri*. Upon cell breakage the majority of methyl-coenzyme reductase is recovered in the soluble fraction. In intact cells, however, the enzyme is localized at the inner surface of cytoplasmic membrane (130). The reductase consists of three subunits with apparent molecular weights of 68 kD, 45 kD, and 38 kD in an $\alpha_2\beta_2\gamma_2$ stoichiometry. Each of the large subunit contains a tightly but not covalently bound coenzyme F_{430}, a nickel tetrapyrrole structure. 7-Mercaptoheptanoylthreonine phosphate (HS-HTP) acts specifically as an electron donor for the methylreductase. Although HS-HTP is a highly specific and effective electron donor in vitro for methyl-coenzyme M reduction, its physiological function is still unknown. In vivo, methyl-CoM could be reduced by a larger molecule with a covalently-bound HS-HTP moiety (113).

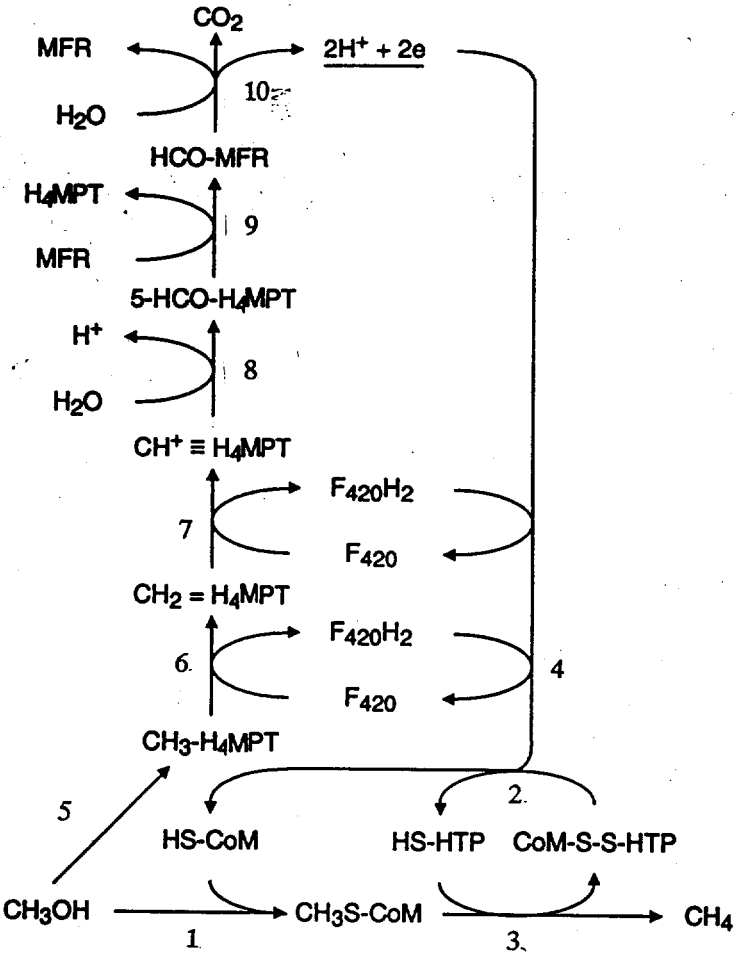


Figure 2. Pathway of methanol degradation by *Methanosarcina barkeri* (48).

HS-CoM = coenzyme M (mercaptoethanesulfonic acid); H_4MPT = 5, 6, 7, 8-tetrahydromethanopterin; MFR = methanofuran; HS-HTP = 7-mercaptoheptanoylthreonine phosphate.

1. MeOH:HS-CoM methyltransferase
2. F_{420}-dependent CoM-S-S-HTP heterodisulfide reductase
3. CH_3-S-CoM reductase
4. $F_{420}H_2$ dehydrogenase
5. CH_3-H_4MPT:HS-CoM methyltransferase ?
6. CH_2=H_4MPT reductase
7. CH_2=H_4MPT dehydrogenase
8. CH≡H_4MPT cyclohydrolase
9. CHO-MFR:H_4MPT transferase
10. CHO-MFR dehydrogenase

The catalytic mechanism of this complex reductase is not well understood. The methylredustase is generally inactive in cell-free extracts. Its activation in vitro requires H_2 and catalytical amount of ATP. Coenzyme F_{420} or vitamin B_{12} is stimulatory although not required. This activation process probably involves a ATP-dependent reduction of the coenzyme F_{430} to the Ni(I) state (3) with electrons donated from H_2 (111). Methyl-coenzyme M is demethylated by the reduced Ni (I) in coenzyme F_{430} to form methyl-Ni(II) F_{430} and CoM-S-S-HTP. The metalloorganic compound is subject to rapid protonolysis to CH_4 and Ni(II)F_{430} (2). CoM-S-S-HTP is reduced by heterodisulfide reductase to regenerate the reducing equivalents HS-HTP for the methyl-coenzyme M reductase. The reductant is $F_{420}H_2$ generated during the oxidation of methanol.

Methanol oxidation to CO_2. Methanol oxidation generally proceeds by the same sequence of reactions as CO_2 reduction to CH_4, albeit in the reverse order. The point at which methanol enters this pathway is not yet resolved. CH_3-H_4MPT is identified as the initial product of methanol oxidation. However, methyltransfer from methanol is independent of ATP and HS-CoM (48). These observations rule out the possibility that CH_3-H_4MPT synthesis occurs via MT1, which is strictly dependent on ATP or HS-CoM. Thus, CH_3S-CoM is not involved in the oxidation route, and a direct transfer of the methyl group to H_4MPT might be involved. It is not known whether the reaction is catalyzed by a separate enzyme, or whether the enzyme methyl-H_4MPT:HS-CoM methyltransferase involved in the CO_2 reduction also accepts the methyl group from methanol. The dependence of methanol oxidation on sodium-motive force (87) supports

the latter possibility because the methyl-H_4MPT:HS-CoM methyltransferase is identified as the primary sodium pump during the CO_2 reduction to CH_4 (8, 9, 85, 86) . Subsequent reactions of methyl group oxidation proceed by the reverse of the pathway for CO_2 reduction. Methylene-H_4MPT reductase, methylene-H_4MPT dehydrogenase, methenyl-H_4MPT cyclohydrolase, formylmethanofuran:H_4MPT fromyltransferase, and formylmethanofuran dehydrogenase are purified from methanol-grown *Methanosarcina barkeri*. Both methylene-H_4MPT reductase and methylene-H_4MPT dehydrogenase use F_{420} as the electron acceptor.

The oxidative pathway is of crucial importance in methylotrophic metabolism. It provides the reducing equivalents in the form of $F_{420}H_2$ for the reduction of CoM-S-S-HTP to HS-CoM and HS-HTP. This reaction is resposible for the generation of the transmembrane electrochemical gradient of proton for ATP synthesis. The oxidative pathway also generates methyl-H_4MPT and CO_2 required for the synthesis of acetyl-CoA, the starting point for cell-carbon synthesis.

Current taxonomy of the methylotrophic methanogens

Based on 16S rRNA sequence data, five orders (16) within the kingdom *Archaeobacteria* were proposed to accommodate all methanogenic bacteria: *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*, *Methanopyrales*, and *Methanosarcinales* (Table 1). All methylotrophic methanogens belong in *Methanosarcinaceae*, one of the two families in the order *Methanosarcinales*. These

methylotrophs are classified into five genera: *Methanolobus*, *Methanococcoides*, *Methanohalophilus*, *Methanohalobium*, and *Methanosarcina*. All these genera except *Methanosarcina* contain halophilic, obligately methylotrophic methanogens which grow exclusively on methanol and methylamines. Some of them can grow on methylated sulfur compounds such as dimethyl sulfide and methane thiol. *Methanolobus* and *Methanococcoides* are slightly halophilic, requiring 0.5 M NaCl for their best growth. *Methanococcoides* contained one species, *Methanococcoides methylutens*. Later, a psychrophilic strain, *Methanococcoides burtonii* (33), was added. *Methanolobus* previously contained three slightly halophilic species: *Methanolobus tindarius*, *Methanolobus siciliae*, and *Methanolobus vulcani* (123). Recently, a proposal was made to transfer *Methanohalophilus oregonensis* to this genus, based on sequence comparisons of 16S rRNA (16); also, strain GS-16 was named *Methanolobus taylorii* (95). *Methanohalophilus* has two moderately halophilic species: *Methanohalophilus mahii* and *Methanohalophilus halophilus*. The genus *Methanohalobium* contains one extremely halophilic species *Methanohalobium evestigatum*.

Methanosarcina species differ morphologically and physiologically from the obligately methylotrophic halophiles. The halophiles grow as irregular cocci, either singly or in small aggregates, throughout their growth stages. In contrast, many *Methanosarcina* strains undergo morphological changes at different growth phases (19, 73, 75, 122). In young cultures of these *Methanosarcina*, individual coccoid cells form aggregates of pseudosarcinal shapes, which then become cysts of various sizes; the cysts have a common external wall of heteropolysaccharide (69, 144). All species assigned to the

genus *Methanosarcina* at the beginning of this study were nonhalophilic species which grow on methylotrophic substrates (methanol and methylamines) and sometimes acetate or H_2-CO_2, but not on methylated sulfur compounds (72, 74). However, results described in part I of this dissertation show that strains HI350 and T4/M^T , previously named *Methanolobus siciliae* (91), belong in the *Methanosarcina* genus. Both strains were slightly halophilic, able to use dimethyl sulfide and methane thiol. Thus, the description for the genus *Methanosarcina* was amended to include obligately methylotrophic halophiles.

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
Methanolobus
M. tindarius, M. siciliae, M. vulcani, M. oregonensis, M. taylorii
Methanococcoides
M. methylutens, M. burtonii
Methanohalophilus
M. mahii, M. halophilus, M. zhilinaeae
Methanosaetaceae
Methanopyrales

MATERIALS AND METHODS

Source of Organisms

All the organisms were obtained from the Oregon Collection of Methanogens (OCM): strain HI350 (OCM 210), *Methanosarcina siciliae* (= *Methanolobus siciliae* T4/M (OCM 156), *Methanolobus tindarius* Tindari 3 (OCM 150), *Methanolobus vulcani* PL-12/M (OCM 157), *Methanococcoides methylutens* TMA-10 (OCM 158), *Methanolobus taylorii* GS-16 (OCM 122), *Methanosarcina mazeii* LYC (OCM 34), *Methanosarcina mazeii* C 16 (OCM 98), *Methanohalophilus halophilus* Z-7982 (OCM 160), and *Methanosarcina acetivorans* C2A (OCM 95).

Media and culture techniques

Modified anaerobic techniques of Hungate (38, 82) were used. The medium (MSH) used throughout this study contained (per liter): 2.0 g of Trypticase peptone (BBL Microbology Systems, Cockeysville, Md.), 2.0 g of yeast extract, 0.5 g of 2-mercapto-ethanesulfonic acid, 29.2 g of NaCl, 4.0 g of NaOH, 2.7 g of $MgCl_2 \cdot 6H_2O$, 0.45 g of KCl, 1.0 g of NH_4Cl, 0.4 g of $K_2PO_4 \cdot 3H_2O$, 0.25 g of $Na_2S \cdot 9H_2O$, 1.0 mg of resazurin, 5.0 mg

of Na-EDTA·$2H_2O$, 1.5 mg of $CoCl_2 \cdot 6H_2O$, 1.0 mg of $MnCl_2 \cdot 4H_2O$, 1.0 mg of $FeSO_4 \cdot 7H_2O$, 1.0 mg of $ZnCl_2$, 0.4 mg of $AlCl_3 \cdot 6H_2O$, 0.3 mg of $Na_2WO_4 \cdot 2H_2O$, 0.2 mg of $CuCl_2 \cdot 2H_2O$, 0.2 mg of $NiSO_4 \cdot 6H_2O$, 0.1 mg of H_2SeO_3, 0.1 mg of H_3BO_3, 0.1 mg of $NaMoO_4 \cdot 2H_2O$. The medium was prepared by dissolving NaOH in O_2-free water and equilibrating the preparation with CO_2; final pH was 6.8. The other constituents (except sulfide) were then added from concentrated stock solutions. The medium was dispensed into serum bottles which were sealed and autoclaved (121°C, 20 min).

MSH medium was sometimes modified. Media of various salinities were prepared by adding sodium or magnesium as their chloride salts. Media having pH values of 8.2 and 7.2 were prepared by replacing the headspace gas in the culture vessels with N_2 and N_2-CO_2 (7:3), respectively. Other pH values were obtained by adding sterile, anoxic 1 M NaOH or HCl to one of these media. In mineral MSH (MMSH), organic constituents such as yeast extract, Trypticase peptone, and mercaptoethansulfonic acid were omitted, and the concentration of $Na_2S \cdot 9H_2O$ was increased to 0.5 g/l. Vitamin supplement (7) was sometimes used to provide the organic growth factors. The vitamin mixture contained (μg/l): biotin, 20; folic acid, 20; pyridoxine hydrochloride, 100; thiamine hydrochloride, 50; riboflavin, 50; nicotinic acid, 50; pantothenate, 50; viatmin B_{12}, 1; *p*-aminobenzoic acid, 50; lipoic acid, 50.

Cultures with dimethyl sulfide and methane thiol were routinely grown in 300 ml of MSH medium contained in a 600-ml bottle. Large culture bottles minimize the effects of loss of those substrates by the absorption of butyl-rubber stoppers. Sulfide and most substrates were added from sterile anoxic stock solutions approximately 1 h prior to

inoculation. Dimethyl sulfide was added as concentrated liquid, and methane thiol was added as a gas. Although we did not measure the pH after addition of catabolic substrate, it is unlikely that addition of dimethyl sulfide or methane thiol changed the pH of the medium; dimethyl sulfide is not known to ionize, and methane thiol does not strongly ionize at the pH of the media (pK_a = 10.75 [22]). Inoculation was 10% in all the experiments. Cultures were tested in triplicate and incubated at 40°C.

Determination of optimal growth conditions

Specific growth rates were determined by measuring the amount of methane formed during growth (15). The specific growth rate during exponential growth was analyzed by linear regression of the logarithm of total methane accumulated (including inoculum-produced methane [107]) and time. When the effects of environmental parameters (pH, temperature, salt concentrations) were tested, growth rates were determined by using cultures adapted to those conditions. We transferred cultures under those conditions at least two or three times sequentially, and we continued making sequential transfers until the specific growth rate was stable. In particular, shifts to a medium of lower osmolarity sometimes caused cells to lyse, even when adapted cells later grew well in that medium. To obtain cultures at a lower osmolarity, it was sometimes necessary to transfer a culture in several steps to media having progressively lower osmolarities.

Preparation of crude cell-free extracts

Strain HI350 was grown in 13-liters of MSH medium with 50 mM methanol, 50 mM trimethylamine, or 30 mM dimethyl sulfide as catabolic substrate. Cells were harvested in the late exponential phase by continuous-flow centrifugation at 10,000 × *g*. A suspension of about 40% wet cells was made in O_2-free 20 mM $KHCO_3$ buffer (pH 7.2) and cells were broken by ultrasonification at 35 W for 5 to 10 min. After centrifugation at 20,000 × *g* for 30 min, the supernatant was used to measure the enzymatic activity for the degradation of dimethyl sulfide, methane thiol, trimethyamine or methanol in a cell-free assay. The assay mixture (final volume, 1.0 ml) contained 10 mM $MgCl_2$, 10 mM coenzyme M, 2 mM ATP, 4 mM titanous chloride, 8 mM sodium citrate (146). The enzyme reaction was started by adding 0.4 ml of cell-free extract (about 20 mg of total protein per ml) to the mixture and incubating at 40°C.

Analytical methods

Methane was determined by gas chromatography with flame ionization detection (71).

Whole-cell proteins were analyzed by SDS (sodium dodecylsulfate) denaturing slab gel electrophoresis (68) by using 35 to 40 μg of protein per lane. The proteins were prepared from exponential-phase cells by extraction with detergent buffer (68).

DNA was isolated from strain HI350 by using the method of Marmur (76).

William B. Whitman (University of Georgia) measured the DNA G + C content by high-performance liquid chromatography after enzymatic hydrolysis (81).

Carl Woese, University of Illinois, sequenced the 16S rRNA of strain T4/M^T by the reverse transcriptase method. He aligned the sequence in a sequence editor against a selected collection of archaeal 16S rRNA sequences (16, 143). Previously aligned near relatives of the new sequence, established secondary structural constraints, and sequence conservation patterns were used to guide the process (143). Corrected pairwise distances (expressed as estimated changes per 100 nucleotides) were computed from percent similarities using the Jukes and Cantor correction (42) as modified by Olsen (140). A dendrogram was constructed from the evolutionary distance matrix by the algorithm of De Soete (25).

Microscopy for colony observation and purity checks was done with an epifluorescence microscopy equipped with a type O2 filter set (Carl Zeiss, Inc., Thornwood, N. Y.). Cells for electron microscopy were fixed at room temperature for 30 min in 2.5% glutaraldehyde, buffered to pH 7.4 with 0.2 M sodium cacodylate. They were then post-fixed in osmium tetroxide for 30 min at 4°C, dehydrated and embedded in Spurr low viscosity resin. Sections were poststained with uranyl acetate and lead citrate, examined, and photographed with a Zeiss model EM-10 transmission electron microscope at 60 kV.

Dimethyl sulfide, methane thiol, and H_2S in the gas phase were determined by combined gas chromatography-mass spectrometry with sulfur hexaflouride (SF_6) as internal standard (35, 39). These compounds were separated on a SPB-1 SULFUR fused

capillary column 30 m long with 0.32 mm internal diameter, with 3 μm-film (Supelco) at 40°C with helium as carrier gas (linear velocity, 30 cm/s). Injection volume was 10 μl with a split ratio to the detector of 35:1. The ionizer of the mass detector was operated at 233°C. Standard gas mixtures were prepared by transferring gaseous dimethyl sulfide or methane thiol in a pressure-lock syringe at room temperature to a sealed serum bottle previously flushed with N_2. Concentrations of dimethyl sulfide, methane thiol, and H_2S were measured only in the gas phase. Henry's constants of those compounds in the culture medium were measured (90.3 mmol per liter of gas per molar concentration in the liquid for dimethyl sulfide, and 118 mmol/L/M). They were used to calculate the total quantity present in culture vessels (gaseous plus aqueous amounts) from the gaseous concentrations.

RESULTS

Strain HI350 was isolated from an oil well at High Island in the Gulf of Mexico. This strain was similar to *Methanolobus siciliae* T4/M^T. However, the previously published characterization of strain T4/M^T is limited to the determination of its temperature optimum and DNA G+C content, so both strain T4/M^T and strain HI350 were characterized .

Morphology

Surface colonies of strain HI350 were circular, convex with entire margins, and dark yellow, and reached a diameter of 2.0 mm in 2 weeks. The cells were irregular, coccoid with a diameter of 1.5 to 3.0 μm (Fig. 3A), and stained gram negative. During the early exponential phase, the cells occurred singly or in pairs. However, when these cultures were later transferred repeatedly in MSH medium with dimethyl sulfide as catabolic substrate, cells grew as massive clumps consisting of pseudosarcinae (Fig. 3B), typical of *Methanosarcina* species (74). Similar clumps were also observed in cultures growing on methanol or trimethylamine if relatively young cultures were transferred repeatedly during mid-exponential growth phase. These clumps could be disrupted into

smaller cell aggregates by physical shaking. Prolonged incubation after exhaustion of substrate induced spontaneous disaggregation, releasing single cells and causing the culture to become turbid. Media with pH below 7 favored clump formation. Electron microscopy of thin-sections, however, did not show continuous wall structure surrounding the pseudosarcinae as is often seen in *Methanosarcina barkeri*, but instead only amorphous wall material at the surface of single cells (Fig. 3C). Cells were not as tightly stuck together as typical *Methanosarcina* cells in their clumps. Strain T4/M^T had similar morphological characteristics to strain HI350. Both individual cells and massive cell clumps of strain HI350 and *Methanolobus siciliae* T4/M^T lysed immediately upon the addition of 2 g of sodium dodecylsulfate per liter, with no visible cellular framework remaining. This suggests that strains HI350 and T4/M^T had proteinaceous cell walls.

DNA composition

The DNA of strain HI350 had a G+C content of 42.80 ± 0.08 mol% and contained no significant modified adenine (-0.14 ± 0.06 mol%). Strain T4/M^T has a G+C content of 41.5 mol%, but the method used to determine this value was not reported (123).

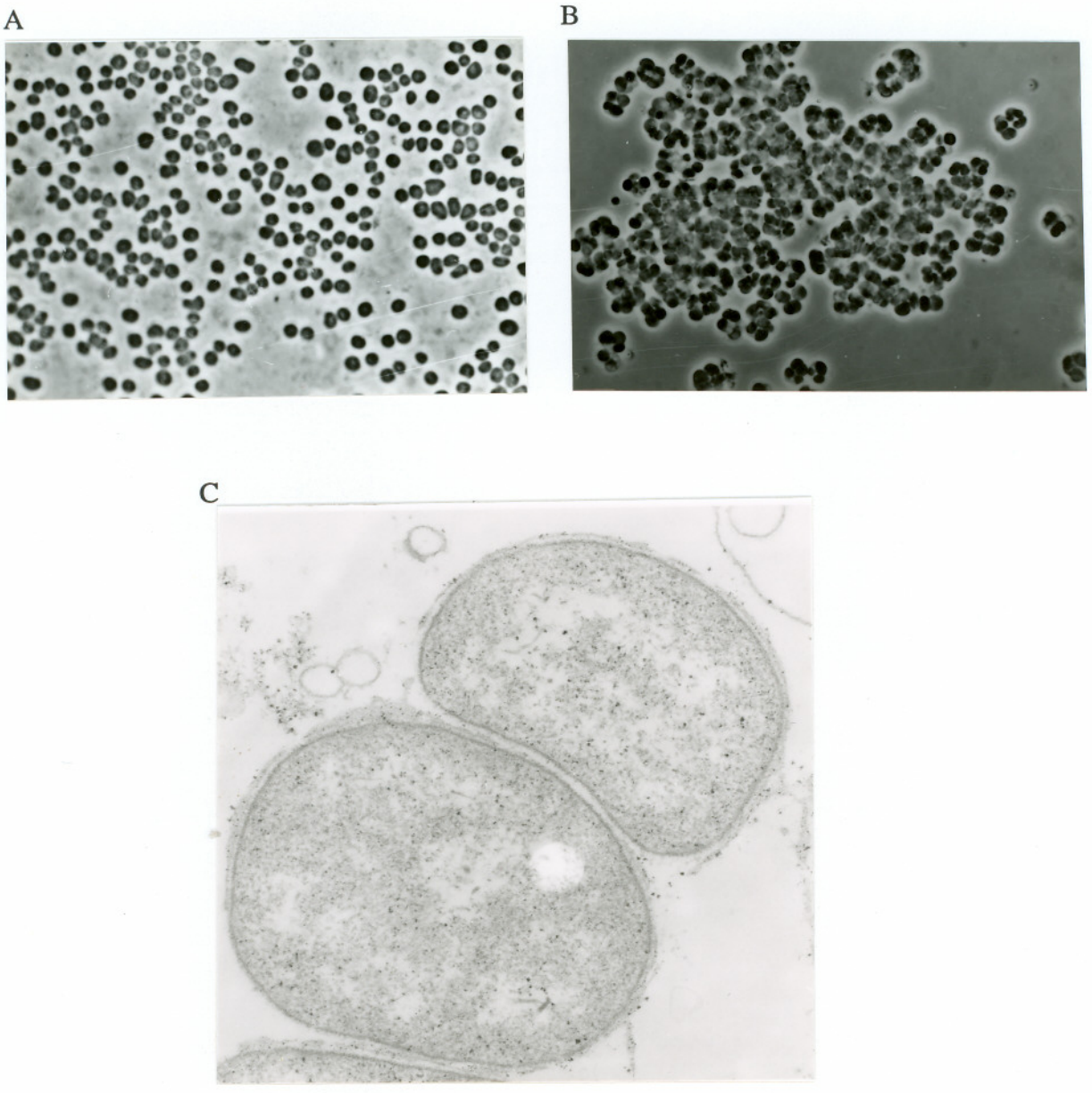


Figure 3. Morphology of strain HI350 shown by phase-contrast micrograph of single cells (A) and cell aggregates (B) of strain HI350, and by thin-section micrograph (C).

Phylogeny

The phylogenetic relationships of strain T4/M^T to other methanogenic strains is shown in Fig. 4. The 16S rRNA sequence similarity analysis indicates that *Methanolobus siciliae* T4/M^T is closely related to members of *Methanosarcina*, especially *Methanosarcina acetivorans* C2A^T. The sequence similarity between these two strains was 98.3%.

Physiology

Each strain exhibited good growth at pH values between 5.8 and 7.2 and grew most rapidly at pH 6.5 to 6.8 (Fig. 5). Figure 6 indicates that each strain required about 0.4 to 0.6 M Na^+ and 60 to 200 mM Mg^{2+} for fastest growth. Each strain required at least small quantities of both of these cations, with no growth occurring whenever one was absent. Figure 6 shows that the growth rates were high only when both Na^+ and Mg^{2+} were present. These cations are not only obligately required, but they also function together to moderate the water activity of the medium. Rapid growth required an Na^+ concentration of ≥80 mM and an Mg^{2+} concentration of ≥20 mM, with the total of the Na^+ and Mg^{2+} concentrations sufficient to provide a water activity between 0.985 and 0.96. When one of the cations was present in small

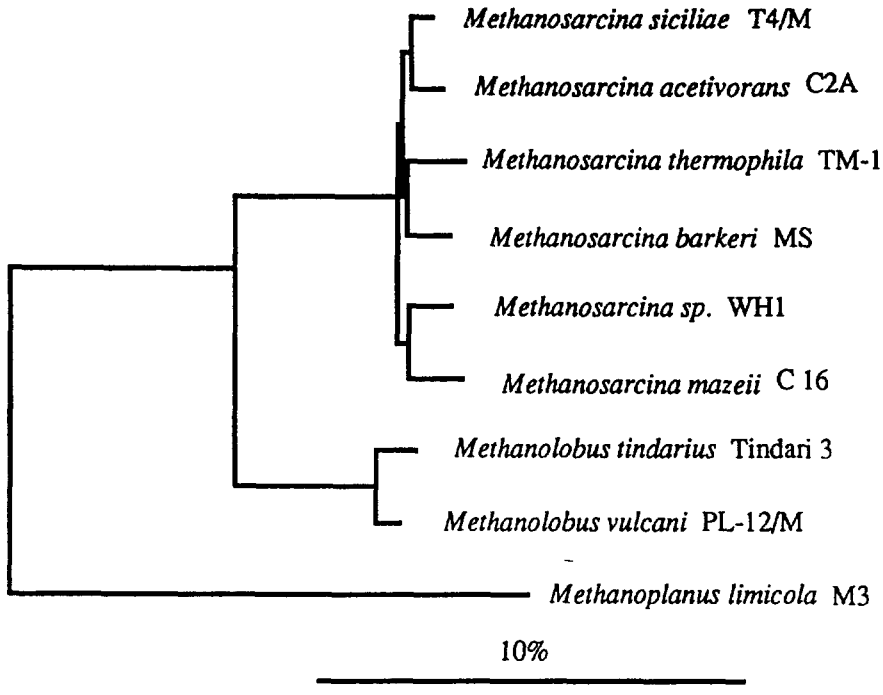


Figure 4 Phylogenetic relationships of *Methanolobus* and *Methanosarcina* species based on 16S rRNA sequences.

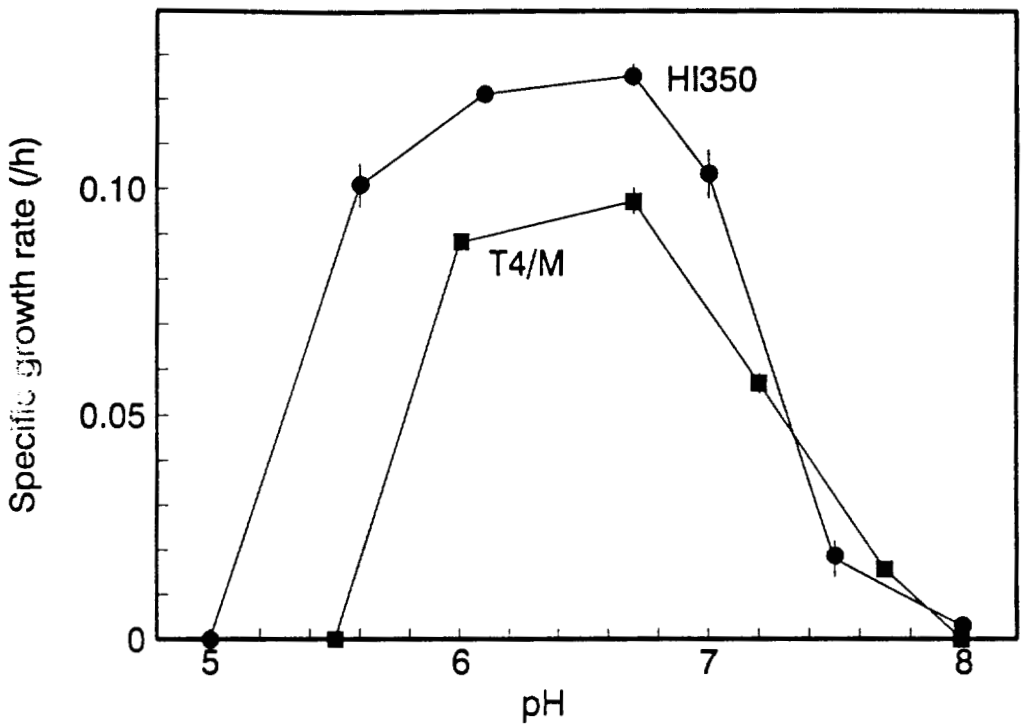


Figure 5. Effect of pH in the growth rates of strain HI350 (grown on 20 mM methanol) and T4/M^T (grown on 20 mM trimethylamine) in MSH medium.

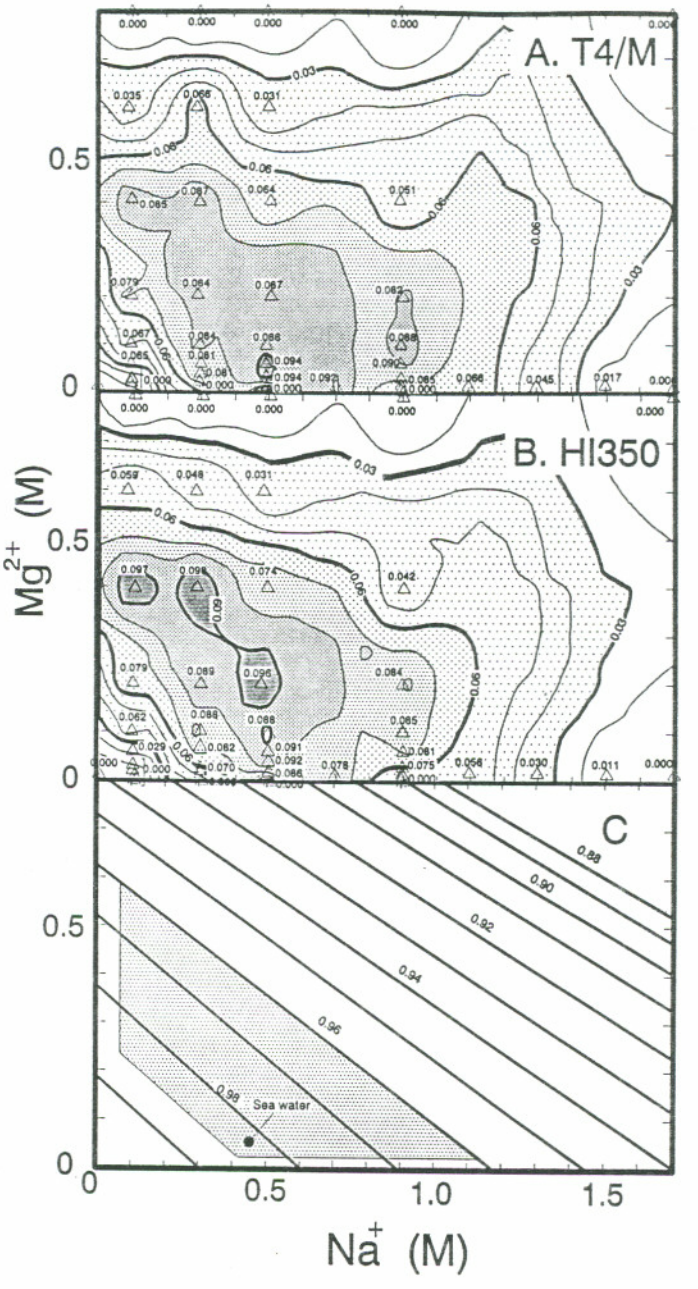


Figure 6 Effect of Na^+ and Mg^{2+} on the growth rates of adapted cultures of strains $T4/M^T$ (A) and HI350 (B). The areas with the darkest shading are those with the fastest growth. (A and B) Contour maps of the specific growth rates at various Na^+ and Mg^{2+} concentrations. Measured specific growth rates, marked with triangles, were used to calculate the contours of specific growth rates with the software Surfer (Golden Software, Inc., Golden, Colo.). (C) Water activities calculated for media containing various concentration of NaCl and $MgCl_2$.

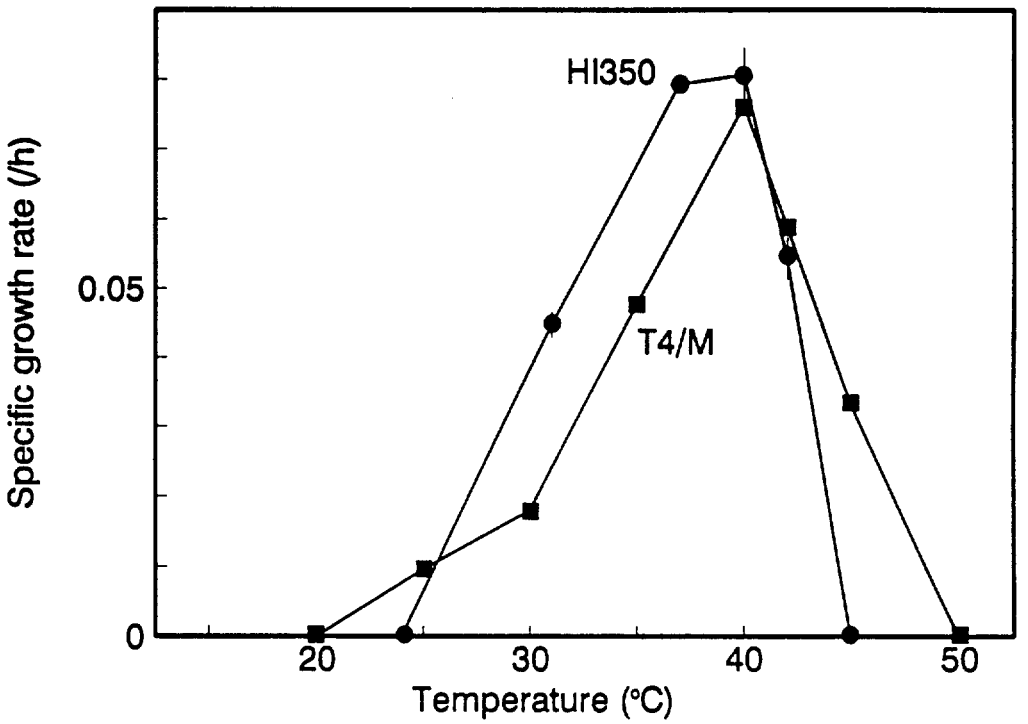


Figure 7 Effect of temperature on the growth rates of strains HI350 and T4/M^T in MSH medium containing 20 mM trimethylamine.

quantities, the other had to present in higher concentrations to provide the optimal water activity of the culture medium. The effects of Na^+ and Mg^{2+} on the growth rates of the nonmethanogenic halophiles *Halobacterium salinarium*, *Halobacterium halobium*, and *Halobacterium volcanii* have been attributed to other effects in addition to water activity, but the proportions of these two cations (whenever the concentration of each was above some minimum level and the water activity was optimal) had little effect on the specific growth rate of strain HI350.

Each strain was mesophilic, with fastest growth occurring at 40°C. No growth was observed at or above 50°C or at or below 20°C (Fig. 7). Previous determinations (123) indicated that the optimum temperature was 37°C and the maximum temperature for growth was 48°C. This maximum is consistent with our data for strain T4/M^T. Both strain T4/M^T and HI350 were able to grow in mineral medium supplemented with a single organic catabolic substrate, but yeast extract simulated growth (Fig. 8).

Growth of strain HI350 on dimethyl sulfide and methane thiol

In addition to methanol and trimethylamine, strain HI350 and strain T4/M^T grew on dimethyl sulfide or methane thiol as substrate as shown in the experiment described below. Each strain was grown in medium containing 5 mM trimethylamine plus 5 mM dimethyl sulfide and in medium containing 5 mM trimethylamine alone. Within 2 to 3 days, each of these cultures produced the quantity of methane stoichiometrically expected by trimethylamine degradation, and methane production stopped. After about 10 days,

the culture containing dimethyl sulfide began producing methane again, this continued until the quantity of methane predicted from dimethyl sulfide degradation was formed. When these cultures grown on dimethyl sulfide were inoculated into media with 2 mM methane thiol as the sole catabolic substrate, they grew exponentially as indicated by the logarithmic increase of CH_4 and obvious increase in turbidity of the cultures.

Initially, cultures of strain HI350 or T4/M^T growing on dimethyl sulfide had lags of about 2 weeks (Figure 9A). The lag was significantly shorter with methane thiol since trimethylamine-grown cultures began to produce methane after a lag of only 3 days when first transferred to 2 mM methane thiol. The lag before growth on dimethyl sulfide was unaffected by the initial concentrations of dimethyl sulfide between 2 mM and 10 mM. Similar lag was also observed when methanol-grown cells were first transferred to dimethyl sulfide or methane thiol. Methane production from either dimethyl sulfide or methane thiol was initially slow, with an estimated specific growth rate of 0.003 h^{-1} (about 3% as fast as growth on trimethylamine or methanol). The lag disappeared after one transfer on dimethyl sulfide when cells were transferred in late exponential phase. Growth rate of subsequent cultures increased to a maximum of 0.087 h^{-1} and 0.068 h^{-1} on 5 mM dimethyl sulfide and methane thiol, respectively. When cultures growing on 5 mM dimethyl sulfide were

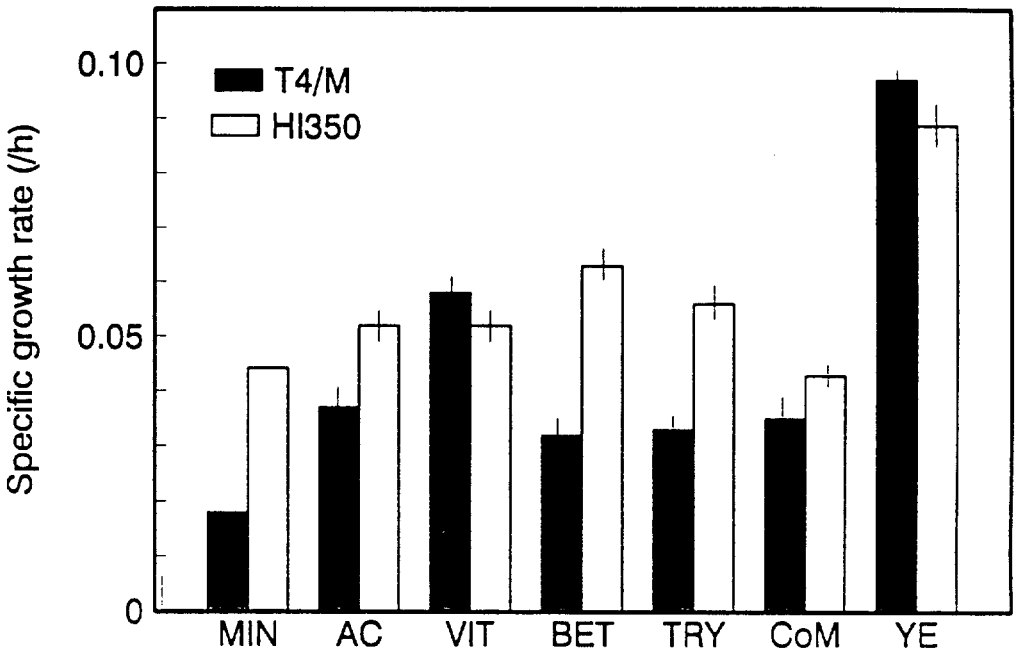


Figure 8. Growth rates of strains HI350 and T4/M^T in the presence of various additions to MMSH medium containing 20 mM trimethylamine. MIN, mineral (no addition); AC, 5 mM acetate; VIT, vitamin mixture; BET, glycine betaine; TRY, 2 g of Trypticase peptone per liter; YE, 2 g of yeast extract per liter; CoM, 0.5 g of mercaptoethanesulfonate per liter.

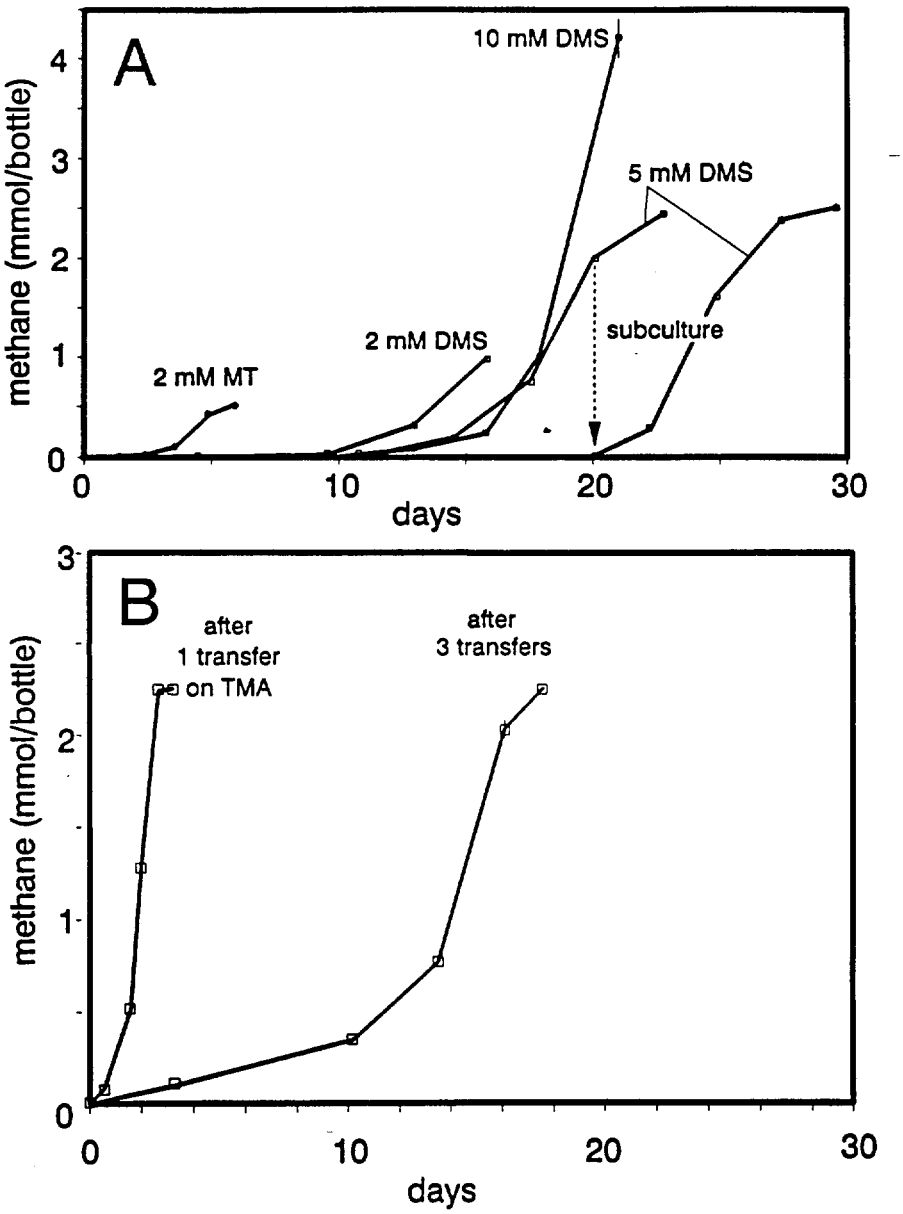


Figure 9. Adaptation of trimethylamine-grown strain HI350 to grow on dimethyl sulfide (DMS) and methane thiol (MT). (A). Cultures grown on 20 mM trimethylamine and not previously exposed to dimethyl sulfide or methane thiol were inoculated into media with dimethyl sulfide or methane thiol. After 20 days incubation, one of the replicate cultures in 5 mM dimethyl sulfide (in late exponential phase) was transferred to fresh medium with 5 mM dimethyl sulfide (indicated by dotted line and arrow). (B). Dimethyl-sulfide-grown cells were transferred and grown once or three times (10% vol/vol inoculum) in medium with 20 mM trimethylamine and then transferred back to medium with 5 mM dimethyl sulfide.

transferred (10% vol/vol) to medium with 50 mM trimethylamine as substrate, growth occurred without a lag (data not shown). When this culture was transferred in late exponential phase to medium with 5 mM dimethyl sulfide, dimethyl sulfide was catabolized without a lag (Fig. 9B). However, if the culture was transferred 3 or more times in medium with trimethylamine, a long lag preceded the utilization of dimethyl sulfide (Fig. 9B). Most surprisingly, this organism could be adapted to high concentrations of dimethyl sulfide or methane thiol. An unadapted (trimethylamine-grown) culture showed no growth on 5 mM methane thiol, but after gradual adaptation, it grew on 25 mM methane thiol and 40 mM dimethyl sulfide. Although cultures grew rapidly on 40 mM dimethyl sulfide, they ceased producing methane before the stoichiometrically expected methane was formed. It is possible that in the latter stages of growth, these cultures were inhibited by accumulating sulfide.

Utilization of dimethyl sulfide and methane thiol by other known methanogens

The following methanogens were selected from different methylotrophic genera and tested: *Methanococcoides methylutens* TMA-10, *Methanolobus tindarius* Tindari 3, *Methanolobus vulcani* PL-12/M, *Methanohalophilus mahii* SLP, *Methanohalophilus halophilus* Z-7982, *Methanosarcina mazeii* LYC, *Methanosarcina acetivorans* C2A^T, and *Methanosarcina frisia* C 16. Trimethylamine-grown cultures were inoculated into medium with 5 mM dimethyl sulfide, as well as three control media with the following catabolic substrates: (i) none; (ii) 5 mM trimethylamine; and (iii) 5 mM trimethylamine

plus 5 mM dimethyl sulfide. For *Methanosarcina acetivorans* C2A^T, within days, cultures with trimethylamine produced approximately the quantity of methane stoichiometrically expected from trimethylamine. After a lag of about one week, bottles with dimethyl sulfide began to produce methane, and this continued until the methane produced was approximately equal to that expected stoichiometrically from the dimethyl sulfide. Methane production was accompanied by an obvious increase in the turbidity of the medium and an increase in cells visible by epifluorescence microscopy. This finding indicates that *M. acetivorans* can degrade dimethyl sulfide. For all other strains, cultures with trimethylamine plus dimethyl sulfide produced similar amounts of methane as cultures with trimethylamine alone; no extra quantities of methane was formed in cultures with dimethyl sulfide only as compared to those without substrate. Thus, none of those strains were able to catabolize dimethyl sulfide. Thus, only a few obligately methylotrophic halotolerant species were capable of catabolizing dimethyl sulfide.

Strain GS-16 grows on dimethyl sulfide, but it was reported to be unable to grow on methane thiol (97). After *Methanolobus siciliae* HI350 was found to grow on methane thiol, strain GS-16 was tested again by inoculating cultures grown on dimethyl sulfide into medium with 5 mM methane thiol. The cultures grew and produced methane in amounts expected by the stoichiometry. When these cultures were subcultured into medium with 5 mM methane thiol, exponential growth occurred. Differences between these results and earlier reports may be due to differences in media or incubation temperature.

Degradation of dimethyl sulfide and methane thiol by strain HI350

Formation of H_2S and CH_4 was monitored during growth of strain HI350 on dimethyl sulfide or methane thiol. Dimethyl sulfide or methane thiol were completely degraded, with expected amounts of H_2S and CH_4 formed (Figs. 10 and 11) according to the following equations:

$$2CH_3SCH_3 + 2H_2O \rightarrow CO_2 + 3CH_4 + 2H_2S \quad (4)$$

$$(\Delta G^{\circ'} = -73.8 \text{ kJ/mol})$$

$$4CH_3SH + 2H_2O \rightarrow 4H_2S + CO_2 + 3CH_4 \quad (5)$$

$$(\Delta G^{\circ'} = -36.6 \text{ kJ/mol})$$

In addition, small amounts of methane thiol were formed during dimethyl sulfide degradation and even smaller amounts of dimethyl sulfide formed during methane thiol degradation.

Catabolism of dimethyl sulfide and methane thiol was further studied by using crude cell-free extracts in the assay mixture contained ATP, titanous citrate, and HS-CoM. ATP and titanous citrate were required for enzymatic activity of methanol degradation in the cell-free extract. Perhaps, titanous citrate acted as a reducing agent for activation of some enzyme systems such as methyltransferase and methyl-CoM reductase. Methane was formed in the cell-free system only when HS-CoM was added. Activity of methane formation was low.

Only the cell-free extract prepared from dimethyl-sulfide- or methane-thiol-grown cells showed enzymatic activities for dimethyl sulfide and methane thiol degradation, and

produced methane from dimethyl sulfide and methane thiol (Fig. 12-13). Cell-free extracts of either methanol- or trimethylamine-grown cells showed no enzymatic activity with either of dimethyl sulfide or methane thiol. However, activities for methanol degradation were present in cell-free extracts of all the substrates (Fig. 14). Similarly, cell-free extracts from trimethylamine, methanol, dimethyl sulfide and methane thiol produced methane from trimethylamine (data not shown). These observations were consistent with ^{14}C-labelling study (97).

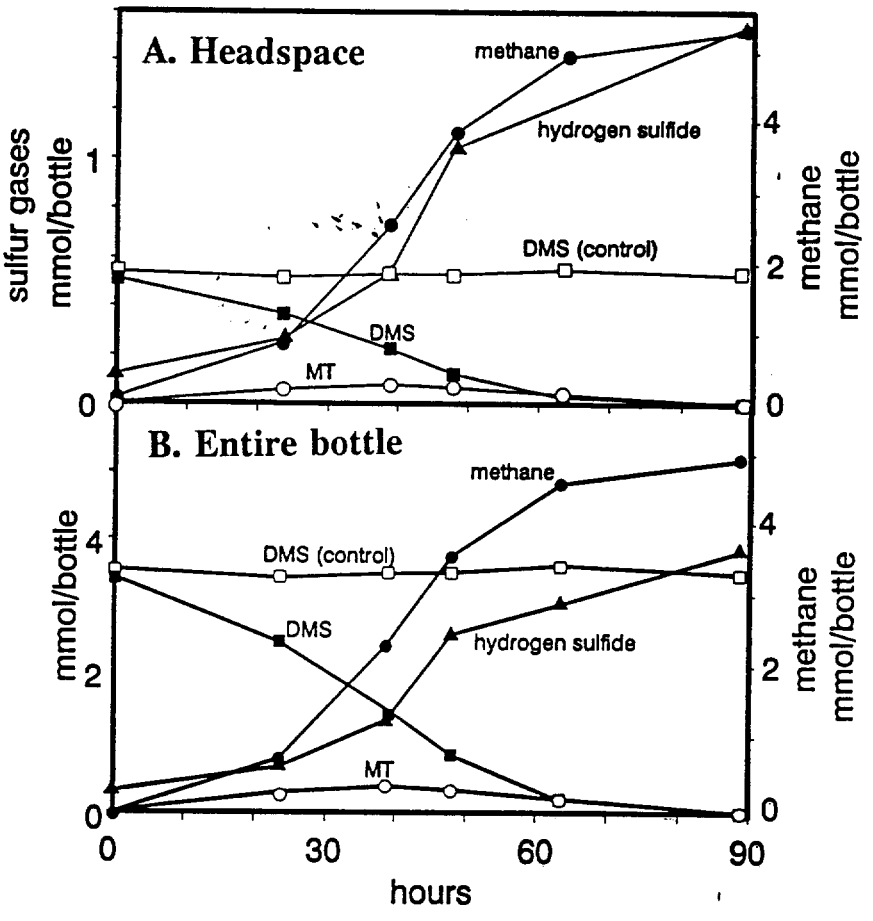


Figure 10. Growth of dimethyl-sulfide-adapted cultures of strain HI350. Total gases measured (A) in the headspace of cultures growing on dimethyl sulfide, or dimethyl sulfide in an uninoculated control, and (B) in the entire bottle (with dissolved gases calculated from the measured gas-phase concentration from Henry's constants of each constituent, which we measured in identical culture medium).

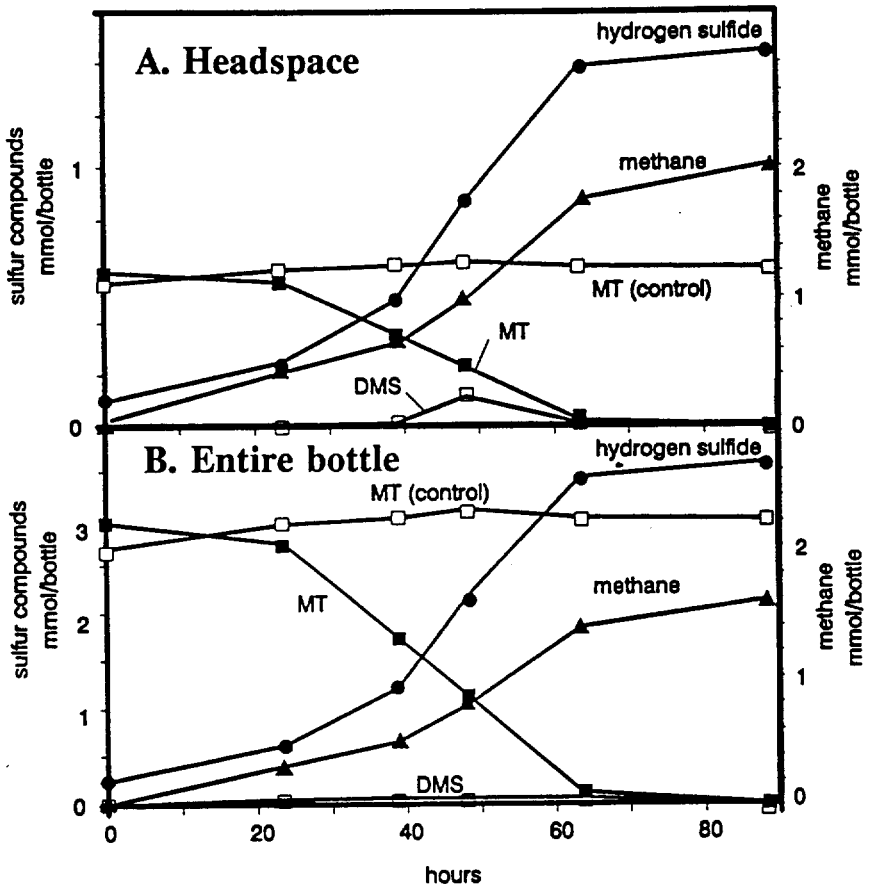


Figure 11. Growth of methane-thiol-adapted cultures of strain HI350. Total gases measured (A) in the headspace of cultures growing on methane thiol, or methane thiol in an uninoculated control, and (B) in the entire bottle (with dissolved gases calculated from the measured gas-phase concentration).

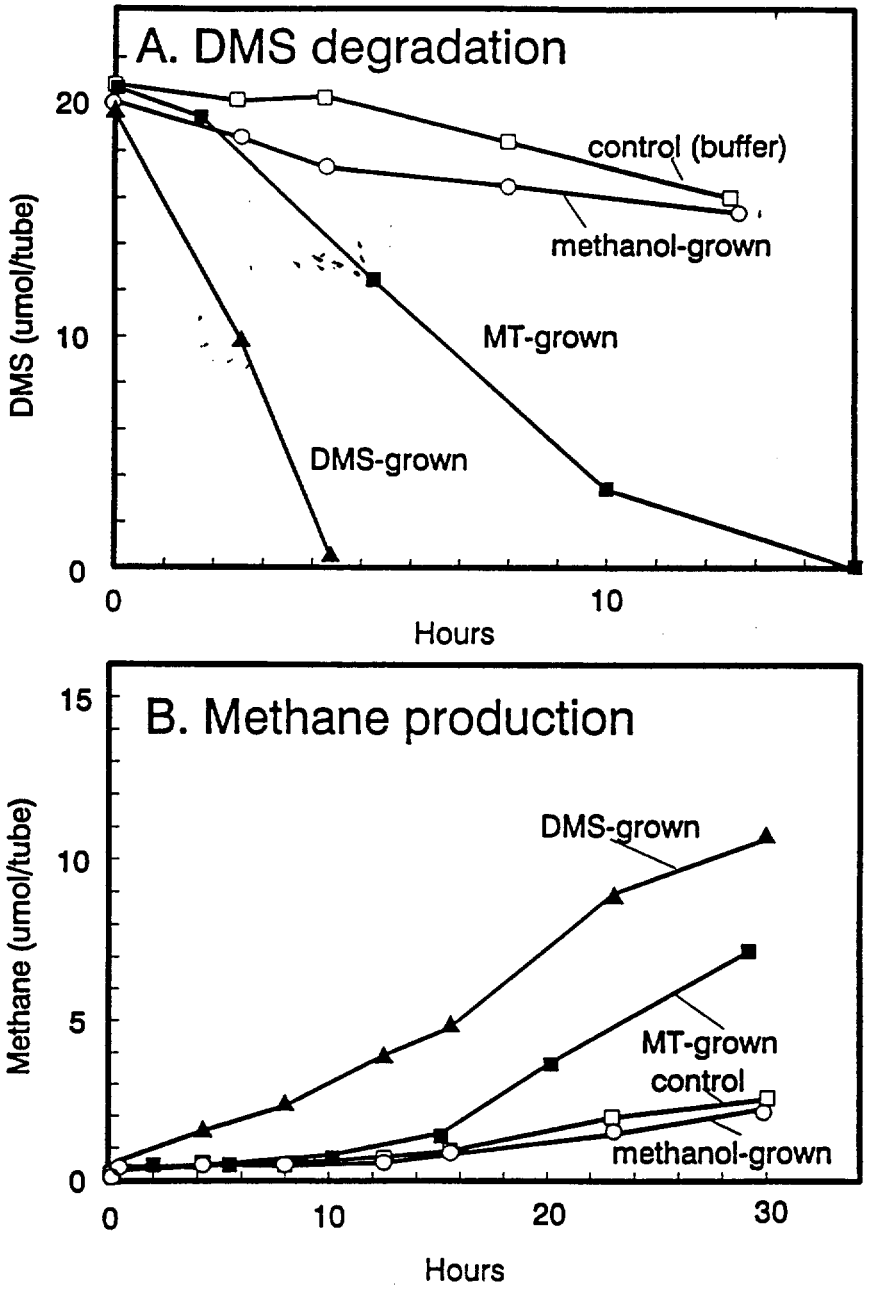


Figure 12. Dimethyl sulfide degradation and CH_4 production by cell-free extracts prepared from cells grown methanol, dimethyl sulfide, and methane thiol.

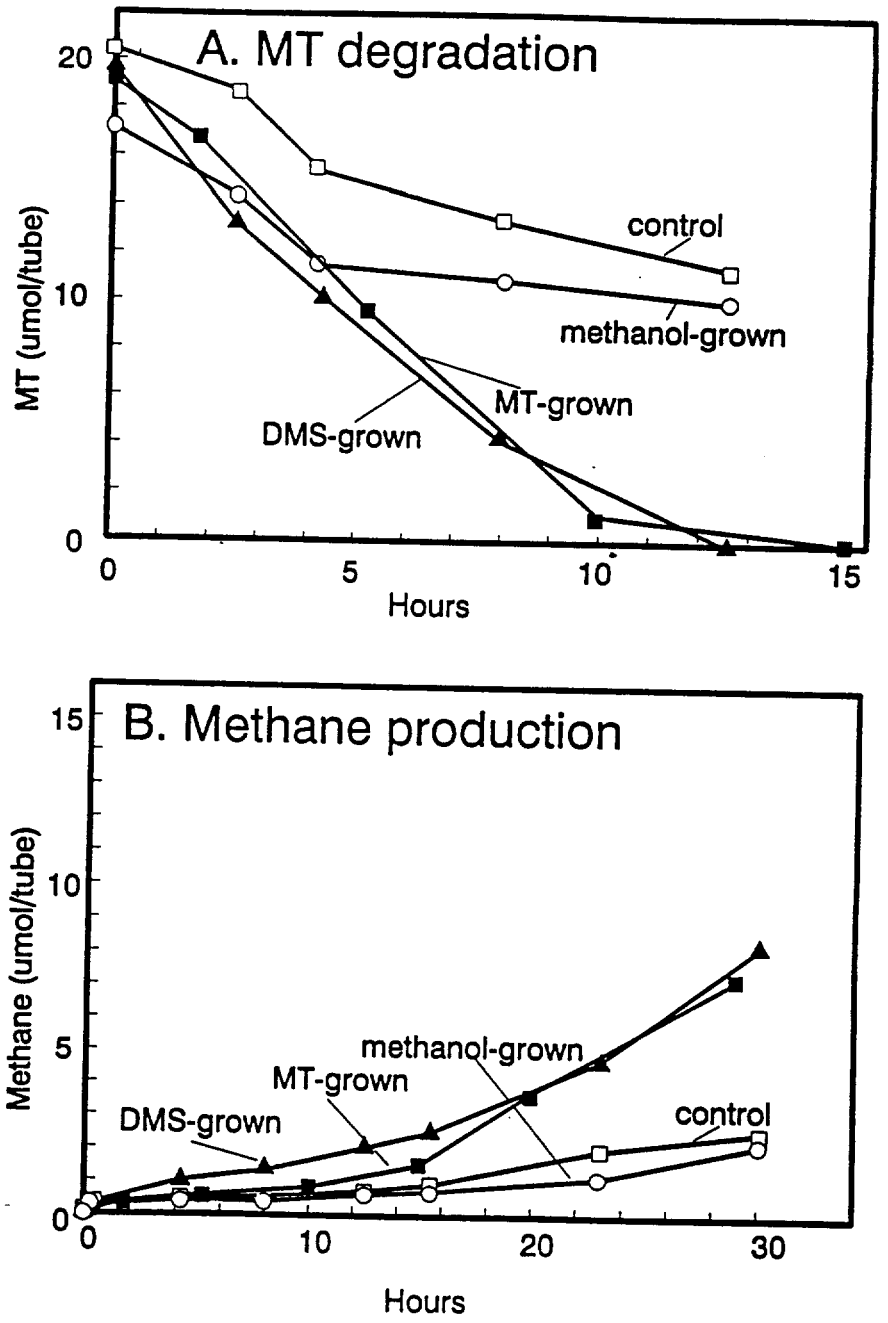


Figure 13. Methane thiol degradation and CH_4 degradation by cell-free extracts of methanol-, dimethyl-sulfide-, and methane-thiol-grown cells of strain HI350.

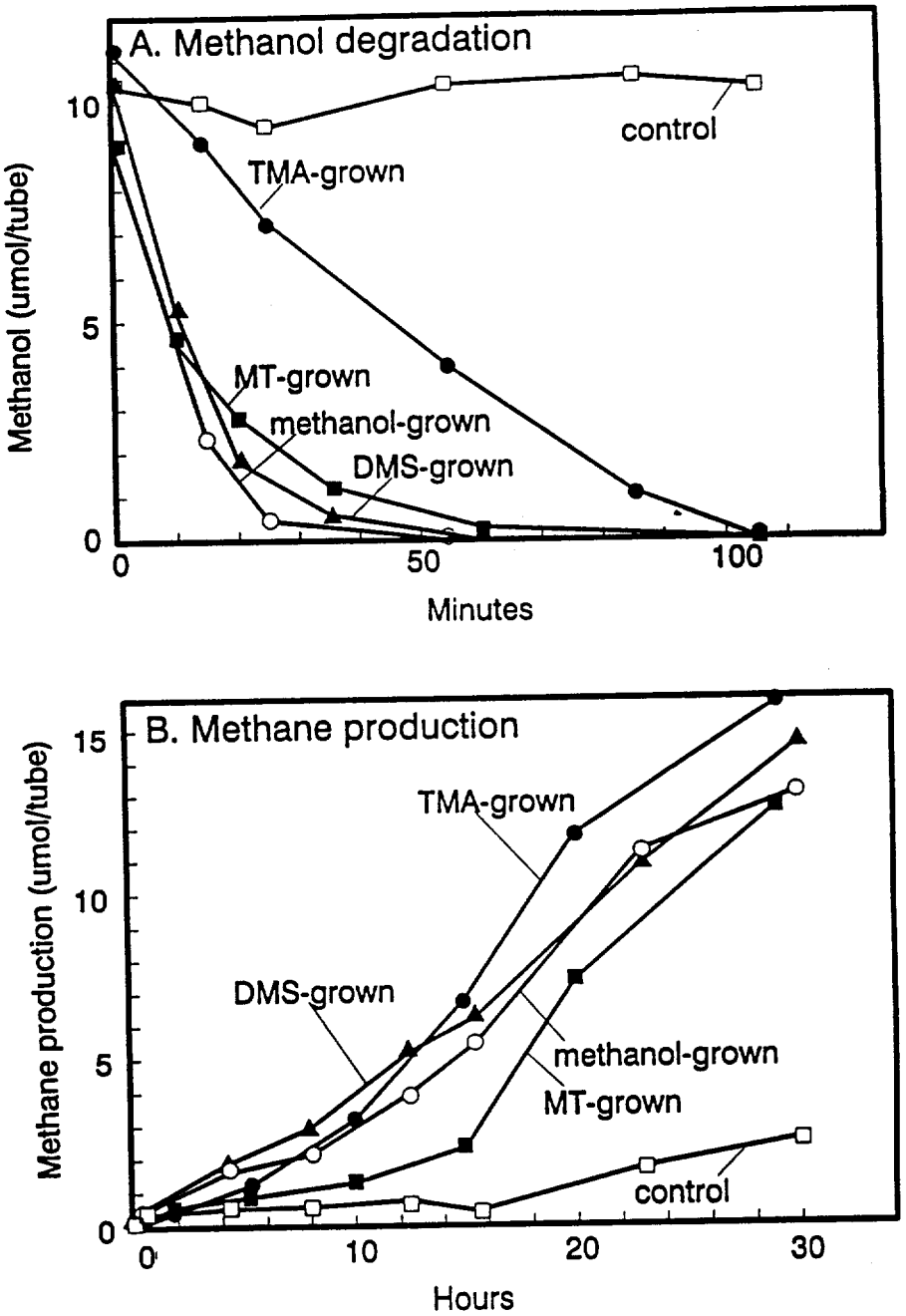


Figure 14. Methanol degradation and CH_4 production by cell-free extracts of strain HI350 cells grown on methanol, trimethylamine (TMA), dimethyl sulfide, and methane thiol.

DISCUSSION

Taxonomy of strains HI350 and T4/M^T

Physiologically, strain HI350 differs from members of genera *Methanohalobium*, *Methanohalophilus*, and *Methanococcoides*. *Methanohalobium evestigatum*, the only species in its genus, requires 4.2 M of NaCl for best growth. The genus *Methanohalophilus* contains three moderately halophilic species: *Methanohalophilus zhilinaeae* is alkaliphilic (77), and the other two species, *Methanohalophilus mahii* (104) and *Methanohalophilus halophilus* (70), require higher salinity (1.2 M Na^+) than strain HI350 does. *Methanococcoides methylutens* TMA-10^T was unable to use dimethyl sulfide. Thus, strain HI350 does not belong in these genera.

The genus *Methanolobus* contains five species. *Methanolobus tindarius* and *Methanolobus vulcani* could not use dimethyl sulfide. *Methanolobus oregonensis* and *Methanolobus taylorii* can use dimethyl sulfide (and methane thiol for strain GS-16), but they prefer to grow at alkaline pH (above 8.0) (68 ,97). Strain HI350 cannot grow at such high pH. Therefore, strain HI350 could not be a member in either of the species *Methanolobus tindarius*, *Methanolobus vulcani*, *Methanolobus oregonensis* or *Methanolobus taylorii.*

Strain HI350 is similar to strain T4/M, the type strain of *Methanolobus siciliae*, in various aspects such as cell size, absence of flagella, substrate range, optimal growth conditions, and nutritional requirements. This suggests that strain HI350 might be a strain of *Methanolobus siciliae*. However, the species *Methanolobus siciliae* was proposed for strain T4/M only based on its limited characterization (94). Result of 16S rRNA sequence comparison in this study indicated that *Methanolobus siciliae* T4/M^T was phylogenetically more closely related to *Methanosarcina* species than to *Methanolobus*.

Strains HI350 and T4/M^T often grew as pseudosarcinae. This morphological characteristic was more typical of members of the genus *Methanosarcina* than members of the genus *Methanolobus*. Cells of both strains had proteinaceous cell walls, and their pseudosarcinae lacked a common external wall of heteropolysaccharide often seen in systs of *Methanosarcina barkeri*. These morphological characteristics, however, was similar to *Methanosarcina acetivorans* C2A^T and *Methanosarcina mazeii* Gö1 and C16 (= *Methanosarcina frisia* C 16^T [72]). Phylogenetically, *Methanolobus siciliae* was also closely related to *Methanosarcina acetivorans*. The 16S rRNA sequences of their type strains exhibit 98.3% similarity. This level of difference is typical of closely related species (16) or even strains within the same species. Therefore, we proposed to transfer *Methanolobus siciliae* to the genus *Methanosarcina*, naming it *Methanosarcina siciliae* (93).

Methanosarcina acetivorans and *Methanosarcina siciliae* differ physiologically. *Methanosarcina acetivorans* C2A^T can grow on acetate in addition to methanol, methylamines (122) and dimethyl sulfide. This strain grows faster in medium with 0.1

M NaCl than 0.5 M NaCl, so this species is not halophilic (72, 122). Thus, in the absence of DNA-DNA reassociation data, we consider *Methanosarcina siciliae* and *Methanosarcina acetivorans* to be separate species of *Methanosarcina*.

With the further characterization of strain T4/M^T and the genus transfer from *Methanolobus* to *Methanosarcina*, we proposed (93) the emended description of the species *Methanosarcina siciliae* given below:

Surface colonies are dark yellow, circular, and convex with entire edges and attain a diameter of 2.0 mm in 2 weeks. Cells are irregular, nonmotile, and coccoid (diameter, 1.5 to 3.0 μm), occur singly, in pairs or sometimes large aggregates of pseudosarcinae. They stain gram negative. They are lysed by 1 g of sodium dodecyl sulfate per liter.

Trimethylamine, methanol, dimethyl sulfide, and methane thiol are catabolic substrates, but H_2-CO_2, formate, and acetate are not. No organic compounds other than a catabolic substrate are required for growth, but yeast extract stimulates growth.

Sodium and magnesium are required for growth. Growth is most rapid in the presence of 400 to 600 mM Na^+, in the presence of 60 to 200 mM Mg^{2+}, at pH 6.5 to 6.8, and at 40°C.

The G+C content of the DNA is 42 to 43 mol%.

The known habitats are anoxic marine sediments and saline subsurface sediments. Strain T4/M (=OCM 156 = DSM 3028), the type strain, was isolated from marine sediment, and strain HI350 (= OCM 210) was isolated from an oil well.

All previous *Methanosarcina* strains are nonhalophilic and catabolize either acetate or H_2-CO_2 (or both) in addition to methanol and methylamines. With the inclusion of

Methanosarcina siciliae in this genus, however, these characteristics cannot be considered as the diagnostic criteria of the genus *Methanosarcina* (74). Thus, we also emend the description of this genus to include slightly halophilic, nonaceticlastic, obligate methylotrophs. The range of catabolic substrates for *Methanosarcina* species is also expanded to include dimethyl sulfide and methane thiol.

Catabolism of Dimethyl Sulfide and Methane Thiol

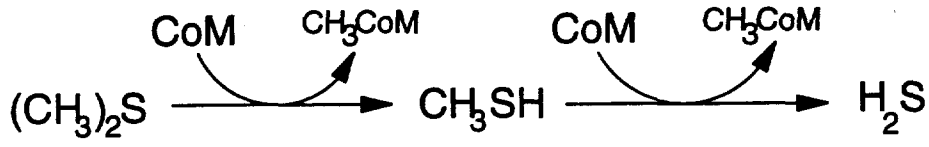
Dimethyl sulfide can be used by only a few species of obligately methylotrophic methanogens (Table 2). These methylated sulfides were completely degraded to methane, hydrogen sulfide, and (presumably) carbon dioxide. When dimethyl sulfide was degraded by strain HI350, small amounts of methane thiol was detected. Dimethyl sulfide was probably degraded via methane thiol as intermediate (equations 6 and 7) since degradation of methane thiol by strain HI350 culture grown on dimethyl sulfide proceeded without a lag. In addition, strain HI350 had shorter lag phase on methane thiol than dimethyl sulfide, indicating that methane thiol was metabolized faster than dimethyl sulfide. This also makes it less likely that methane thiol is converted to dimethyl sulfide first.

$$(CH_3)_2S + \tfrac{1}{2}H_2O \rightarrow CH_3SH + \tfrac{1}{4}CO_2 + \tfrac{3}{4}CH_4 \quad (6)$$

$$CH_3SH + \tfrac{1}{2}H_2O \rightarrow H_2S + \tfrac{1}{4}CO_2 + \tfrac{3}{4}CH_4 \quad (7)$$

Enzymes for the catabolism of dimethyl sulfide and methane thiol were inducible. This was indicated by the absence of activities on dimethyl sulfide and methane thiol in cell-free extracts prepared from either methanol- or trimethylamine-grown cells of strain

HI350. This was also consistent with the long lags we observed. Those inducible enzymes might include dimethyl sulfide:HS-CoM methyltransferases or methane thiol:HS-CoM methyltransferase. Such enzymes transfer methyl group from dimethyl sulfide or methane thiol to the methyl carrier HS-CoM to form CH_3-CoM:



(8)

The methyltransferases induced by either dimethyl sulfide or methane thiol were active on both dimethyl sulfide and methane thiol. In *Methanosarcina barkeri*, the inducible trimethylamine:HS-CoM transferase is also active on di- or monomethylamine (90).

Another less likely possibility is that dimethyl sulfide and sulfide are first converted to two methane thiol molecules and that methane thiol is the precursor for methanogenesis (equations 9 and 10):

$$(CH_3)_2S + H_2S \rightarrow 2CH_3SH \quad (9)$$

$$2CH_3SH + H_2O \rightarrow 2H_2S + \tfrac{1}{2}CO_2 + 1\tfrac{1}{2}CH_4 \quad (10)$$

In this case, methane thiol could still serve as an intermediate. However, this pathway would require a dimethyl sulfide:hydrogen sulfide methyltransferase, which could not be induced by methane thiol. Yet, cell-free extract prepared from methane-thiol-grown HI350 was also able to degrade dimethyl sulfide as well as methane thiol.

Table 2. Physiological characteristics of dimethyl-sulfide-using methanogens

Species	Substrates[a]	Optimal Growth Conditions			Growth factors[b]
		°C	pH	NaCl (M)	
Methanosarcina siciliae	MeOH, TMA, DMS, MT	38-40	6.5-6.8	0.5	(YE)
Methanosarcina acetivorans[c]	MeOH, TMA, DMS, Ace	35-40	6.5-7.0	0.1-0.2	(YE, Casamino acids)
Methanolobus oregonensis[c]	MeOH, TMA, DMS	40	8.2-8.6	0.5	thiamine, (YE)
Methanolobus taylorii	MeOH, TMA, DMS, MT	40	8.2	0.5	biotin
Methanohalophilus zhilinaeae[c]	MeOH, TMA, DMS	45	9.2	0.7	none

[a]Substrates: MeOH = methanol; TMA = trimethylamine; DMS = dimethyl sulfide; MT = methane thiol; Ace = acetate.
[b]Growth factor: YE = yeast extract. Parentheses indicate that factors are stimulatory for growth.
[c]These strains were not tested on MT.

PART II

POTASSIUM EXTRUSION BY THE MODERATELY ALKALIPHILIC METHYLOTROPHIC METHANOGEN *Methanolobus taylorii* GS-16 FOR THE POSSIBLE ROLE OF pH HOMEOSTASIS

Portions of these results were submitted in an abstract to the 93rd General Meeting of the American Society for Microbiology, Las Vegas, Nevada, 1994.

BACKGROUND

Bioenergetics and ion transport in methylotrophic methanogens

Methanogens synthesize ATP by coupling their exergonic catabolic reactions with electron transport phosphorylation (11, 12). Electrons generated from the oxidation of substrates are passed along membrane-bound electron carriers with increasing redox potentials to a terminal acceptor. The flow of electrons through this electron transport chain is accompanied by the extrusion of protons from the inside of the bacterial cell to the outside medium. As a result, a transmembrane electrical potential and proton chemical gradient are formed. These two components make up the proton-motive force which drives the influx of protons into the cells through ATP synthase to generate ATP.

Thermodynamically, methyl-CoM reduction to CH_4 is the most favorable reaction in the CO_2 reduction pathway. This reaction consists of two partial reactions. At first it was thought that the reduction of methyl-coenzyme M with H-S-HTP is the site of energy conservation. This idea was abandoned when evidence became available that the reduction of CoM-S-S-HTP with H_2 by H_2-dependent heterodisulfide reductase system is responsible for the formation of the proton-motive force (23). Methylotrophic methanogens derive the reducing equivalant from the oxidation of methyl group. They

contain a F_{420}-dependent heterodisulfide reductase system. Oxidation of $F_{420}H_2$ is catalyzed by a membrane-bound $F_{420}H_2$ dehydrogenase. This enzyme has been isolated from *Methanolobus tindarius* (24). Its apparent molecular mass of the native enzyme is 120 kDa, consisting of five different subunits of 45, 40, 22, 18, and 17 kDa. It contains 16 mol Fe and 16 mol acid-labile S per mol of enzyme, but flavin is not detectable. The electrons from the oxidation of $F_{420}H_2$ pass through some unknown electron carriers (47) to the heterodisulfide reductase (Fig. 15). Unfortunately, the membrane-bound heterodisulfide reductase has not been purified from obligately-methylotrophic methanogens, and the exact site of proton pumping is not located in this system.

In addition to the coupling of proton-motive force with the ATP synthesis, methanogensis from methanol and possibly other methylated substrates also involves an electrochemical Na^+ gradient which is coupled to the endergonic conversion of the methyl group of methanol or other methylated compounds to CH_3-H_4MPT (87). The Na^+ gradient is probably generated by a secondary Na^+ transport via Na^+/H^+ antiporter (86). Na^+/H^+ antiporter activity is detected in *Methanosarcina barkeri* by measuring the Na^+-dependent acidification of a weakly buffered cell suspension (86, 117). Addition of NaCl to such suspensions results in a sudden drop of the extracellular pH. Similar activity can also be elicited by the addition of Li^+. Furthermore, the acidification is inhibited by amiloride and harmaline, compounds known to inhibit the Na^+/H^+ antiporter in eucaryotic cells.

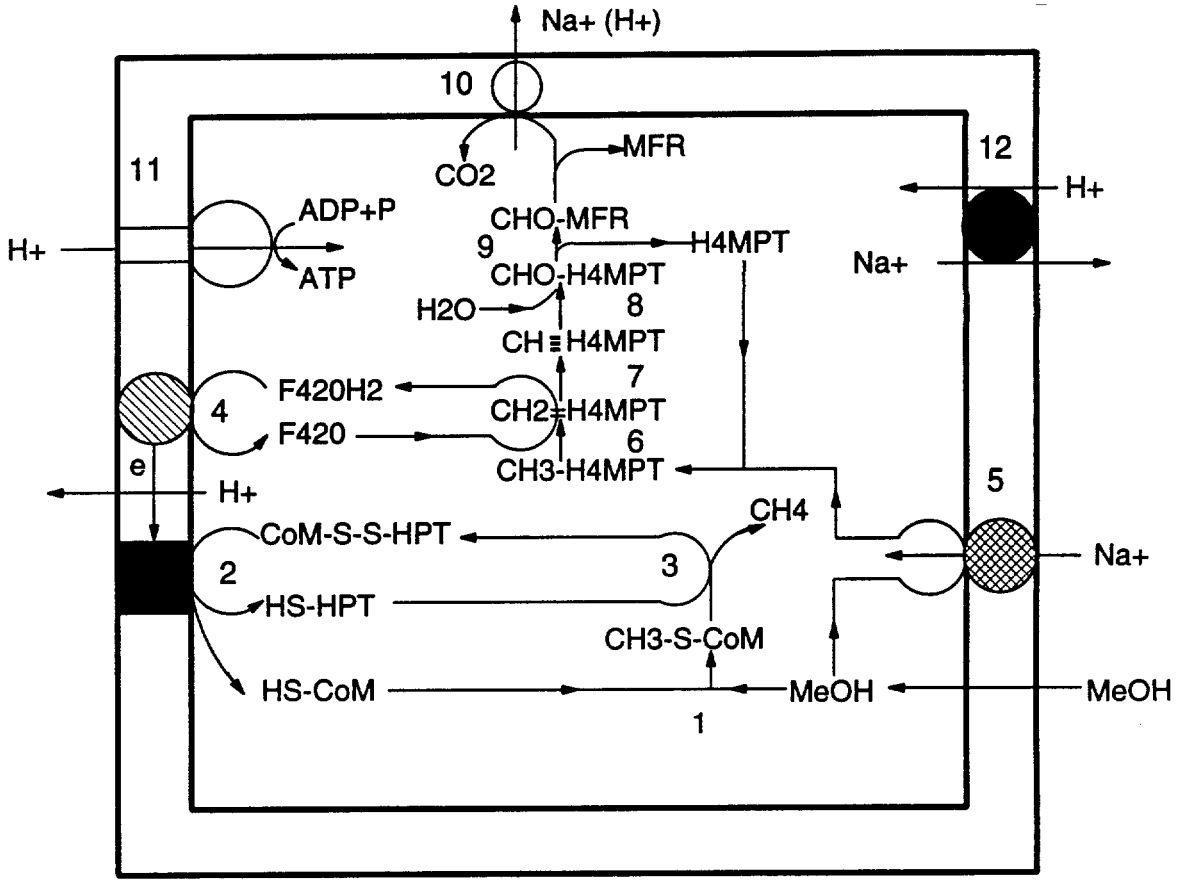


Figure 15. Energy coupling in *Methanosarcina barkeri* during methanogenesis from methanol (29).

1. MeOH:HS-CoM methyltransferase
2. F_{420}-dependent CoM-S-S-HTP heterodisulfide reductase
3. CH_3-S-CoM reductase
4. $F_{420}H_2$ dehydrogenase
5. CH_3-H_4MPT:HS-CoM methyltransferase ?
6. CH_2=H_4MPT reductase
7. CH_2=H_4MPT dehydrogenase
8. CH≡H_4MPT cyclohydrolase
9. CHO-MFR:H_4MPT transferase
10. CHO-MFR dehydrogenase
11. F_0F_1 ATP synthase
12. Na^+/H^+ antiporter

Homeostasis of pH in nonmethanogenic bacteria

There is substantial evidence that eubacteria maintain their cytosolic pH within a relatively narrow range, near neutrality. The cytosolic pH of most acidophilic bacteria is 6.0 to 7.0, but the cytosolic pH is 7.5 to 8.0 for neutrophilic bacteria, and 8.5 to 9.0 for alkaliphilic bacteria (5). In order to keep the cytosolic pH near neutral during growth at extremes of external pH, cells must modify their transmembrane pH gradient (ΔpH). The chemiosmotic model (83) indicates that the proton-motive force comprises the ΔpH together with the electric membrane potential ($\Delta\psi$). The proton-motive force is usually coupled to ATP synthesis and thus remains relatively stable, regardless of the external pH. Therefore, cells compensate deviations in ΔpH by adjusting the magnitude of their $\Delta\psi$ through the activity of various cation transport systems, including both uptake and extrusion of specific cations (6, 61).

Electrogenic K^+ uptake by *Escherichia coli* decreases the $\Delta\psi$ generated by H^+ extrusion via the proton pumps of the cells. This depolarization allows the cells to extrude more protons, causing the cells to maintain a cytosolic pH slightly more alkaline than the medium (5, 6, 17, 61). An extreme situation occurs in acidophilic bacteria. At about pH 2, the electrogenic uptake of K^+ is so strong that the cells develop reversed $\Delta\psi$ (interior positive). This allows the cells at the existing proton-motive force of 220 mV to maintain their internal pH value close to 6 (5).

At alkaline pH values, on the other hand, cells must acidify their cytosol so that they can maintain the cytosolic pH near neutral. This results in a reversed pH gradient.

In order for the proton-motive force to be strong enough for ATP synthesis by ATPase under these conditions, the $\Delta\psi$ must be very strong with the outside of the cell positive. Cells may achieve such strong $\Delta\psi$ by extruding cations. A Na^+/H^+ antiporter was proposed for pH homeostasis in *E. coli* under alkaline conditions (101, 118, 119). However, the Na^+/H^+ antiporter mutants without the antiporter genes of *nhaA* and *nhaB* (94, 100) are able to grow at alkaline pH even in the absence of Na^+. Alternatively, a K^+/H^+ antiporter could play a role in the acidification of cytosolic pH of *E. coli* (18, 62). The antiporter has an alkaline pH optimum between 7.8 and 8.2, consistent with a role in adjusting the cytosolic pH, and resting cells of *E. coli* extrude K^+ when the external pH is high (145). Mutants lacking this antiporter cannot grow at alkaline pH (105).

Although it remains controversial whether Na^+ or K^+ ions are exchanged with protons for the cytosolic acidification by neutrophilic organisms such as *E. coli* under alkaline conditions, different organisms appear to use either Na^+/H^+ or K^+/H^+ antiporters to acidify their cytosolic pH. A Na^+/H^+ antiporter regulates the cytosolic pH in some nonhalophilic alkaliphiles such as *Bacillus firmus* and *Exiguobacterium auranticum* (63, 65, 78, 79). The marine bacterium *Vibrio alginolyticus* uses K^+/H^+ antiporters for pH homeostasis (88, 89). The K^+/H^+ antiporter in *V. alginolyticus* is strongly dependent on the internal pH. When the cytosol is alkalized by the membrane permeable weak base diethanolamine to pH above 7.8, this antiporter begins to functions and cytosolic K^+ is released until the internal pH becomes about 7.8. The outwardly directed K^+ gradient is essential for the cytosolic acidification by the K^+/H^+ antiporter.

MATERIALS AND METHODS

All the organisms used in this work were obtained from the Oregon Collection of Methanogens (OCM). Cultural techniques, media preparation, determination of specific growth rate, and cell collection were described in part I of this dissertation.

Determination of $\Delta\psi$ and ΔpH

$\Delta\psi$ was determined by the transmembrane equilibrium distribution of the ^{14}C-labeled lipophilic cation tetraphenylphosphonium (TPP^+) and the magnitude of ΔpH was estimated from the distribution of ^{14}C-labeled benzoic acid (110). We incubated a cell suspension (20 mg of wet cells per ml) in MMSH medium with 1 μM TPP^+ or 1.7 μM benzoic acid. After 15 min, 1 ml of the suspension was placed in a 1.5-ml centrifuge tube containing 0.1 ml of deoxygenated silicone oil. The tube was centrifuged at 16,000 × *g*, 4°C for 1 min. The supernatant was removed and the top part (above the silicone oil) of the centrifuge tube was washed with MMSH medium to remove the residual radioactivity. After the silicone oil was decanted, the bottom part of the tube was sliced off and immersed in 0.1% (wt/vol) sodium dodecyl sulfate solution to lyse the cell pellet. Radioactivity in the lysate and in the supernatant fluid was counted by adding 100 μl to

4 ml of Ecolite scintillation fluid. Nonspecific binding of TPP^+ and benzoic acid was determined in the toluene-treated (116) suspension of the same cell density. The corrected concentrations of intracellular and extracellular TPP^+ and benzoic acid were used to calculate $\Delta\psi$ and ΔpH (110).

Determination of cell volume

Cells were suspended in MMSH medium with 10 mM cellobiose. The amount of cellobiose retained in the pellet after centrifugation was measured enzymatically (109). The weight and volume of the cell pellet were determined by adding the pellet to a pre-weighed 25-ml volumetric flask. The pellet was weighed, and then the flask was filled with water and re-weighed. This allowed calculation of the density of the cell pellet. The total volume of the cell pellet was calculated from the mass of the pellet and its density. We assumed that the volume of the nonaqueous cell fraction was negligible, so the cytosolic volume in the cell pellet was the total volume of each cell pellet minus the volume of buffer retained in the intercellular space (80).

Preparation of aqueous cell extracts for intracellular K^+ analysis

Cell pellets from 1 ml of dense culture (containing about 20 mg of wet cells) were lysed in 0.5 ml of deionized water. The lysate was boiled in water bath for 10 min and centrifuged at 3000 × g for 5 min. The supernatant was analyzed for K^+.

Analytical methods

Cellobiose was determined colorimetrically by using cellobiose dehydrogenase (109). K^+ was quantified with an atomic absorption spectrophotometer with an acetylene-and-air flame. Intracellular ATP content was determined by the luciferase assay (114). pH was measured with an Orion pH meter model SA520 with a combination electrode.

RESULTS

Effect of antibiotics on the growth of various methanogens

Six representative strains of methylotrophic methanogenic species were examined for sensitivity to valinomycin, an antibiotic that acts as a K^+ uniporter (99). The growth rates of these six strains were measured in the presence and absence of 0.8 μM valinomycin in media with 20 mM trimethylamine as the sole energy source (Table 3). Strains S-6, HI350, and SLP, each growing at pH 6.8, were insensitive to valinomycin. The growth of the alkaliphilic strains WeN5 and WAL1 at pH 8.5 were completely inhibited by valinomycin. Strain GS-16, which can grow at neutral or alkaline pH, was sensitive to valinomycin only during growth at alkaline pH. Strain GS-16 was sensitive to other membrane-active antibiotics, such as 0.15 μM monensin, 1.7 μM nigericin or 10 μM 3,3',4',5'-tetrachlorosalicylanilide (TCS), during growth at neutral and alkaline pH values. The pH-dependent sensitivity of strain GS-16 towards valinomycin suggested that K^+ transport plays an important role in growth under alkaline conditions. Because strain GS-16 grows at neutral and alkaline pH values, this strain was selected for studies of the possible involvement of K^+ transport in adaption to high pH.

Table 3. Effect of valinomycin on the specific growth rate of methylotrophic methanogens

Species names	pH	specific growth rate (1/h)	
		val	no val
Methanosarcina mazeii S-6	6.8	0.069 ± 0.006	0.07 ± 0.0004
Methanosarcina siciliae HI350	6.8	0.074 ± 0.0024	0.083 ± 0.0002
Methanohalophilus mahii SLP	6.8	0.042 ± 0.0052	0.048 ± 0.0005
Methanohalophilus zhilinaeae WeN5	8.5	0	0.026 ± 0.007
Methanolobus oregonensis WAL1	8.5	0	0.04 ± 0.0063
Methanolobus taylorii GS-16	7.2	0.03 ± 0.0001	0.035 ± 0.0018
Methanolobus taylorii GS-16	8.5	0	0.054 ± 0.004

Effect of external pH on the cytosolic K^+ concentrations and internal pH

Methanol-grown cells of strain GS-16 were harvested and resuspended in MMSH medium at various pH values and with 20 mM methanol. These cell suspensions were incubated for 20 to 40 min at 38°C, by which time significant amounts of CH_4 were produced. Cells maintained a relatively constant cytosolic pH (7.7 to 8.2) over the growth pH range (Fig. 16A). When the external pH was below 8.0, the cytosolic pH was slightly higher than the external pH. But at higher external pH values, the cytosol became more acidic than the medium. Cells at alkaline pH had lower concentrations of cytosolic K^+. For instance, cells suspended at pH 8.7 had only half as much K^+ as those at neutral pH (Fig. 16A).

Composition and magnitude of the proton-motive force

The proton-motive force of cells suspended at pH values between 7.5 and 8.8 was mainly due to $\Delta\psi$ (Fig. 16B). At pH values below 8, the ΔpH contributed a small amount to the proton-motive force (up to 15 mV at external pH 7.0). However, at external pH values above 8.0, the ΔpH was reversed, and the $\Delta\psi$ was higher than at external pH values of 8.0 or higher. This increased $\Delta\psi$ at alkaline pH values helped to keep the proton-motive force constant.

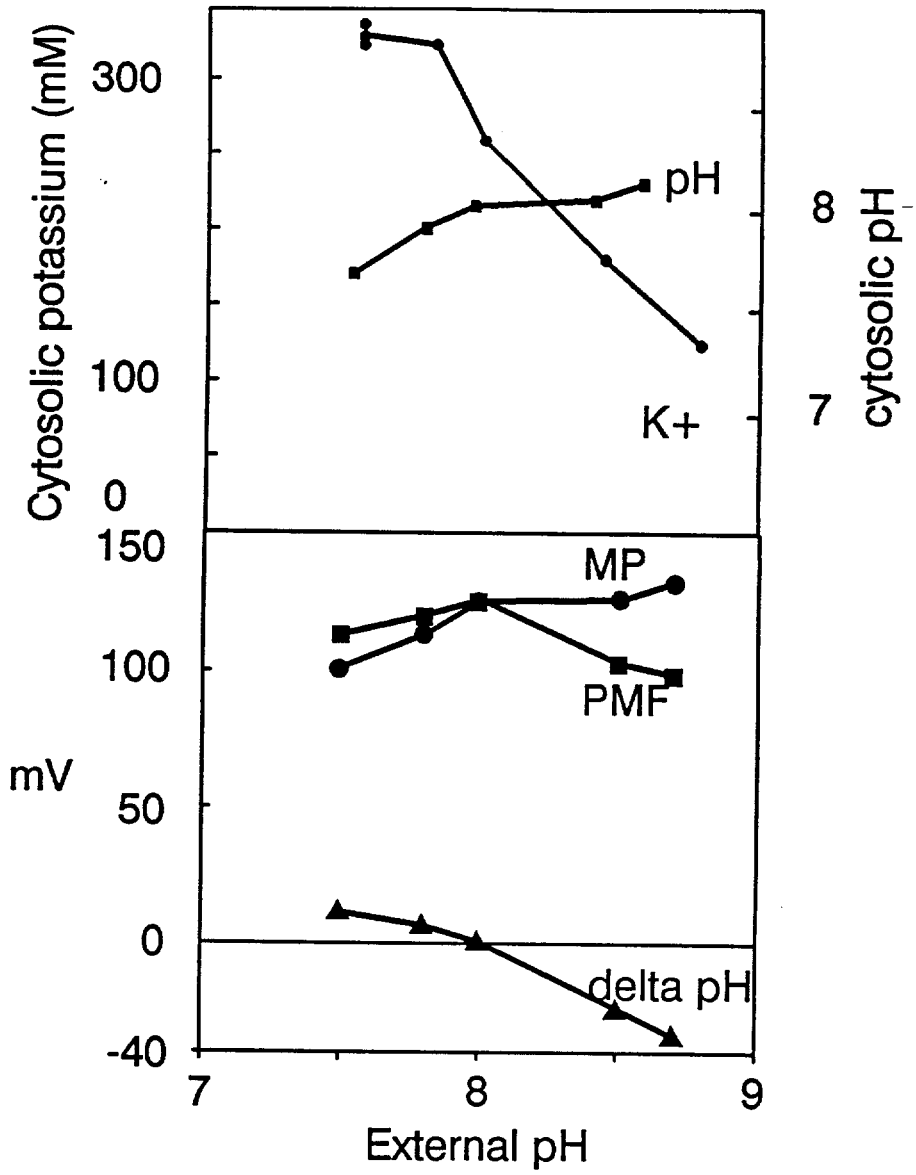


Figure 16. Effect of pH on the proton motive force (PMF) and cytosolic K^+ concentrations of *Methanolobus taylorii* GS-16. Cells grown on methanol at pH 8.2 were suspended (20 mg of wet cells per ml) in 5 ml of MMSH medium with 50 mM methanol at various pH values. MP = membrane potential

The membrane potential of strain GS-16, normally in the range of 100 to 130 mV, was only partially dissipated to about 85 mV by 8 μM TCS; but completely collapsed by 8 μM monensin (data not shown), a antibiotic that acts as an electroneutral Na^+/H^+ antiporter (36). Valinomycin had no effect on $\Delta\psi$ of cells resuspended in medium with neutral pH. At pH 8.7, valinomycin reduced the membrane potential from 136 mV to about 60 mV, but the negative ΔpH remained (internal pH around 8.2).

Effect of external K^+ concentration on growth

We measured the specific growth rate of strain GS-16 at various pH values. Cells grew in MMSH medium supplemented with a vitamin mixture (14) and with no added K^+. This medium contained about 2 mM K^+ as contaminant of NaCl. After four sequential transfers (2.5%) in medium without added K^+, strain GS-16 grew fastest (specific growth rate of 0.025 /h) at pH 8.2, and growth occurred at pH values between 7.0 and 9.0.

High external K^+ concentrations, on the other hand, inhibited growth at high pH (Fig. 17). In this experiment, the concentrations of K^+ and Na^+ in MSH medium were varied while keeping their total unchanged to minimize changes in the ionic strength of the medium. With 10 mM K^+, strain GS-16 grew at pH values between 6.8 and 9.0, with fastest growth near pH 8.2. With 210 mM K^+, growth pH range became narrower, and fastest growth occurred at pH 7.5.

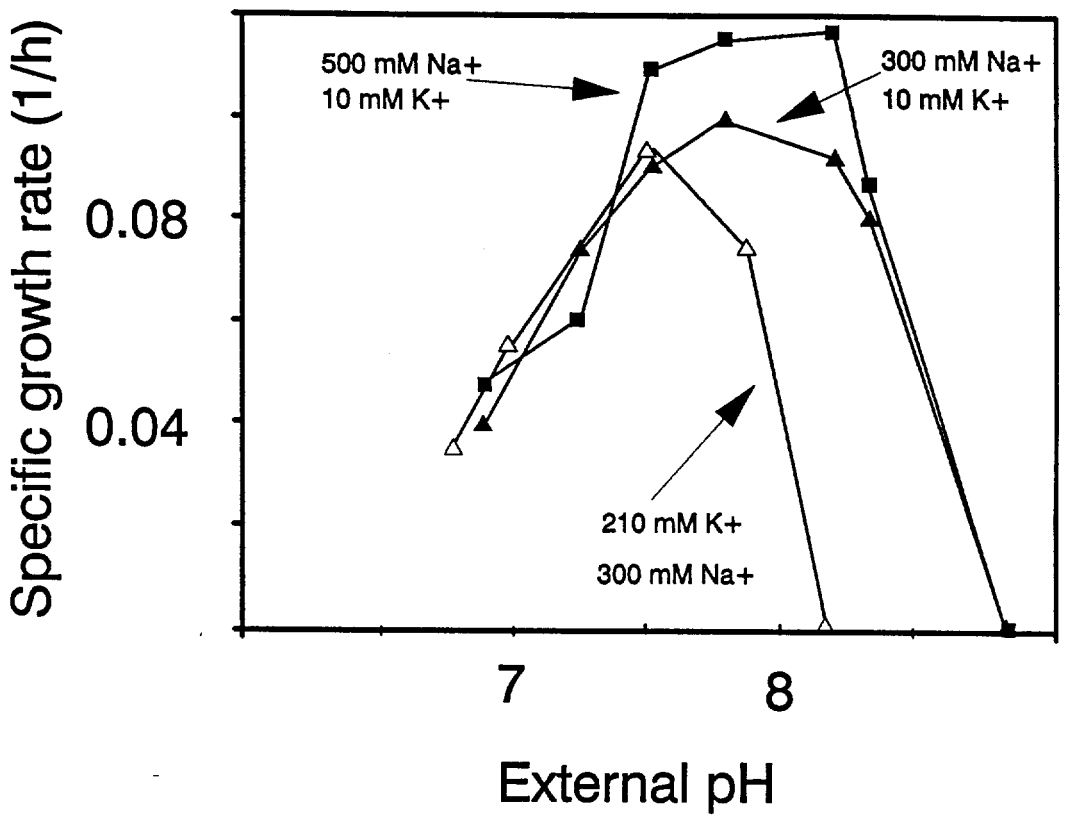


Figure 17. Effect of high K^+ concentration on the pH optimum of *Methanolobus* strain GS-16. Methanol-grown culture was inoculated into 20 ml of MSH medium with 200 mM NaCl being either replaced by 200 mM KCl or omitted.

K^+ at a high concentration also resulted in the failure of cytosolic acidification by strain GS-16 suspended in media at pH 8.2. Cells suspended in medium with 10 mM K^+ had cytosol slightly more acidic (pH 8.0) than the medium. However, when cells were suspended in medium with 300 mM K^+, the cytosolic pH was 9.1.

Effect of valinomycin on growth and methanogenesis

Cells were suspended in 5 ml of MSH medium with or without 3.2 μM valinomycin at pH values of 7.2 and 8.2. Figure 18 shows that CH_4 production and turbidity increase were not inhibited by valinomycin at pH 7.2. However, at pH 8.6, valinomycin inhibited growth. Turbidity of these cultures with valinomycin remained unchanged, and methane appeared to be produced by non-growing cells. In a similar experiment, valinomycin was added after CH_4 production had begun (Fig. 19). The addition of valinomycin caused a transient increase in CH_4 production only in alkaline medium. These conditions also caused a dramatic decrease in cytosolic ATP content (Fig. 20). At alkaline pH, valinomycin elicited a similar effect on cytosolic ATP concentration as did TCS, a potent protonophore. These results were indicative of typical uncoupling between the primary proton pump and ATP synthesis.

Net proton extrusion upon addition of K^+ or Na^+

Cells of strain GS-16 were suspended in 5 ml of 0.7 M anoxic sucrose solution, which had a similar osmotic strength to 0.5 M NaCl. The cell suspension was incubated at 38 °C for 20 min, and then 0.1 M KCl or NaCl was added from a concentrated stock solution of the same pH as the assay mixture. The cell suspension was continuously stirred under a steady stream of O_2-free N_2, and kept at 38 °C during pH measurement. As shown in Figure 21, addition of K^+ or Na^+ resulted in immediate acidification of the medium by cells suspended at alkaline pH; after 2 min, the Na^+-acidified medium returned to the starting pH. At neutral pH, addition of K^+ or Na^+ elicited little or no acidification. The pH-dependent acidification of the bulk medium by sudden increase of K^+ was indicative of the activity of a K^+/H^+ antiporter.

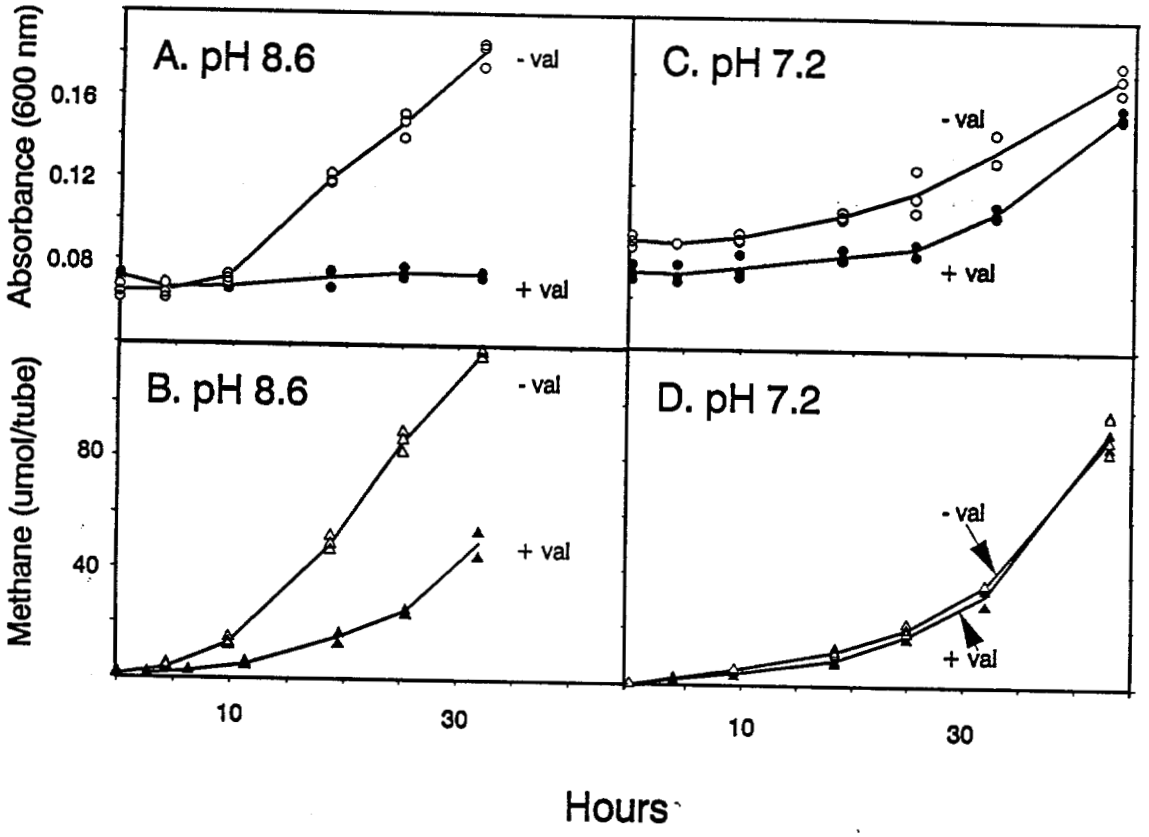


Figure 18. Effect of valinomycin on cell growth and CH_4 production by strain GS-16 at neutral and alkaline pH values. Strain GS-16 grown on methanol at pH 8.2 was inoculated (10% by volume) into 5 ml of MSH medium with or without 3.2 μM valinomycin at pH values of 7.2 and 8.6.

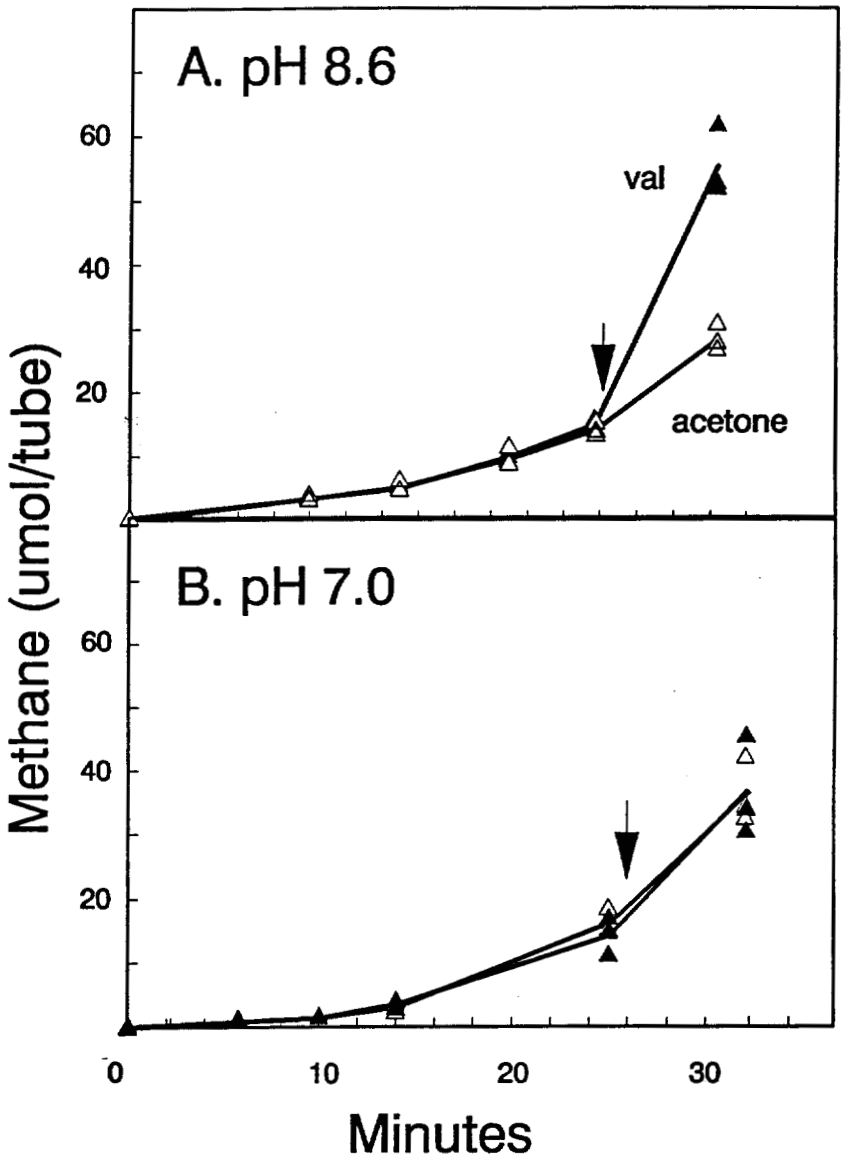


Figure 19. Effect of valinomycin on the transient CH_4 production by strain GS-16. Cell suspensions were prepared as in Figure 16, but valinomycin (3.2 μM) was added as an acetone solution. Controls received acetone without valinomycin.

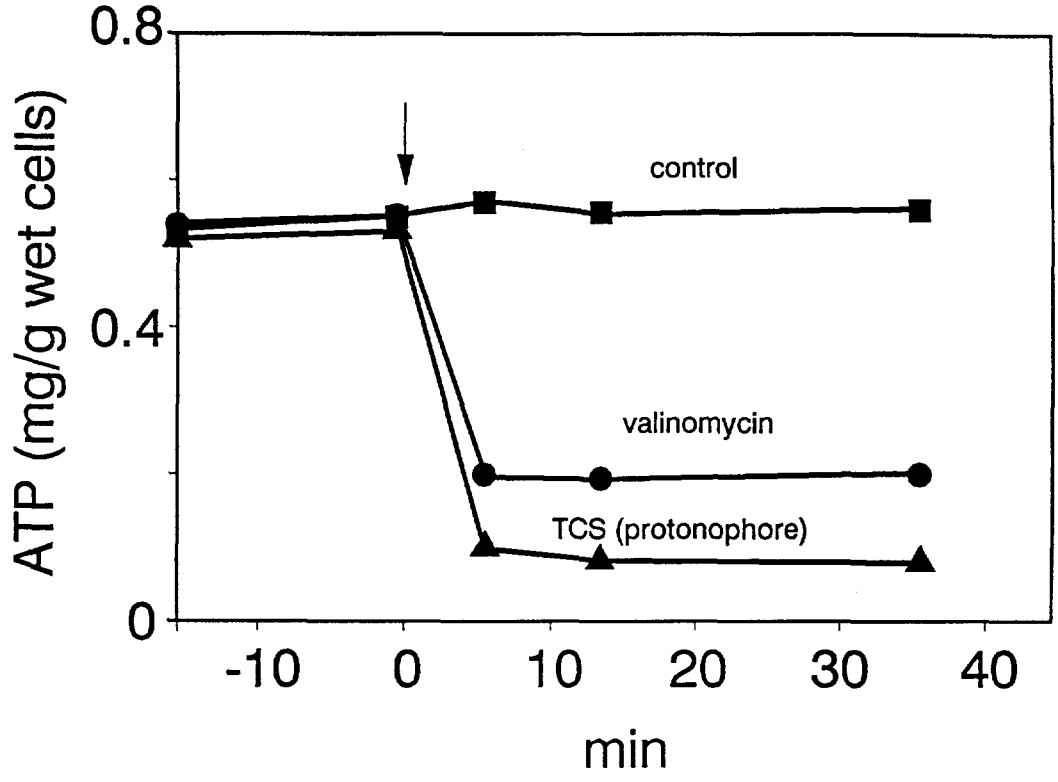


Figure 20. Effect of valinomycin on the cytosolic ATP contents of strain GS-16 at pH 8.7. Cells grown on methanol at pH 8.2 were suspended (100 mg of wet cells per ml) in MSH medium at pH 8.7 with 50 mM methanol. Valinomycin (5 μM) or TCS (15 μM) was added as indicated by the arrow. Aliquots (0.5 ml) of the cell suspension were placed in 2.0 ml of boiling glycine (pH 7.2) buffer to inactivate enzymatic activity, and then 0.15 ml of the sample was mixed with 50 μl of luciferase-luciferin agent (Sigma).

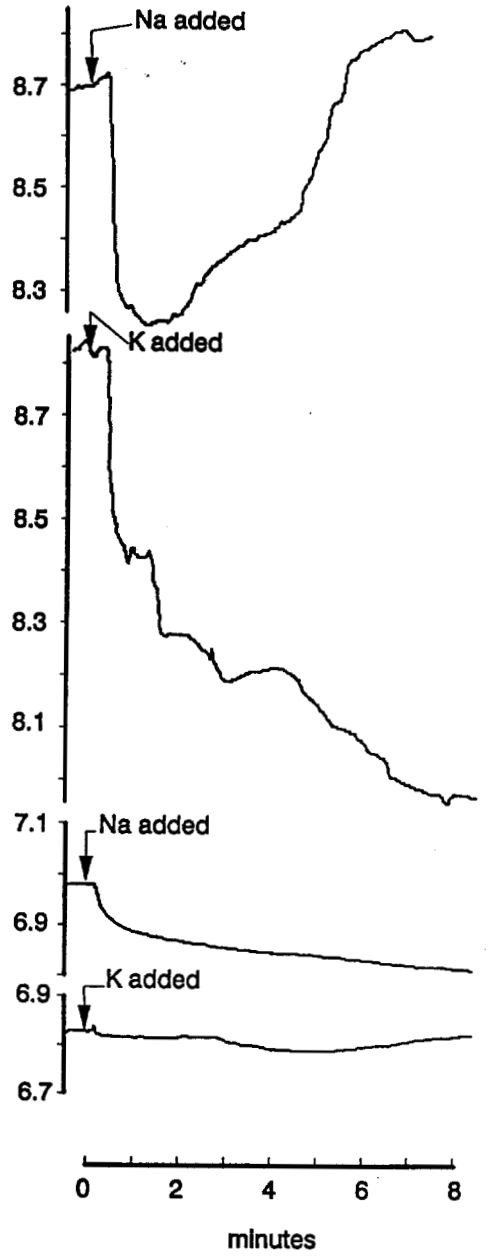


Figure 21. Acidification of the bulk medium of cell suspension of strain GS-16 upon the addition of K^+ or Na^+. Cells grown on methanol at pH 8.2 were washed and resuspended (100 mg wet cells per ml) at neutral and alkaline pH values in 0.7 M of anoxic sucrose solution, which had similar salinity as 0.5 M NaCl. After 20 min incubation at 38°C, KCl or NaCl (0.1 M) was added as indicated by arrows.

DISCUSSION

Calculations based on the measured cytosolic K^+ concentration and $\Delta\psi$ indicate that the electrochemical gradient of K^+ could amount to -56 mV in cells suspended in medium with pH 8.7. Thus, K^+ extrusion by strain GS-16 at alkaline external pH requires energy to overcome the inwardly-directed electrochemical gradient of K^+. Bacteria usually use secondary antiporters to provide energy, although at least one organism (*Enterococcus hirae*, = *Streptococcus faecalis*) uses a K^+-ATPase (45). In several bacteria, K^+ extrusion for cytosolic acidification has been ascribed to secondary K^+/H^+ antiporters (18, 88, 89). We also demonstrated the presence of such antiporter activity in strain GS-16. Upon the addition of KCl to the cell suspension of strain GS-16, the bulk medium was acidified by H^+ ejection from the whole cells. This activity was detected in cells suspended in alkaline medium, but not in cells suspended in neutral medium, in accordance with a function in regulating cytosolic pH. The addition of 0.1 M Na^+ to the cell suspension at alkaline pH also elicited acidification of the bulk medium. This probably indicated that the K^+/H^+ antiporter activity of strain GS-16, as in other organisms (18, 41, 44), was nonselective for cations, and that Na^+ could be a substrate of the K^+/H^+ antiporter activity. However, K^+ is believed to be the physiological substrate for this antiporter activity because K^+ is the most predominant cation inside the cells (66).

This model (Fig. 22) is consistent with the pH-dependent uncoupling effect of valinomycin on the chemosmotic proton circulation. Such short-circuiting could be caused by the futile K^+ cycling between the K^+/H^+ antiporter activity and valinomycin, the unregulated K^+ uniporter. These phenomena, however, are also consistent with the combined activities of a H^+-ATPase and a K^+-ATPase. We could not exclude the existence of such systems in strain GS-16.

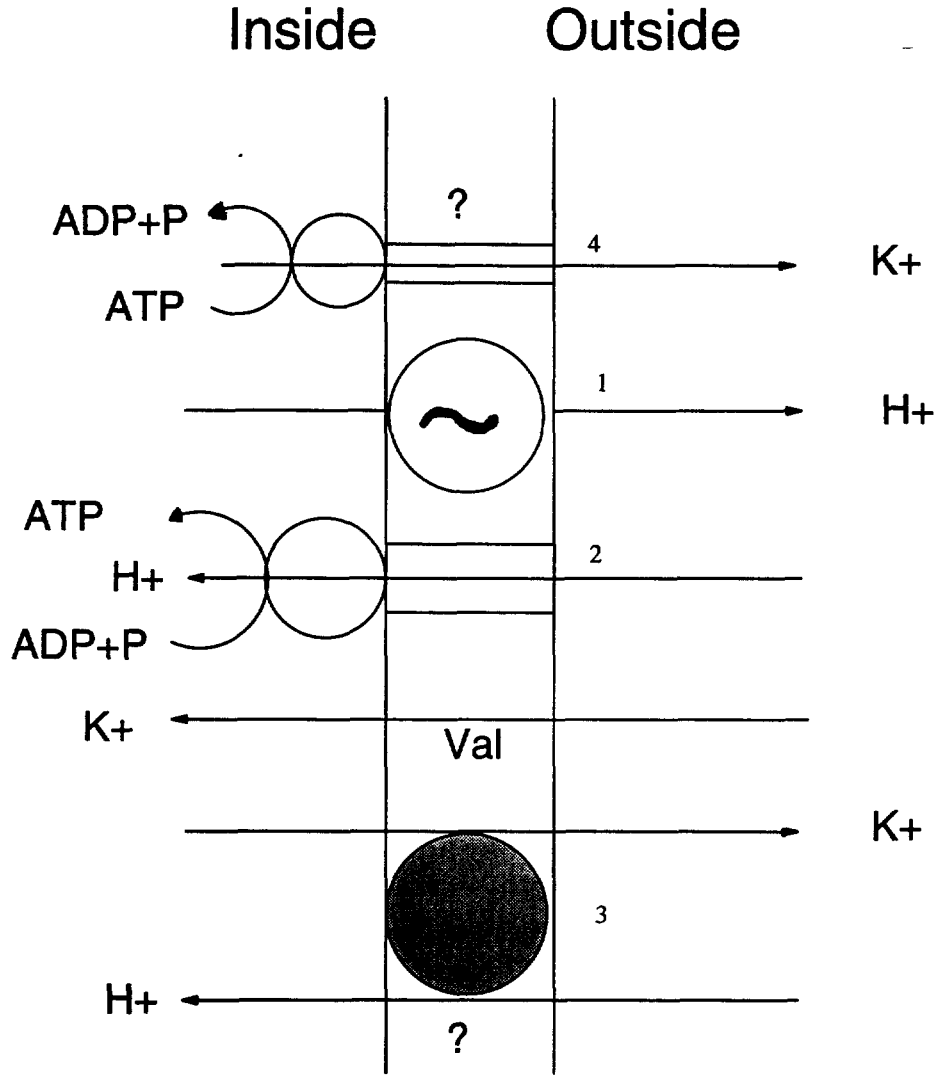


Figure 22. Proposed model for pH homeostasis in *Methanolobus taylorii* GS-16. (1, primary proton pump; 2, ATP synthase; 3, putative K^+/H^+ antiporter; 4, putative K^+-ATPase; Val, valinomycin acting as K^+ uniporter)

CONCLUSIONS

1. Strain HI350 was similar to *Methanolobus siciliae* T4/M^T.
2. Phylogenetically, *Methanolobus siciliae* T4/M^T was more closely rlated to members of the genus *Methanosarcina* than those of *Methanolobus*.
3. *Methanolobus siciliae* T4/M^T was proposed to be transferred to *Methanosarcina* as *Methanosarcina siciliae* with strain HI350 as its reference strain.
4. Only a few obligately methylotrophic methanogens are capable of catabolizing dimethyl sulfide. These include *Methanolobus oregonensis*, *Methanolobus taylorii* and *Methanohalophilus zhilinaeae* as previously reported. The present study expanded this list of dimethyl-sulfide-using methanogens to include *Methanosarcina siciliae* and *Methanosarcina acetivorans*.
5. In *Methanosarcina siciliae* HI350, enzymes for the utilization of both dimethyl sulfide and methane thiol were inducible. Those enzymes might include methyltransferases which transfer the methyl group from dimethyl sulfide or methane thiol to the C_1 carrier such as coenzyme M.
6. When either methane thiol or dimethyl sulfide was degraded by *Methanosarcina siciliae* HI350, small amounts of the other were detected. However, it is not clear which, if either, of these compounds is an intermediate in the degradation pathway

of the other. Methyltransferases catalyzing reversible reactions might be responsible for transitory appearance of these compounds during degradation.

7. When suspended at alkaline pH above 8.2, cells of *Methanolobus taylorii* GS-16 adjusted the strength of their proton-motive force by maintaining a stronger $\Delta\Psi$ to partially compensate for the reversed ΔpH.

8. *Methanosarcina taylorii* GS-16 achieved a stronger $\Delta\Psi$ probably by extruding cytosolic K^+ in exchange of H^+ through the K^+/H^+ antiporter activity observed.

FUTURE DIRECTIONS

Further study on the methyltransferase systems in methylotrophic methanogens

Methyltransferases catalyze the initial reactions in the degradation pathway of methylated substrates in the methylotrophic methanogen *Methanosarcina* (Fig. 2). There are at least two methyltransferase systems involved. One of them uses HS-CoM as the methyl acceptor to form CH_3-S-CoM and initiates the reductive branch of the pathway. The other methyltransferase is responsible for the initiation of the oxidative branch and does not depend on HS-CoM as methyl carrier (48). It has been postulated that this methyltransferase used H_4MPT as the methyl carrier (48).

This study suggested that dimethyl sulfide:HS-CoM methyltransferase was not present in methanol- or trimethylamine-grown cells of strain HI350, and that it was induced by dimethyl sulfide or methane thiol. The coenzyme-M-dependent methyltransferse systems was the major contributor of the observed enzymatic activity of substrate degradation in the cell-free extract of strain HI350, since coenzyme M was added in the assay mixture. In this study, however, activity of the inducible methyltransferase was measured only by the disappearance of the substrate, but not by the formation of CH_3-S-CoM. The assay mixture also contained the methyl carrier, H_4MPT,

due to the carry-over from the cell-free extract. Thus, to a less extent, the H_4MPT-dependent methyltransferase might also contribute to the degradation of the substrates in the cell-free extract of strain HI350. The fact that degradation of methylated substrates occurred faster than the CH_4 production in the cell-free extract was indicative of the two methyltransferases.

Future efforts should focus on the identification of the two transferase in separate systems. Two assay mixtures containing H_4MPT or HS-CoM as the methyl carrier will probably resolve these methyltransferase. This will allow further study on the H_4MPT-dependent methyltransferase to see if dimethyl sulfide:H_4MPT methyltransferase is inducible or not. Such efforts could finally facilitate the purification of these methyltransferase.

Further study on the potassium-transporting enzyme in *Methanolobus taylorii* GS-16

Preliminary results in part II of this dissertation indicated that alkaline pH of the medium activated a putative K^+/H^+ antiporter in the alkaliphilic strain GS-16. This antiporter activity was then proposed to account for the loss of cytosolic K^+ and acidification of the cytosolic pH. This proposal was based on the assumptions that increase in the external pH would also lead to the cytosolic alkalinization, and that the cytosolic alkalinization would in turn activate the K^+/H^+ antiporter. These assumptions should be proved to be valid while or before the antiporter is further pursued in the future study.

A K^+/H^+ antiporter is proposed for cytosolic pH regulation in the marine bacterium *Vibrio alginolyticus* (89). This antiporter is activated above cytosolic pH 7.8. Cytosolic alkalinization as well as K^+ extrusion in this organism does not occur in the alkaline medium unless diethanolamine, a membrane permeable weak base is also present in the medium. At alkaline pH, unprotonated diethanolamine can enter the cells, where it gets protonated and raises the cytosolic pH. The MSH medium used in this study contains about 20 mM NH_4Cl, which can behave similarly as diethanolamine at alkaline pH. It would be interesting to test if K^+ extrusion from cells of strain GS-16 in the alkaline medium is also dependent on the presence of NH_4Cl.

Future study of the K^+/H^+ antiporter will also be greatly facilitated by using everted vesicles instead of whole cells of strain GS-16. Everted vesicles can be prepared from the whole cells of strain GS-16 (97). Cells of strain GS-16 have proteinaceous cell wall. Thus, treating the cells with pronase in 0.5 M sucrose solution can form protoplasts (43, 102). Everted vesicles can then be generated by subjecting the protoplasts to osmotic shock. The everted vesicles, if prepared in the presence of K^+-specific fluorescent probe (44, 67), will contain the probe. Since the vesicles are everted, what was the external medium in the whole cell culture has now become the lumen of the vesicles. Thus, the directionality of the putative K^+/H^+ is reversed. At alkaline pH, the vesicles will accumulate K^+ in their lumen. The K^+-specific probe will respond to the uptake of K^+ by emitting fluorescence. Such systems will allow us to detect and quantify the activity of the antiporter. This will also allow further study on the kinetics of the antiporter.

REFERENCES

1. **Abbanat, D. R., and J. G. Ferry.** 1991. Resolution of component proteins in an enzyme complex from *Methanosarcina thermophila* catalyzing the synthesis or cleavage of acetyl-CoA. Proc. Natl. Acad. Sci. USA **88:**3272-3276.

2. **Ahn, Y. H., J. A. Krrzycki, and H. G. Floss.** 1991. Steric course of the reduction of ethyl coenzyme M to ethane catalyzed by methyl coenzyme M reductase from *Methanosarcina barkeri.* J. Am. Chem. Soc. **113:**4700-4701.

3. **Albracht, S. P. J., D. Ankel-Fuchs, R. Böcher, J. Ellermann, J. Moll, J. W. van der Zwaan, and R. K. Thauer.** 1988. Five new EPR signals assigned to nickel to methyl-coenzyme M reductase from *Methanobacterium thermoautotrophicum*, strain Marburg. Biochim. Biophus. Acta. **955:**86-102.

4. **Andreae, M. O., and H. Raemdonck.** 1983. Dimethyl sulfide in the surface ocean and the marine atmosphere: a global view. Science **221:**744-747.

5. **Bakker, E. P.** 1990. The role of alkali-cation transport in energy coupling of neutrophilic and acidophilic bacteria: an assessment of methods and concepts. FEMS Microbiol. Rev. **75:**319-334.

6. **Bakker, E. R., and W. E. Mangerich.** 1981. Interconversion of components of the bacterial proton motive force by electrogenic potassium transport. J. Bacteriol. **147:**820-826.

7. **Balch, W. E., G. E. Fox, L. J. Magrum, C. R. Woese, and R. S. Wolfe.** 1979. Methanogens: reevaluation of a unique biological group. Microbiol. Rev. **43:**260-296.

8. **Becher, B. V. Müller, and G. Gottschalk.** 1992. The methyl-tetrahydromethanopterin:coenzyme M methyltransferase of *Methanosarcina* strain Gö1 is a primary sodium pump. FEMS Microbiol. Lett. **99:**234-244.

9. **Becher, B. V. Müller, and G. Gottschalk.** 1992. N^5-methyl-tetrahydromethanopterin:coenzyme M methyltransferase of *Methanosarcina* strain Gö1 is a Na^+ translocating membrane protein. J. Bacteriol. **174:**7656-7660.

10. **Berkessel, A.** 1991. Methyl-coenzyme reductase: model studies on pentadentate nickel complexes and a hypothetical mechanism. Bioorg. Chem. **19:**101-115.

11. **Blaut, M., and G. Gottschalk.** 1984. Coupling of ATP synthesis and methane formation from methanol and molecular hydrogen in *Methanosarcina barkeri.* Eur. J. Biochem. **141:**217-222.

12. **Blaut, M., and G. Gottschalk.** 1984. Proton motive force-driven synthesis of ATP during methane formation from molecular hydrogen and formaldehyde or carbon dioxide in *Methanosarcina barkeri* FEMS Microbiol. Lett. **24:**103-107.

13. **Boone, D. R.** 1991. Ecology of methanogenesis, p. 57-70. *In* J. E. Rogers and W. B. Whitman (ed.), Microbial production and consumption of greenhouse gases: methane, nitrogen oxides, and halomethanes. American Society for microbiology, Washington, DC.

14. **Boone, D. R., R. L. Johnson, and Y. Liu.** 1989. Diffusion of the interspecies electron carriers H_2 and formate in methanogenic ecosystems and its implications in the measurement of K_m for H_2 or formate uptake. Appl. Environ. Microbiol. **55:**1735-1741.

15. **Boone, D. R., and W. B. Whitmann.** 1988. Proposal of minimal standards for describing new taxa of methanogenic bacteria. Int. J. Syst. Bacteriol. **38:**212-219.

16. **Boone, D. R., W. B. Whitman, and P. Rouvière.** 1993. Diversity and taxonomy of methanogens, p. 35-80. *In* J. G. Ferry (ed.) Methanogenesis. Chapman & Hall, New York.

17. **Booth, I. R.** 1985. Regulation of cytoplasmic pH in bacteria. Microbiol. Rev. **49:**359-378.

18. **Brey, R. N., B. P. Rosen, and E. N. Sorensen.** 1980. Cation/proton antiport systems in *Escherichia coli.* Properties of the potassium/proton antiport. J. Biol. Chem. **255:**39-44.

19. **Bryant, M. P., and D. R. Boone.** 1987. Emended description of strain MS^T ($DMS\ 800^T$), the type strain of *Methanosarcina barkeri.* Int. J. Syst. Bacteriol. **37:**169-170.

20. **Charlson, R. J., J. E. Lovelock, M. O. Andreae, and S. G. Warren.** 1987. Oceanic phytoplankton, atmospheric sulfur, cloud albedo and climate. Nature (London) **326:**655-661.

21. **Cooper, W. J., and P. A. Matrai.** 1989. Distribution of dimethyl sulfide in the

oceans, p. 140-151. *In* E. S. Saltzman and W. J. Cooper (ed.), Biogenic sulfur in the environment. American Chemical Society.

22. **Dean, J. A.** 1987. Handbook of organic chemistry, section 8, p. 37. McGraw Hill, New York.

23. **Deppenmeier, U., M. Blaut, A. Mahlmann, and G. Gottschalk.** 1990. Reduced coenzyme F_{420} heterodiculfide oxidoreductase, a proton translocating redox system in methanogenic bacteria. Proc. Natl. Acad. Sci. USA **87:**9449-9453.

24. **Deppenmeier, U., M. Blaut, A. Mahlmann, and G. Gottschalk.** 1990. Membrane-bound $F_{420}H_2$-dependent heterodisulfide reductase in methanogenic bacterium strain Gö1 and *Methanolobus tindarius*. FEBS Lett. **261:**199-203.

25. **De Soete, G.** 1983. A least squares algorithm for fitting additive trees to proximity data. Psychometrika **48:**621-626.

26. **DiMarco, A. A., T. A. Bobik, and R. S. Wolfe.** 1990. Unusual coenzyme of methanogenesis. Annu. Rev. Biochem. **59:**355-394.

27. **Drotar, A., G. Allen Burton Jr., J. E. Tavernier, and R. Fall.** 1987. Widespread occurrence of bacterial thiol methyltransferases and the biogenic emission of methylated sulfur gases. Appl. Environ. Microbiol. **53:**1626-1631.

28. **Edgerton, M. E., and P. Brimblecombe.** 1981. Thermodynamics of halobacterial environments. Can. J. Microbiol. **27:**899-909.

29. **Ferry, J. G.** 1993. Methanogenesis: ecology, physiology, biochemistry and genetics. Chapman & Hall.

30. **Fiebig, K., and G. Gottschalk.** 1983. Methanogenesis from choline by a coculture of *Desulfovibrio* sp. and *Methanosarcina barkeri*. Appl. environ. Microbiol. **45:**161-168.

31. **Fiebig, K., and B. Friedrich.** 1989. Purification of the F_{420}-reducing hydrogenase from *Methanosarcina barkeri* (strain Fusaro). Eur. J. Biochem. **184:**79-88.

32. **Finster K., F. Bak, G. M. King.** 1991. Formation of methylmercaptan and dimethyl sulfide from methoxylated aromatic compounds anoxic marine and freshwater sediments. FEMS Microbiol. Ecol. **74:**295-302.

33. **Franzmann, P. D., N. Springer, W. Ludwig, E. C. D. Macario, and M. Rohde.** 1992. A methanogenic aechaeon from Ace Lake, Antarctica: *Methanococcoides*

burtonii sp. nov.. System. Appl. Microbiol. **15:**573-581.

34. **Hartzell, P. L., and R. S. Wolfe.** 1986. Comparative studies of component C from the methylreductase system of different methanogens. Syst. Appl. Microbiol. **7:**376-382.

35. **Henatsch, J. J., and F. Jüttner.** 1990. Occurrence and distribution of methane thiol and other volatile organic sulphur compounds in a stratified lake with anoxic hypolimnion. Arch. Hydrobiol. **119:**314-323.

36. **Harold, F. M.** 1991. The vital force: a study of bioenergetics. Freeman Publications, San Francisco.

37. **Höllriegl, V., P. Scherer, and P. Renz.** 1983. Isolation and characterization of the Co-methyl and Co-aquo derivative of 5-hydroxybenzimidazolylcobamide (Factor III) from *Methanosarcina barkeri* grown on methanol. FEBS Lett. **151:**156-158.

38. **Hungate, R. E.** 1969. A roll tube method for cultivation of strict anaerobes, p. 117-132. *In* R. Norris and D. W. Ribbons (ed.), Methods in microbiology, vol. 3B. Academic Press, Inc., New York.

39. **Hutte, R. S., N. G. Johansen, and M. F. Legier.** 1990. Column selection and optimization for sulfur compound analyses by gas chromatography. J. High Resolution Chromatogr. **13:**421-426.

40. **Jablonski, P. E., and J. G. Ferry.** 1991. Purification and properties of methyl coenzyme M methylreductase from acetate-grown *Methanosarcina thermophila.* J. Bacteriol. **173:**2481-2487.

41. **Jezek P., F. Mahdi and K. D. Garlid.** 1990. Reconstitution of the beef heart and rat liver mitochondrial K^+/H^+ (Na^+/H^+) antiporter. J. Biol. Chem. **265:**10522-10526.

42. **Jukes, T. H., and C. R. Cantor.** 1969. Evolution of protein molecules, p. 21-132. *In* H. N. Munro (ed.), Mammalian protein metabolism. Academic Press, New York.

43. **Jussofie, A., F. Mayer, and G. Gottschalk.** 1986. Methane formation from methanol and molecular hydrogen by protoplasts of new methanogenic isolates and inhibition by dicyclohexylcarbodiiminde. Arch. Microbiol. **146:**245-249.

44. **Kakar, S. S., F. Mahdi, X. Li, and K. Garlid.** 1989. Reconstitution of the mitochondrial non-selective Na^+/H^+ (K^+/H^+) antiporter into proteoliposomes. J.

Biol. Chem. **264:**5846-5851.

45. **Kakinuma, Y., and K. Igarashi.** 1988. Active potassium extrusion regulated by intracellular pH in *Streptococcus faecalis*. J. Biol. Chem. **263:**14166-14170.

46. **Keller, M. D.** 1989. Dimethyl sulfide production and marine phytoplankton: the importance of species composition and cell size. Biol. Oceanogr. **6:**375-382.

47. **Keltjens, J. T., and C. van der Drift.** 1986. Electron transfer reactions in methanogens. FEMS Microbiol. Rev. **39:**259-303.

48. **Keltjens, J. T., and G. D. Vogels.** 1993. Conversion of methanol and methylamines to methane and carbon dioxide, p. 253-303. *In* J. G. Ferry (ed.) Methanogenesis. Chapman & Hall, New York.

49. **Kengen, S. W. M., J. J. Mosterd, R.L. H. Nelissen, J. T. Keltjens, C. van der Drift, and G. D. Vogels.** 1988. Reductive activation of the methyl-tetrahydromethanopterin: coenzyme M methyltransferase from *Methanobacterium thermoautotrophicum* strain ΔH. Arch. Microbiol. **150:**405-412.

50. **Kengen, S. W. M., P. J. H. Daas, E. F. G. Duits, J. T. Keltjens, C. Van der Drift, and G. D. Vogels.** 1992. Isolation of a 5-hydroxybenzimidazolyl cobamide-containing enzyme involve in the dethyltetrahydromethanopterin:coenzyme M methyltransferase reaction in *Methanobacterium thermoautotrophicum*. Biochim. Biophys. Acta. **1118:**249-260.

51. **Kiene, R. P.** 1988. Dimethyl sulfide metabolism in salt marsh sediments. FEMS Microb. Ecol. **53:**71-78.

52. **Kiene, R. P., and D. G. Capone.** 1988. Microbial transformations of methylated sulfur compounds in anoxic salt marsh sediments. Microb. Ecol. **15:**275-291.

53. **Kiene, R. P., R. S. Oremland, A. Catena, L. G. Miller, and D. G. Capone.** 1986. Metabolism of reduced methylated sulfur compounds in anaerobic sediments and by a pure culture of an estuarine methanogen. Appl. Environ. Microbiol. **52:**1037-1045.

54. **Kiene, R. P., and P. T. Visscher.** 1987. Production and fate of methylated sulfur compounds from methionine and dimethylsulfoniopropionate in anoxic salt marsh sediments. Appl. Environ. Microbiol. **53:**2426-2434.

55. **King, G. M.** 1983. Utilization of hydrogen, acetate, and 'non-competitive' substrates by methanogenic bacteria in marine sediments Geomicrobiol. J. **3:**275-306.

56. **Klein, A. R., J. Breitung, D. Linder, K. O. Stetter, and R. K. Thauer.** 1993. N^5,N^{10}-Methenyltetrahydromethanopterin cyclohydrolase from the extremely thermophilic sulfate reducing *Archaeoglobus fulgidus*: comparison of its properties with those of the cyclohydrolase from the extremely thermophilic *Methanopyrus kandleri*. Arch. Microbiol. **159:**213-219.

57. **Koga, Y., M. Nishihara, H. Morii, and M. Akagawa-matsushita.** 1993. Ether polar lipids of methanogenic bacteria: structures, comparative aspects, and biosyntheses. Microbiol. Rev. **57:**164-182.

58. **König, H., and K. O. Stetter.** 1982. Isolation and characterization of *Methanolobus tindarius* sp. nov., a coccoid methanogen growing only on methanol and methylamines. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe C **3:**478-490.

59. **König, H., and K. O. Stetter.** 1986. Studies on archaebacterial S-layers. Syst. Appl. Microbiol. **7:**300-309.

60. **Krist, G. O.** 1989. Salinity tolerance of eucaryotic marine algae. Annu. Rev. Plant Physiol. Plant Mol. Biol. **40:**21-53.

61. **Kroll, R. G., and I. R. Booth.** 1981. The role of potassium transport in the generation of a pH gradient in *Escherichia coli*. Biochem. J. **198:**691-698.

62. **Kroll, R. G., and I. R. Booth.** 1983. The relationship between intracellular pH, the pH gradient and potassium transport in *Escherichia coli*. Biochem. J. **216:**709-716.

63. **Krulwich, T. A., A. A. Guffanti, R. F. Bornstein, and T. Hoffstein.** 1982. A sodium requirement for growth, solute transport and pH homeostasis in *Bacillus firmus* RAB. J. Biol. Chem. **257:**1885-1889.

64. **Krulwich, T. A., and D. M. Ivey.** 1990. Bioenergetics in extreme environments, p. 417-447. *In* T. A. Krulwich (ed.), Bacterial energetics. Academic Press, Inc. New York.

65. **Krulwich, T. A., J. G. Federbush, and A. A. Guffanti.** 1985. Presence of a non-metabolizable solute that is translocated with Na^+-dependent pH homeostasis in an alkaliphilic *Bacillus*. J. Biol. Chem. **260:**4055-4058.

66. **Lai, M., K. R. Sowers, D. E. Robertson, M. F. Roberts, and R. P. Gunsalus.** 1991. Distribution of compatible solutes in the halophilic methanogenic archaebacteria. J. Bacteriol. **173:**5352-5358.

67. **Li, X., M. G. Hegazy, F. Mahdi, P. Jezek, R. D. Lane, and K. D. Garlid.** 1990. Purification of a reconstitutively active K^+/H^+ antiporter from rat liver mitochondria. J. Biol. Chem. **265:**15316-15322.

68. **Liu, Y., D. R. Boone, and C. Choy.** 1990. *Methanohalophilus oregonense* sp. nov., a methylotrophic methanogen from an alkaline, saline aquifer. Int. J. Syst. Bacteriol. **40:**111-116.

69. **Liu, Y., D. R. Boone, R. Sleat, and R. A. Mah.** 1985. *Methanosarcina mazei* LYC, a new methanogenic isolate which produces a disaggregating enzyme. Appl. Environ. Microbiol. **49:**608-613.

70. **Lysenko, A. M., and T. N. Zhilina.** 1985. Taxonomic position of *Methanosarcina vacuolata* and *Methanococcus halophilus* determined by the technique of DNA-DNA hybridization. Mikrobiologiya **54:**501-502.

71. **Maestrojuán, G. M., and D. R. Boone.** 1991. Characterization of *Methanosarcina barkeri* strains MS^T and 227, *Methanosarcina mazei* S-6^T, and *Methanosarcina vacuolata* Z-761^T Int. J. Syst. Bacteriol. **41:**267-274.

72. **Maestrojuán, G. M., J. E. Boone, R. A. Mah, J. A. G. F. Menaia, M. S. Sachs, and D. R. Boone.** 1992. Taxonomy and halotolerance of mesophilic *Methanosarcina* strains, assignment of strains to species, and synonymy of *Methanosarcina mazei* and *Methanosarcina frisia.* Int. J. Syst. Bacteriol. **42:**561-567.

73. **Mah, R. A.** 1980. Isolation and characterization of *Methanococcus mazei.* Curr. Microbiol. **3:**321-326.

74. **Mah, R. A., and D. R. Boone.** 1989. Genus *I. Methanosarcina* Kluyver and van Niel 1936, 400,^{AL} emend. Mah and Kuhn 1984, 266 (Nom. Cons. Opin. 63 Jud. Comm. 1986, 492), p. 2199-2205. *In* J. T. Staley, M. P. Bryant, N. Pfennig, and H. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 3. The William & Wilkins Co., Baltimore.

75. **Mah, R. A., and D. A. Kuhn.** 1984. Transfer of the type species of the genus *Methanococcus* to the genus *Methanosarcina*, naming it *Methanosarcina mazei* (Barker 1936) comb. nov. et emend., and conservation of the genus *Methanococcus* with *Methanococcus vannielii* as the type species. Int. J. Syst. Bacteriol. **34:**263-265.

76. **Marmur, J.** 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. **3:**208-218.

77. **Mathrani, I. M., D. R. Boone, R. A. Mah, G. E. Fox, and P. P. Lau.** 1988. *Methanohalophilus zhilinaeae* sp. nov., an alkaliphilic, methylotrophic methanogen. Int. J. Syst. Bacteriol. **38:**139-142.

78. **McLaggan, D. M., J. Selwyn, and A. P. Dawson.** 1984. Dependence on Na^+ of control of cytoplasmic pH in a facultative alkalophile. FEBS lett. **165:**254-258.

79. **McLaggan, D. M., J. Selwyn, A. P. Dawson, I. R. Booth.** 1991. Role of Na^+ in pH homeostasis by the alkalophilic bacterium *Exiguobacterium auranticum.* J. Gen. Microbiol. **137:**1709-1714.

80. **Menaia, J. A. G. F.** 1992. Osmotics of halophilic methanogenic archaeobacteria. Ph. D. dissertation, Oregon Graduate Institute of Science and Technology.

81. **Mesbah, M., U. Premachandran, and W. B. Whitman.** 1989. Precise measurement of the guanine-plus-cytosine content of deoxyribonucleic acid by high-performance liquid chromatography. Int. J. Syst. Bacteriol. **39:**159-167.

82. **Miller, T. L., and M. J. Wolin.** 1983. A serum bottle modification of the Hungate technique for cultivation of obligate anaerobes. Appl. Microbiol. **27:**985-987.

83. **Mitchell, P.** 1966. Chemosmotic coupling in oxidative phosphorylation. Biol. Rev. **41:**445-501.

84. **Moura, I., J. J. G. Moura, H. Santos, A. V. Xavier, G. Burch, H. D. Peck Jr., and J. LeGall.** 1983. Proteins containing the factor F_{430} from *Methanosarcina barkeri* and *Methanobacterium thermoautotrophicum.* Isolation and properties. Biochim. Biophys. Acta **742:**84-90.

85. **Müller, V., C. Winner, and G. Gottschalk.** 1988. Electron transport-driven sodium extrusion during methanogenesis from formaldehyde + H_2 by *Methanosarcina barkeri.* Eur. J. Biochem. **178:**519-525.

86. **Müller, V., M. Blaut, and G. Gottschalk.** 1987. Generation of transmembrane gradient of Na^+ in *Methanosarcina barkeri.* Eur. J. Biochem. **162:**461-466.

87. **Müller, V., M. Blaut, and G. Gottschalk.** 1988. The transmembrane electrochemical gradient of Na^+ as driving force for methanol oxidation in *Methanosarcina barkeri.* Eur. J. Biochem. **172:**601-606.

88. **Nakamura, Y., H. Tokuda, and T. Unemoto.** 1982. Effect of pH and monovalent cations on the potassium ion exit from the marine bacterium, *Vibrio alginolyticus*, and the manipulation of cellular cation contents. Biochim. Biophys.

Acta. **692:**389-396.

89. **Nakamura, Y., H. Tokuda, and T. Unemoto.** 1984. K^+/H^+ antiporter functions as a regulator cytoplasmic pH in a marine bacterium *Vibrio alginolyticus*. Biochim. Biophys. Acta. **776:**330-336.

90. **Naumann, E. K. Fahlbusch, and G. Gottschalk.** 1984. Presence of a trimethylamine: HS-coenzyme M methyltransferase in *Methanosarcina barkeri*. Arch. Microbiol. **138:**79-83.

91. **Ni, S., and D. R. Boone.** 1991. Isolation and characterization of a dimethyl-sulfide-degrading methanogen, *Methanolobus siciliae* HI350, from an oil well, characterization of *M. siciliae* T4/M^T, and emendation of *M. siciliae*. Int. J. Syst. Bacteriol. **41:**410-416.

92. **Ni, S., and D. R. Boone.** 1993. Catabolism of dimethyl-sulfide and methane thiol by methylotrophic methanogens, p. 796-810. *In* R. S. Oremland (ed.), Biogeochemistry of global change. Chapman & Hall, Inc., New York.

93. **Ni, S., C. R. Woese, H. C. Aldrich, and D. R. Boone.** 1993. Transfer of *Methanolobus siciliae* to the genus *Methanosarcina*, naming it *Methanosarcina siciliae*, and emendation of the genus of *Methanosarcina*. Submitted to Int. J. Syst. Bacteriol.

94. **Ohyama, T., R. Imaizumi, K. Igarashi, and H. Kobayashi.** 1992. *Escherichia coli* is able to grow with negligible sodium ion extrusion activity at alkaline pH. J. Bacteriol. **174:**7743-7749.

95. **Oremland, R. S., and D. R. Boone.** 1994. *Methanolobus taylorii*, a new species of methylotrophic, estuarine methanogen. Int J. Syst. Bacteriol. (submitted)

96. **Oremland, R. S., L. Marsh, and D. J. DesMarais.** 1982. Methanogenesis in Big Soda Lake, Nevada: an alkaline, moderately hypersaline desert lake. Appl. Environ. Microbiol. **43:**462-468.

97. **Oremland, R. S., R. P. Kiene, M. J. Whiticar, and D. R. Boone.** 1989. Description of an estuarine methylotrophic methanogen which grows on dimethyl sulfide. Appl. Environ. Microbiol. **55:**944-1022.

98. **Oremland, R. S., S. P. Polcin.** 1982. Methanogenesis and sulfate reduction: competitive and noncompetitive substrates in estuarine sediments. Appl. Environ. Microbiol. **44:**1270-1276.

99. **Ovchinnokov, Y. A., and T. V. Ivanov.** 1982. The cyclic peptides: structure,

conformation, and function, p.563. *In* H. Neurath and R. L. Hill (ed.), The proteins, Vol. 5. Academic Press, Inc., New York.

100. **Padan, E., N. Maisler, D. Taglicht, R. Karpel, and S. Schuldiner.** 1989. Deletion of *ant* in *E. coli* reveals its function in adaptation to high salinity and an alternative Na^+/H^+ antiporter system(s). J. Biol. Chem. **264:**20297-20302.

101. **Padan, E., D. Zilberstein, and S. Schuldiner.** 1981. pH homeostasis in bacteria. Biochim. Biophys. Acta **650:**151-166.

102. **Patel, G. B., C. G. Choquet, J. H. E. Nash, and G. D. Sprott.** 1993. Formation and regeneration of *Methanococcus voltae* protoplast. Appl. Environ. Microbiol. **59:**27-33.

103. **Paterek, J. R., and P. H. Smith.** 1985. Isolation and characterization of a halophilic methanogen from Great Salt Lake. Appl. Environ. Microbiol. **50:**877-881.

104. **Paterek, J. R., and P. H. Smith.** 1988. *Methanohalophilus mahii* gen. nov., sp. nov., a methylotrophic halophilic methanogen. Int. J. Syst. Bacteriol. **38:**122-132.

105. **Plack, R. H., and B. P. Rosen.** 1980. Cation/proton antiport systems in *Escherichia coli*. Absence of potassium/proton antiporter activity in a pH-sensitive mutant. J Biol. Chem. **255:**3824-3825.

106. **Pol, A., R. A. Gage, J. M. Neis, J. W. M. Reijnen, C. van der Drift, and G. D. Vogels.** 1984. Corrinoids from *Methanosarcina barkeri*: the β-ligands. Biochimica. Biophysica Acta **797:**83-93.

107. **Powell, G. E.** 1983. Interpreting gas kinetics of batch cultures. Biotechnol. Lett. **5:**437-440.

108. **Reed, R. H.** 1983. Measurement and osmotic significance of β-dimethylsulfoniopropionate in marine macroalgae. Marine Biol. Lett **4:**173-181.

109. **Renganathan, V., S. N. Usha, and F. Lindenburg.** 1990. Cellobiose-oxidizing enzymes from the lignocellulose-degrading basidiomycete *Phanerochaete chrysosporium*: interaction with microcrystalline cellulose. Appl. Microbiol. Biotechnol. **32:**609-613.

110. **Rottenberg, H.** 1979. The measurement of membrane potential and ΔpH in cells, organelles, and vesicles, p. 547-569. *In* (ed.) Methods in enzymology. vol. LV. Academic Press, Inc., New York.

111. **Rouvière, P. E., and R. S. Wolfe.** 1988. Novel biochemistry of methanogenesis. J. Bacteriol. **263:**7913-7916.

112. **Salsbury, R. L., and D. L. Merricks.** 1975. Production of methane thiol and dimethyl sulfide by rumen microorganisms. Plant Soil **43:**191-209.

113. **Sauer, F. D., B. A. Blackwell, J. K. G. Kramer, and B. J. Marsden.** 1990. Structure of a novel cofactor containing *N*-(7-mercaptoheptanoyl)-*O*-3-phosphothreonine. Biochemistry. **29:**7593--7600.

114. **Scheel, E., and G. Schäfer.** 1990. Chemosmotic energy conversion and the membrane ATPase of *Methanolobus tindarius*. Eur. J. Biochem. **187:**727-735.

115. **Schink, b., and J. G. Zeikus.** 1982. Microbial ecology of pectin decomposition in anoxic lake sediments. J. Gen. Microbiol. **128:**393-404.

116. **Schönheit, P., D. Beimborn, and H. Perski.** 1984. Potassium accumulation in growing *Methanobacterium thermoautotrophicum* and its relation to the electrochemical proton gradient. Arch. Microbiol. **140:**247-251.

117. **Schönheit, P., and D. Beimborn.** 1985. Presence of a Na^+/H^+ antiporter in *Methanobacterium thermoautotrophicum* and its role in Na^+ dependent methanogenesis. Arch. Microbiol. **142:**354-361.

118. **Schuldiner, S., and E. Padan.** 1993. Na^+/H^+ antiporters in *Escherichia coli*, p. 25-51. *In* E. P. Bakker (ed.), Alkali cation transport systems in prokaryotes. CRC Press, Inc., Florida.

119. **Schuldiner, S., and H. Fishkes.** 1978. Sodium-proton antiport in isolated membrane vesicles of *E. coli*. Biochemistry. **17:**706-711.

120. **Schwörer, B., and R. K. Thauer.** 1991. Activities of formylmethanofuran dehydrogenase, methylenetetrahydromethanopterin dehydrogenase, methylenetetrahydromethanopterin reductase, and heterodisulfide reductase in methanogenic bacteria. Arch. Microbiol. **155:**459-465.

121. **Sowers, K. R., and J. G. Ferry.** 1983. Isolation and characterization of a methylotrophic marine methanogen, *Methanococcoides methylutens* gen. nov., sp. nov. Appl. Environ. Microbiol. **45:**684-690.

122. **Sowers, K. R., S. F. Baron, and J. G. Ferry.** 1984. *Methanosarcina acetivorans* sp. nov., an acetotrophic methane-producing bacterium isolated from marine sediments. Appl. Environ. Microbiol. **47:**971-978.

123. **Stetter, K. O.** 1989. Genus II. *Methanolobus* König and Stetter 1983, 439, p. 2205-2297. *In* J. T. Staley, M. P. Bryant, N. Pfennig, and H. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 3. The William & Wilkins Co., Baltimore.

124. **StrØm, A. R., J. A. Olafsen, and H. Larsen.** 1979. Trimethylamine oxide: a terminal electron acceptor in anaerobic respiration of bacteria. J. Gen. Microbiol. **112:**315-320.

125. **Suylen, G. M. H., P. J. Large, J. P. van Dijken, and J. G. Kuenen.** 1987. J. Gen. Microbiol. **133**:2989-1997.

126. **Taylor, B. F.** 1993. Bacterial transformations of organic sulfur compounds in marine environments, p. 745-781. *In* R. S. Oremland (ed.), Biogeochemistry of global change. Chapman & Hall, Inc., New York.

127. **Taylor, B. F., and D. C. Gilchrist.** 1991. New routes fro aerobic biodegradation of dimethylsulfoniopropionate. Appl. Environ. Microbiol. **57:**3581-3584.

128. **Taylor, B. F., and R. P. Kiene.** 1987. Microbial metabolism of dimethyl sulfide, p. 202-221. *In* E. S. Saltzman and W. J. Cooper (ed.), Biogenic sulfur in the environment. American Chemical Society.

129. **Taylor, C. D., and R. S. Wolfe.** 1974. Structure and methylation of coenzyme M ($HSCH_2CH_2SO_3^-$). J. Biol. Chem. **249:**4879-4885.

130. **Thomas, I., H. C. Dubourguier, G. Prensier, P. Debeire, and G. Albagnac.** 1987. Purification of component C from *Methanosarcina mazei* and immunolocalization in *Methanosarcinaceae*. Arch. Microbiol. **148:**193-201.

131. **Trüper, H. G., and H. G. Schlegel.** 1964. Sulfur metabolism in *Thiorhodaceae*. I. Quantitative measurements on growing cells of *Chromatium okeii*. Antonie van Leeuwenhoek J. Microbiol. Serol. **30:**225-238.

132. **Van der Meijden, P., C. van der Lest, V. van der Drift, and G. D. Vogels.** 1984. Reductive activation of methanol: 5-hydroxybenzimidazolylcobamide methyltransferase of *Methanosarcina barkeri*. Bichem. Biophys. Res. Commun. **118:**760-766.

133. **Van der Meijden, P., H. J. Heythuysen, A. Powels, F. Houwen, C. van der Drift, and G. D. Vogels.** 1984. Methyltransferases involved in methanol conversion by *Methanosarcina barkeri*. Arch. Microbiol. **134:**238-242.

134. **Van der Meijden, P., H. J. Heythuysen, H. T., H. T. Sliepenbeek, F. P.**

Houwen, C. van der Drift, G. D. Vogels. 1983. Activation and inactivation of methanol:2-mercaptoethanesulfonic acid methyltransferase from *Methanosarcina barkeri*. FEMS Microbiol. Lett. **19:**247-251.

135. **Van der Meijden, P., L. P. J. M. Jansen, C. van der Drift, and G. D. Vogels.** 1983. Involvement of corrinoids in the methylation of coenzyme M (2-mercaptoethanesulfonic acid) by methanol and enzymes from *Methanosarcina barkeri*. FEMS Microbiol. Lett. **19:**247-251.

136. **Van der Meijden, P., te Brömmelstroet, C. M. Poirot, C. van der Drift, and G. D. Vogels.** 1984. Purification and properties of methanol:5-hydroxybenz-imidazolylcobamide methyltransferase from *Methanosarcina barkeri*. J. Bacteriol. **160:**629-635.

137. **Visscher, P. T., and H. van Gemerden.** 1991. Production and consumption of dimethylsulfoniopropionate in marine microbial mats. Appl. Environ. Microbiol. **57:**3237-3242.

138. **Visscher, P. T., M. R. Diaz, and B. F. Taylor.** 1992. Enumeration of bacteria which cleave or demethylate dimethylsulfoniopropionate in the Caribbean Sea. Marine Ecol. Progr. Ser. (in press)

139. **Wakeham, S. G., and J. W. H. Dacey.** 1989. Biogeochemical Cycling of dimethyl sulfide in marine environments, p. 152-166. *In* E. S. Saltzman and W. J. Cooper (ed.), Biogenic sulfur in the environment. American Chemical Society.

140. **Weisburg, W. G., J. G. Tully, D. L. Rose, J. P. Petzel, H. Oyaizu, D. Yang, L. Mandelco, J. Sechrest, T. G. Lawrence, J. van Etten, J. Maniloff, and C. R. Woese.** 1989. A phylogenetic analysis of the mycoplasmas: basis for their classification. J. Bacteriol. **171:**6455-6467.

141. **Woese, C. R., L. J. Magrum, and G. E. Fox.** 1978. Archaebacteria. J. Mol. Evol. **11:**245-252.

142. **Woese, C. R.,O. Kandler, and M. L. Wheelis.** 1990. Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. Proc. Natl. Acad. Sci. USA **87:**457-466.

143. **Woese, C. R., R. Gutell, R. Gupta, and H. F. Noller.** 1983. Detailed analysis of the higher-order structure of 16S-like ribosomal ribonucleic acids. Microbiol. Rev. **47:**621-669.

144. **Xun, L., R. A. Mah, and D. R. Boone.** 1990. Isolation and Characterization of disaggregatase from *Methanosarcina mazei* LYC. Appl. Environ. Microbiol.

56:3693-3698.

145. **Yamasaki, K., Y. Moriyama, M. Futai, and T. Tsuchya.** 1980. Uptake and extrusion of K^+ regulated by extracellular pH in *Escherichia coli.* FEBS Lett. **120:**125-127.

146. **Zehnder, A. J. B., and K. Wuhrmann.** 1976. Titanium (III) citrate as a nontoxic oxidation-reduction buffering system for the culture of obligate anaerobes. Nature **194:**1165-1166.

147. **Zeyer, J., P. Eicher, S. G. Wakeham, and R. P. Schwarzenbach.** 1987. Appl. Environ. Microbiol. **53:**2026-2032.

148. **Zhilina, T. N., and G. A. Zavarzin.** 1987. *Methanohalobium evistigatus*, n. gen., n. sp., the extremely halophilic methanogenic archaebacterium. Dokl. Akad. Nauk SSSR **293:**464-468.

149. **Zinder, S. H., and T. D. Brock.** 1978. Production of methane and carbon dioxide from methane thiol and dimethyl sulfide by anaerobic lake sediments. Nature **273:**226-228.

150. **Zinder, S. H., and T. D. Brock.** 1978. Methane, carbon dioxide, and hydrogen sulfide production from the terminal methiol group of methionine by anaerobic lake sediments. Appl. Environ. Microbiol. **35:**344-352.

151. **Zinder, S. H., W. N. Doemel, and T. D. Brock.** 1977. Production of volatile sulfur compounds during the decomposition of algal mats. Appl. Environ. Microbiol. **34:**859-860.

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PUBLICATIONS

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2. **Ni, S., and D. R. Boone.** 1991. Isolation and characterization of a dimethyl-sulfide-degrading methanogen, *Methanolobus siciliae* HI350, from an oil well, characterization of *M. siciliae* T4/M^T, and emendation of *M. siciliae*. Int. J. Syst. Bacteriol. **41:**410-416.
3. **Ni, S., C. R. Woese, H. C. Aldrich, and D. R. Boone.** 1993. Transfer of *Methanolobus siciliae* to the genus *Methanosarcina*, naming it *Methanosarcina siciliae*, and emendation of the genus of *Methanosarcina*. Submitted to Int. J. Syst. Bacteriol.
4. **Ni, S., J. E. Boone, and D. R. Boone.** 1993. Potassium extrusion by the moderately halophilic and alkaliphilic methanogen, *Methanolobus taylorii* GS-16, and homeostasis of cytosolic pH. Manuscript in preparation.