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Physiological and ^{15}N-NMR analysis of molecular nitrogen fixation by *Methanococcus thermolithotrophicus, Methanobacterium bryantii* and *Methanospirillum hungatei*

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Two mesophilic methanogenic bacteria, *Methanobacterium bryantii* strain MOH and *Methanospirillum hungatei* strain GP1 were demonstrated, using several different experimental approaches, to fix dinitrogen. Evidence includes (1) growth with N_2 as the sole nitrogen source; (2) incorporation of $^{15}N_2$ into cellular material (both soluble amino acid pools and insoluble cell protein and other macromolecules) detected by ^{15}N-NMR spectroscopy; (3) acetylene reduction to ethylene by the cells, and inhibition of this reaction by bromoethanesulfonic acid (BES), a methanogen inhibitor. High-resolution ^{15}N-NMR analysis of ethanol extracts of these organisms and cross-polarization magic-angle sample spinning analysis of the solid debris from these extracts are compared to labeled material from *Methanococcus thermolithotrophicus*, a methanogen previously determined to fix dinitrogen.

Introduction

Recent evidence suggests living organisms are most appropriately divided into three kingdoms, the eubacteria, archaebacteria and eukaryotes [1,2]. While dinitrogen fixation (diazotrophy) occurs in most of the major divisions of the eubacterial kingdom [3], archaebacteria (which include the extreme halophiles, methanogens and sulfurophiles) have been little studied with respect to diazotrophy.

Pine and Barker [4] reported that *Methanobacterium omelianskii* fixed nitrogen. This strain was later shown to be a co-culture of a methanogen later named *Methanobacterium bryantii* M.O.H. and a syntrophic organism [5] so that the validity of the original nitrogen fixation data was doubted. Recently, however, several laboratories have demonstrated dinitrogen fixation in methanogen genera: *Methanococcus thermolithotrophicus* [6], *Methanosarcina barkeri* strain 227 [7] and strain Fusaro [8], *Methanolobus tindarius* [9], and *Methanobacterium invanovi* [10]. Concurrently, Sibold and co-workers have demonstrated that several methanogens possess, in their genome, sequences that hybridize with the *nifHDK* genes of *Klebsiella pneumoniae* and *Anabaena* [10–14]. Comparison of *nif* H gene sequences between methanogens and eubacteria agrees with the dis-

Abbreviations: CP-MASS, cross-polarization magic-angle sample spinning; BES, bromoethanesulfonic acid.

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tant phylogenetic position of archaebacteria and with an ancient origin for *nif* genes. However, it has not always been possible to show growth with N_2 as the nitrogen source with all methanogens shown to contain *nifHDK* genes [10]. While all methanogens tested show DNA sequences similar to the *Anabaena nif* H gene, none of the sulfurophilic archaebacteria tested showed similarity [13] to *nif H*. The nitrogen fixation phenotype has not been examined in non-methanogenic archaebacteria.

We report here our studies on diazotrophy in two mesophilic methanogens, *Mb. bryantii* strain M.O.H. and *Msp. hungatei* strain GP1. The addition of these species to the list of diazotrophic methanogens demonstrates that the ability to fix dinitrogen is widespread among this group of archaebacteria, with members of four families of methanogens [2,15] proven to fix dinitrogen. Our proof of diazotrophy includes: (i) growth with N_2 as sole nitrogen source, (ii) acetylene reduction and (iii) incorporation of $^{15}N_2$ into cell material. Ultimate proof that the methanogens (rather than any hypothetical contaminant) were responsible for the uptake of N_2 is that the addition of BES, an inhibitor of methanogenesis [16,17], caused immediate and total cessation of acetylene reduction. Our results also indicate the utility of ^{15}N-NMR as a method for studying N_2 fixation in bacteria.

Some of these results have been briefly described by Sparling, R; Belay, N. and Daniels, L. (1986) Abstracts from the Annual Meeting of the Canadian Society for Microbiology MP10, p. 94.

Materials and Methods

Organisms. Mb. bryantii strain M.O.H. [5] and *Msp. hungatei* GP1 [18] were obtained from G.D. Sprott. *Mc. thermolithotrophicus* [19] was from K.O. Stetter. Diazotrophic *Clostridium* sp. and *Rhodopseudomonas* sp. were isolated from soil samples in Iowa City.

Media and growth conditions. All organisms were grown anaerobically using techniques described before [20,21]. *Mc. thermolithotrophicus* and *Msp. hungatei* were grown as described by Daniels et al. [21]. *Mb. bryantii* was grown as described previously [22] except that 20 mM $MgCl_2 \cdot 6H_2O$ was included when ammonium was deleted for N_2 fixation experiments.

For N_2 fixation growth experiments, the methanogens were grown in 50 ml amounts in 250 ml serum bottles (Wheaton Scientific, No. 223950) sealed with cut-off No. 1 black stoppers and aluminum seals (Wheaton, No. 224187). The diazotrophic soil isolates were isolated and purified using media described by Phillips and Brock [23] except that 10 ml/l of mineral elixir [21] was used as the source of iron and molybdenum. Vitamin mix (10 ml/l) [24] was also added. Glucose (10 g/l) was the carbon source used for the clostridia while 1.5 g/l acetate was used as the electron donor for the phototrophically grown purple non-sulphur bacterium. All the bacteria listed were grown anaerobically under atmospheres of 140 kPa H_2/CO_2 (80 : 20), $N_2/H_2/CO_2$ (50 : 40 : 10) or 35 kPa N_2/CO_2 (80 : 20) depending on whether H_2 was used as an electron source and whether N_2 was the nitrogen source. Purity of the methanogen cultures was confirmed by inoculation into heterotrophic media and media containing antibiotics as previously described [21] as well as by microscopic examination (phase contrast).

For the calculation of the K_m value of whole-cell N_2 for *Mc. thermolithotrophicus*, inoculated aluminum seal tubes (No. 2048-00150, Bellco Glass, Vineland, NJ) were pressurized to 105 kPa (gauge pressure) with H_2/CO_2 (80 : 20), then filled further to higher pressure (112–180 kPa) with N_2 to give N_2 contents in the tubes of 0.07–0.76 atm. The slope of the exponential growth phase was used as representative of the velocity of N_2 fixation.

For ^{15}N incorporation experiments the diazotrophic methanogens were grown to mid logarithmic phase in 500 ml serum bottles (Wheaton, No. 223952). The gas was then exchanged and replaced with H_2/CO_2 (80 : 20). $^{15}N_2$ (60 ml) was injected using a syringe fitted with a Millex-GV 0.22 μm filter (Millipore, Bedford, MA) and a Mininert syringe valve (Alltech, Deerfield, IL); this prevented both gas escape and contamination. The bottles were pressurized to 140 kPa with H_2/CO_2 and the cells were then incubated for approx. 0.25–3 generations. ^{15}N labeling of cells grown on ammonium was carried out by addition of 20 mM $^{15}NH_4$ into cultures grown on 7.5 mM

$^{14}NH_4$ to the mid-logarithmic phase. The labeled cells were then centrifuged, and the pellet was extracted according to the procedures of Choi et al. [25] for NMR analysis.

Culture turbidity was measured by absorbance at 600 nm with a Perkin Elmer 552-A spectrophotometer or spectronic 20 spectrophotometer (Bausch and Lomb). Protein concentration was determined by the Bradford method [26]. All data are the average of duplicate determinations, except those from the whole-cell N_2 K_m experiment which are average values from triplicate tubes.

Chemicals. BES (sodium salt) was purchased from Sigma, St. Louis, MO. $^{15}N_2$ (greater than 99% ^{15}N) and $^{15}NH_4Cl$ (greater than 99% ^{15}N) were obtained from Cambridge Isotopes Labs., Boston, MA. Acetylene was produced by reacting CaC_2 with water.

Gas chromatographic analysis. Methane, ethylene and acetylene were assayed by gas chromatography as described previously [27].

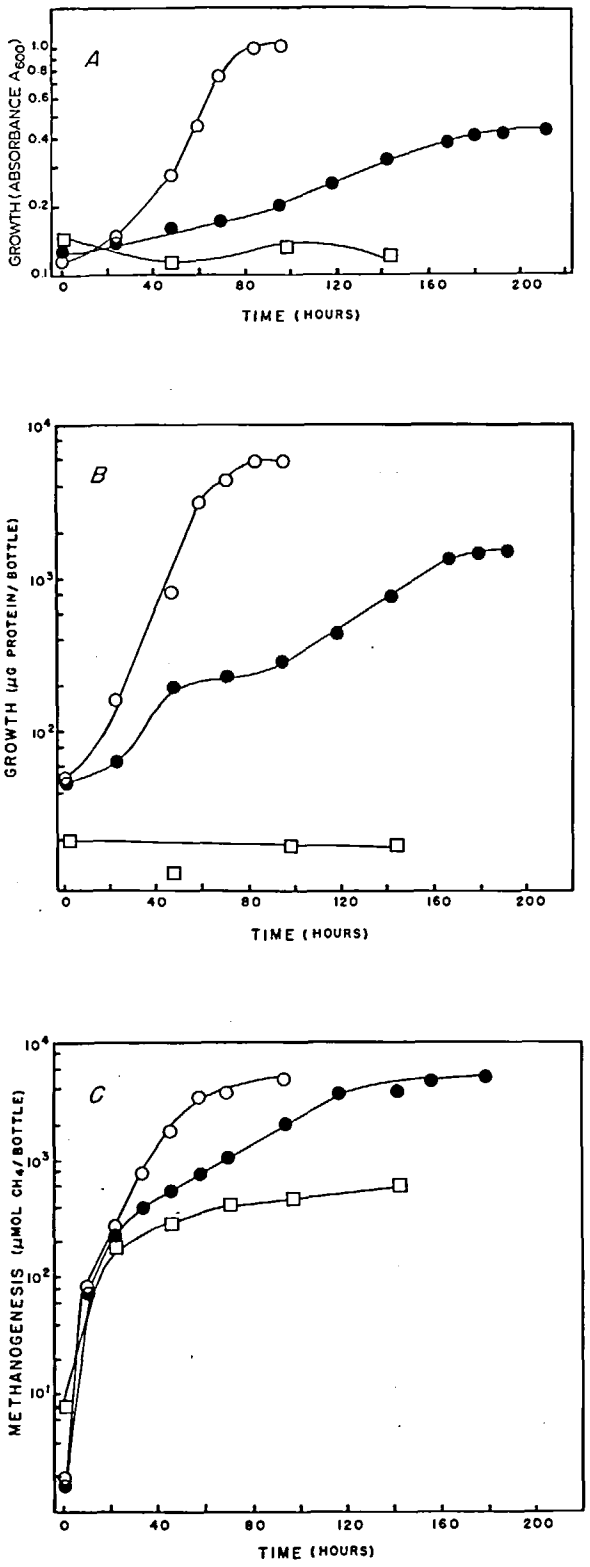
^{15}N-NMR spectroscopy. High-resolution ^{15}N-NMR spectra of ethanol extracts of methanogens were obtained at 40.55 MHz on a Bruker AM-400 spectrometer (Tufts University Medical School). Proton-decoupled ^{15}N-NMR spectra were acquired as described previously [25]. Samples were run in 50 mM sodium phosphate, 10 mM EDTA, (pH 7.0), 6% ^2H_2O, with 10 mM Na $^{15}NO_3$ as an internal shift and intensity reference. Spectra were acquired at room temperature unless otherwise noted. ^{15}N-CP-MASS NMR spectra of the cell debris remaining from the ethanol extraction procedure were obtained on a General Electric NMR Instruments GN-33 spectrometer operating at 300.1 MHz for ^1H and 30.41 MHz for ^{15}N. The ^{15}N rf field strength was approx. 21 kHz; the maximum ^1H rf field strength (used for decoupling) was 65 kHz. Powdered samples of 13–26 mg (for ^{15}N-enriched material) or 100–175 mg (for ^{15}N natural abundance controls) were packed into ceramic rotors and spun at rotor speeds from 3.2 to 4.1 kHz. A cross-polarization contact time of 1.5 ms was used; the time between successive accumulations was 5 s. ^{15}N chemical shifts were referenced to external ammonium chloride. At each spectrometer session, the sample from *Mc. thermolithotrophicus* grown for three generation times on $^{15}NH_4Cl$ was run either before or after each sample to confirm that cross-polarization conditions did not change significantly. Samples for a given organism grown on $^{15}N_2$ or $^{15}NH_4Cl$ were run successively. Sample weights were measured by weighing the rotor and endcaps before and after packing the sample in the rotor. Samples with a relatively low ^{15}N enrichment were run up to four times to obtain a reproducible signal-to-noise (S/N) ratio for the amide nitrogen peak. The latter was calculated by measuring the peak height divided by the peak-to-peak noise times 2.5. A quantitative analysis of ^{15}N content is presented as a hypothetical S/N ratio for 1 transient obtained on 1 mg of material. This was done by measuring the observed S/N in a given spectrum, normalizing for the number of transients (i.e., dividing by the square root of the number of transients), and dividing by the sample weight in mg. Given the low levels of enrichment in many of the $^{15}N_2$ samples, there could be as much as 20–30% error in S/N. For more enriched samples, the S/N was determined with greater accuracy.

Results

Growth and methanogenesis

Cultures of *Mb. bryantii* and *Msp. hungatei* grew in $N_2/H_2/CO_2$ (50:40:10) medium containing essentially no fixed nitrogen (100 μM of nitrilotriacetic acid was present as a chelator in the mineral elixir). When initially acclimated to N_2 conditions, a long lag (3–4 weeks for *Mb. bryantii* and 5–7 months for *Msp. hungatei*) occurred. After several transfers under N_2-fixing conditions, growth studies were performed.

Fig. 1 shows a comparison of rates of methanogenesis and growth (as determined either by A_{600} or protein concentration) of *Msp. hungatei* when grown using ammonium or N_2 as the major nitrogen source. Fig. 2 shows a comparison of these rates under the same conditions as those for *Mb. bryantii*. Data derived from these figures are shown in Table I and are compared with growth data from other studies on diazotrophic methanogens (Refs. 6, 8, 9 and 28 and Lobo and Zinder, personal communication). Invariably, maximal A_{600} and cell yields were lower and the generation time increased for the nitrogen-fixing



cultures compared to the same strains growing in the presence of NH_4^+.

Purity of cultures

Microscopic examination of our cultures revealed no contamination. Methanogen cultures, when transferred to heterotrophic media or media permitting growth of sulphate reducers, showed no growth in the absence of H_2/CO_2. The growth of methanogens on dinitrogen was not impaired by the additions of streptomycin and vancomycin, antibiotics known to kill a wide variety of known eubacteria but not methanogens.

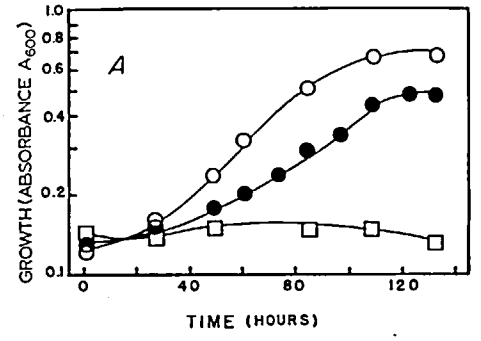
Acetylene reduction

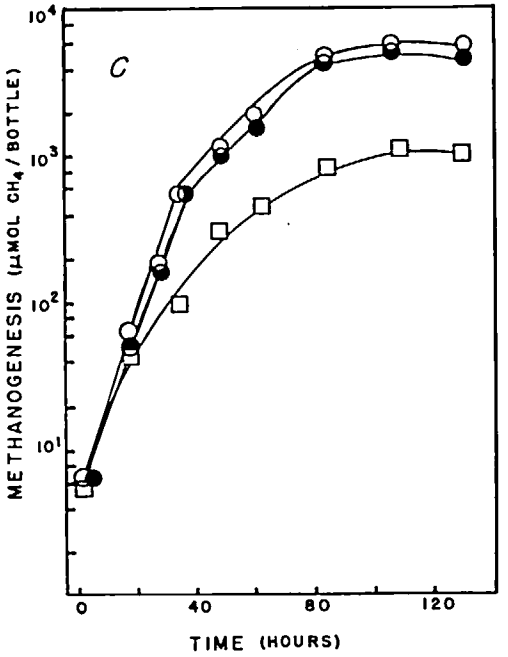
A classical method of determining the presence of nitrogenase activity is to monitor the reduction of acetylene to ethylene [29]. One drawback of this method is the fact that acetylene is itself a potent inhibitor of methanogenesis [30]. One can, however, use low concentrations of acetylene (0.1–0.5%, v/v) such that methanogenesis is only partially inhibited. In this case (Fig. 3), ethylene was produced by *Mb. bryantii* at a rate of 0.43 nmol ethylene per µmol CH_4. Both methanogenesis and ethylene production were significantly reduced in the presence of 25 mM BES. Similar results were observed for both *Msp. hungatei* and *Mc. thermolithotrophicus* (Table II). No ethylene was detected in the gas phase of these cultures in the absence of acetylene nor was ethylene detected from ammonium grown cultures in the presence of acetylene. Acetylene reduction by eubacterial diazotrophs was not inhibited by BES (Table II); these organisms grew at similar rates and maximal density in the absence or presence of 25 mM BES. In all cases, very little (if any) ethylene was produced in the absence of acetylene.

$^{15}N_2$ Incorporation

Before $^{15}N_2$ incorporation experiments were carried out, it was necessary to determine the level

Fig. 1. Growth and methanogenesis by *Msp. hungatei* with NH_4^+ or N_2 as nitrogen source. (A) Growth as observed by increase of A_{600}, (B) growth as observed by an increase in protein concentration, (C) methanogenesis. ○, NH_4^+ grown cells; ●, N_2 grown cells; □, cells grown in the absence of N_2 and NH_4^+.





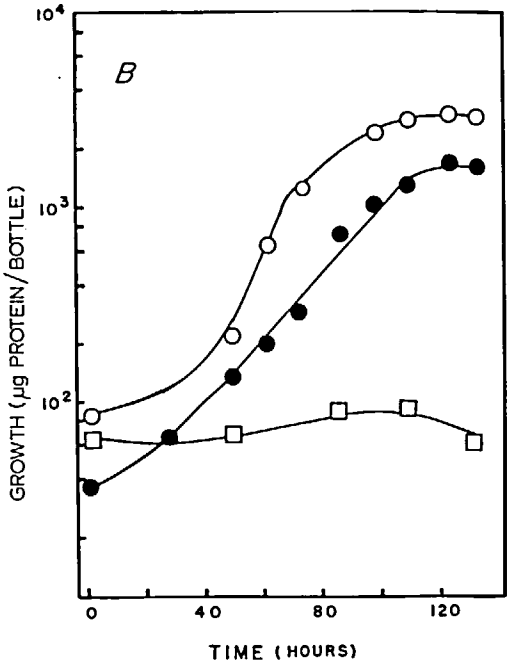


Fig. 2. Growth and methanogenesis by *Mb. bryantii* with NH_4^+ or N_2 as nitrogen source. (A) Growth as observed by increase of A_{600}, (B) growth as observed by protein concentration increase, (C) methanogenesis. ○, NH_4^+ grown cells; ●, N_2 grown cells; □, cells grown in the absence of N_2 and NH_4^+.

of N_2 needed for growth. Preliminary experiments showed no growth of *Mc. thermolithotrophicus* when 0.05 atm of N_2 was used, and reduced growth at 0.09 atm N_2. From further experiments, the whole-cell K_m value for growth on N_2 was estimated to be 0.11 ± 0.05 atm. From these data, a level of 0.15 atm N_2 was selected for the incorporation studies using $^{15}N_2$. To insure proper interpretation of results, the $^{15}N_2$ was assayed by mass spectrometry. It was 99.95% enriched for ^{15}N with a maximal concentration of ^{15}NO ($m/z = 31$) of 1% as the major ^{15}N-contaminating mass.

Both high-resolution and solid-state ^{15}N-NMR data demonstrated that $^{15}N_2$ was assimilated by the cells. High-resolution spectra of the soluble fractions of ethanol extracts of *Mc. thermolithotrophicus* grown on $^{15}NH_4Cl$ or $^{15}N_2$ are shown in Fig. 4. In cells growing exponentially on $^{15}NH_4Cl$, the α-NH of glutamate (−335 ppm) was the major soluble ^{15}N-labeled species (Fig. 4A). When cells entered the stationary phase, a resonance ('X') at −336 ppm became equivalent in intensity to glutamate (Fig. 4B). The chemical shift of this species did not overlap with known amino acids; hence, its identity is unknown at present. Significantly more transients were accumulated to detect ^{15}N-labeled amino acids in *Mc. thermolithotrophicus* grown on $^{15}N_2$ (Fig. 4C and D). The

TABLE I

GROWTH PARAMETERS OF SEVERAL METHANOGENIC DIAZOTROPHS

The generation time is that of one doubling in protein concentration. The cell yield is expressed as g dry cell weight per mol CH_4, measured during logarithmic growth. Yields were calculated as the difference of protein concentrations divided by that of the methane concentrations over the period of logarithmic growth. To obtain dry cell weights it was assumed that protein comprises 50% of the cells dry weight. Under methanogenesis, the specific activity expressed as μmol CH_4 per min per mg protein is given, and is the increase in methane during logarithmic growth divided by the average concentration of protein over this time.

Organism	Methanogenic substrate	Nitrogen source	A_{600} at late logarithmic phase	Generation time (h)	Cell yield (g cell/ mol CH_4)	Methanogenesis (μmol per min per mg protein)
Msp. hungatei	H_2/CO_2	N_2	0.42	27	0.26	3.1
		NH_4	1.00	8	1.11	2.1
Mb. bryantii	H_2/CO_2	N_2	0.48	23	0.23	5.8
		NH_4	0.66	10	0.99	3.4
Mc. thermolitho-trophicus	H_2/CO_2 [a]	N_2	0.40	4.5	0.36	11.5
		NH_4	1.1	0.6	0.94	20.2
	HCOOH [b]	N_2	0.28	4.5	0.73	10.7
		NH_4	0.76	0.6	1.84	21.1
		NO_3	1.0	0.6	–	–
Ms. barkeri strain Fusaro	CH_3OH [c]	N_2	0.25 [d]	54	1.64	–
		NH_4	0.42 [d]	38	4.55	–
Ms. barkeri	CH_3OH [e]	N_2	–	22	2.3	–
		NH_4	–	14	3.7	–
	acetate [e]	N_2	–	72	1.2	–
		NH_4	–	30	2.8	–
Ml. tindarius	CH_3OH [f]	N_2	0.80 [g]	22.5	–	–
		NH_4	1.10 [g]	16.0	–	–

[a] Data from Belay et al. [6]; [b] data from Belay et al. [28]; [c] data from Bomar et al. [8]; [d] cells monitored at 500 nm; [e] data from Steve Zinder and A.L. Lobo, personal communication; [f] calculated from data in König et al. [9]; [g] cells monitored at 578 nm.

relative distribution of ^{15}N among different compounds in the soluble fraction was the same for both nitrogen sources (e.g., compare A with C, and B with D in Fig. 4). If cells grown on $^{15}N_2$ were harvested, frozen immediately, then allowed to thaw slowly in the NMR tube, an ^{15}N NMR spectrum acquired at 4°C showed an additional intense ^{15}N-labeled species at −343 ppm. This procedure should maintain any intracellular ammonia and possibly intermediates produced by reduction of N_2. The chemical shift of the new species was in the region for R-NH_2, free amino groups as in the ϵ-amino group of lysine or polyamines; it did not represent free ammonium ions whose chemical shift is approx. −354 ppm. When cells containing this signal were extracted and processed by rotary evaporation, neither the soluble pool nor the cell debris retained the signal. Given its volatile nature, it may be an intermediate in ^{14}N metabolism by *Mc. thermolithotrophicus*. A very minor additional unidentified signal was seen at −298 ppm in all the spectra in Fig. 4.

The success with *Mc. thermolithotrophicus* prompted us to examine ^{15}N uptake into the soluble fraction of other methanogens grown on $^{15}N_2$. However, due to slower growth by these other organisms on N_2 (as shown in Table II, 0.2 as fast) labeling was carried out for only approx. 0.3 generations. While the signal-to-noise ratio was poor (primarily due to the very low cell yield), distinct ^{15}N-enriched resonances were observed consistent with the α-NH of glutamate and aspartate in ethanol extracts of *Msp. hungatei* and *Mb. bryantii* incubated with $^{15}N_2$ (data not shown). Both were above the natural abundance back-

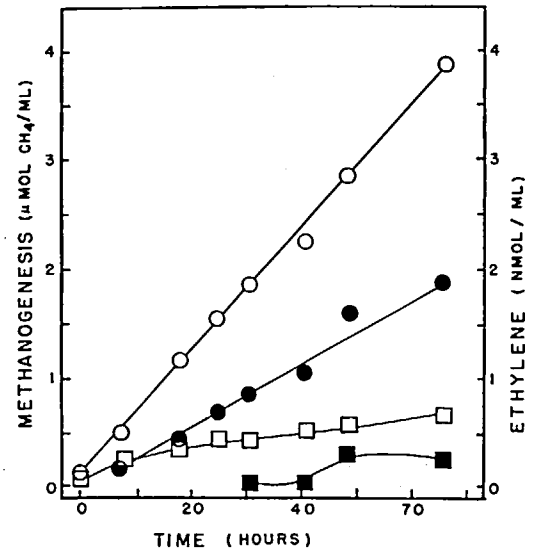


Fig. 3. Methanogenesis and acetylene reduction for diazotrophic *Mb. bryantii* in the presence or absence of BrCoM. ○, methanogenesis; ●, ethylene production in the absence of BrCoM; □, methanogenesis; ■, ethylene productions in the presence of 25 mM BrCoM. Cells were grown to the stationary phase (approximate absorbance of 0.5), gased well with H_2/CO_2 and BrCoM and acetylene were added to the appropriate cultures, after which the gas phase was analyzed at intervals.

TABLE III

THE INCORPORATION OF ^{15}N INTO AMIDE GROUPS IN PARTICULATE CELL MATERIAL OF DIFFERENT METHANOGENS

The equivalent generation time of the organism was converted from the incubation time with ^{15}N source. Normalized signal-to-noise ratio was calculated as described in Materials and Methods. The natural abundance ^{15}N background (0.37%) of particulate material was estimated from cell residue of cells grown on $^{14}NH_4Cl$.

Methanogen	Nitrogen source	Generation time	Normalized signal-to-noise
Mc. thermolitho-trophicus	$^{14}NH_4Cl$	–	0.0005
	$^{15}NH_4Cl$	0.17	0.005
		0.5	0.016
		3.0	0.038
	$^{15}N_2$	0.5	0.0016
		3.0	0.026
Msp. hungatei	$^{14}NH_4Cl$	–	0.0007
	$^{15}NH_4Cl$	0.5	0.019
	$^{15}N_2$	0.3	0.0016
Mb. bryantii	$^{14}NH_4Cl$	–	0.0006
	$^{15}NH_4Cl$	0.5	0.024
	$^{15}N_2$	0.3	0.001

ground and no resonances were detected if a comparable extract from ^{14}N-grown cells was examined.

Solid-state ^{15}N-NMR spectra of lyophilized powders are generally broad and not very informative in terms of identifying labeled species,

TABLE II

EFFECT OF BROMOETHANESULFONATE ON BACTERIAL GROWTH AND NITROGEN FIXATION

Initial concentrations of acetylene in the gas phase were 0.5% for eubacterial diazotrophs and 0.1% for methanogens; data points represent the ethylene concentration at full growth for the eubacteria and for the methanogens then represent ethylene concentrations corresponding to the methane productions indicated. No or insignificant levels of ethylene were produced (not above 0.03 nmol/ml) in the absence of acetylene.

Organism	Generation time (h)		Acetylene reduction and methanogenesis (nmol/ml gas phase)			
	no BES	25 mM BES	no BES		25 mM BES	
			methane	ethylene	methane	ethylene
Clostridium 3-1	4.0	3.5	n.d.	113	n.d.	117
Clostridium 4-1	4.0	4.0	n.d.	116	n.d.	167
Clostridium 4-2	6.0	6.0	n.d.	197	n.d.	226
Rhodopseudomonas 5-1	9.5	9.5	n.d.	100	n.d.	160
Mc. thermolithotrophicus	4.5	n.g.	14800	3.2	3100	1.2
Msp. hungatei	27	n.g.	3000	0.41	1200	0.06
Mb. bryantii	23	n.g.	4000	1.85	700	0.03

n.d., not detectable; less than 1.5 nmol CH_4/ml of gas phase; n.g., no growth occurred at this concentration of BES.

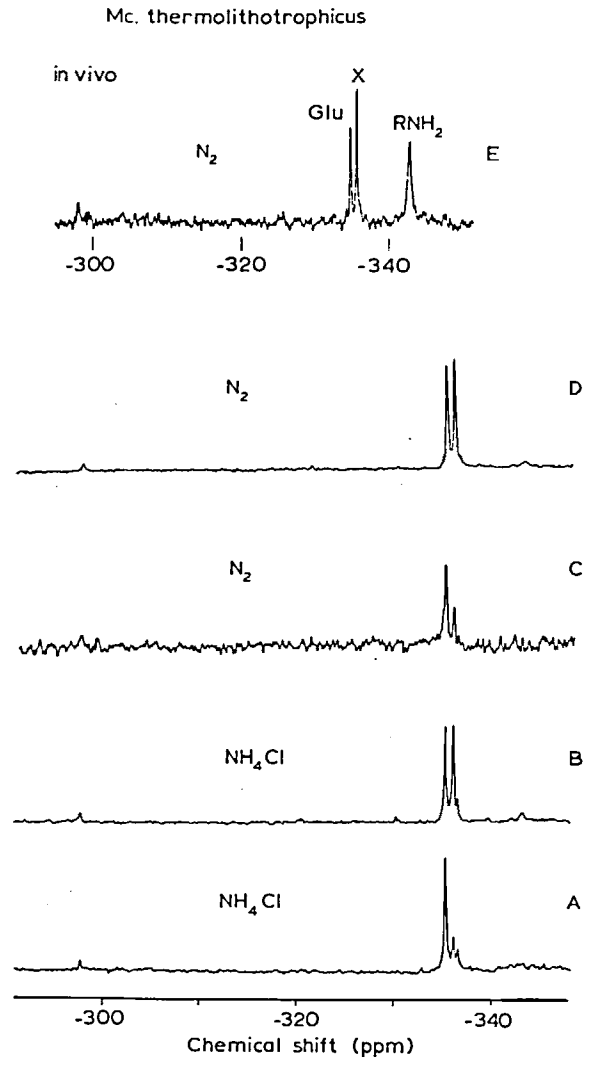


Fig. 4. ^{1}H-decoupled 40.55 MHz ^{15}N-NMR spectra of ethanol extracts of *Mc. thermolithotrophicus* grown on $^{15}NH_4Cl$ for (A) 0.5 generation times (5432 transients) and (B) 3 generation times (6401 transients) and on $^{15}N_2$ for (C) 0.5 generation time (11,300 transients) and (D) 3 generation times (9413 transients). The ^{15}N spectrum (at 4°C) of non-extracted cells grown for 3.0 generation times on $^{15}N_2$ is shown in (E) which was acquired with 8010 transients. A line-broadening of 5Hz was applied to the free induction decays prior to Fourier transformation in A–D; a 10 Hz line-broadening was used in E.

because of large dipolar interactions and chemical shift anisotropies. Magic-angle sample spinning (MASS) with high-power proton decoupling removes the dipolar broadening and averages the chemical shift anisotropies leading to relatively narrow resonances. Extra sensitivity in detection is provided by cross-polarization techniques. A combination of the two techniques, CP-MASS [31,32], is an excellent method to quantify uptake of ^{15}N into cell particulate material. Spectra of cell debris will monitor ^{15}N incorporation into protein (via the intense amide nitrogen resonance at 90 ppm and side-chain nitrogens of the arginine guanidino group and the ϵ-amino group of lysine), cell wall (*N*-acetyl nitrogens will be part of the large amide peak) and nucleic acids. Fig. 5 shows CP-MASS spectra of cell debris of *Mc. thermolithotrophicus* grown for 0.5 and 3 generation times on $^{15}N_2$ (Fig. 5A and B, respectively) compared to growth of the organism on $^{15}NH_4Cl$ for 0.5 and 3 generation times (Fig. 5C and D, respectively). The amide resonance was quite intense; because of residual chemical shift anisotropy it is flanked by rotational sidebands. Essentially the same amount of material was examined in Fig. 5A and C, yet many more transients were required to obtain a reasonable spectrum of the $^{15}N_2$-grown cells. As described in Materials and Methods the signal intensity of the amide nitrogen can be used to quantify ^{15}N uptake in different samples. A normalized S/N ratio was computed and values for *Mc. thermolithotrophicus* are given in Table III. That this technique does indeed monitor ^{15}N uptake associated with cell growth was shown with cells grown for longer times on $^{15}N_2$. Incorporation as monitored by the normalized S/N ratio clearly increased with the age of the culture (Table III). Cells grown for 0.5 generation time on $^{15}NH_4Cl$ (approx. 99% enrichment with ^{15}N) had an ^{15}N incorporation about 30-fold above the

TABLE IV

RELATIVE INCORPORATION OF $^{15}N_2$ VERSUS $^{15}NH_4Cl$ BY METHANOGENS

Data using CP-MASS spectra of particulate cell material.

Methanogen	Generation time	$\frac{^{15}N_2}{^{14}NH_4Cl}$	$\frac{^{15}N_2}{^{15}NH_4Cl}$
Mc. thermolitho-	0.5	3	0.1
trophicus	3.0	50	0.7
Msp. hungatei	0.3	2	0.07
Mb. bryantii	0.3	2	0.05

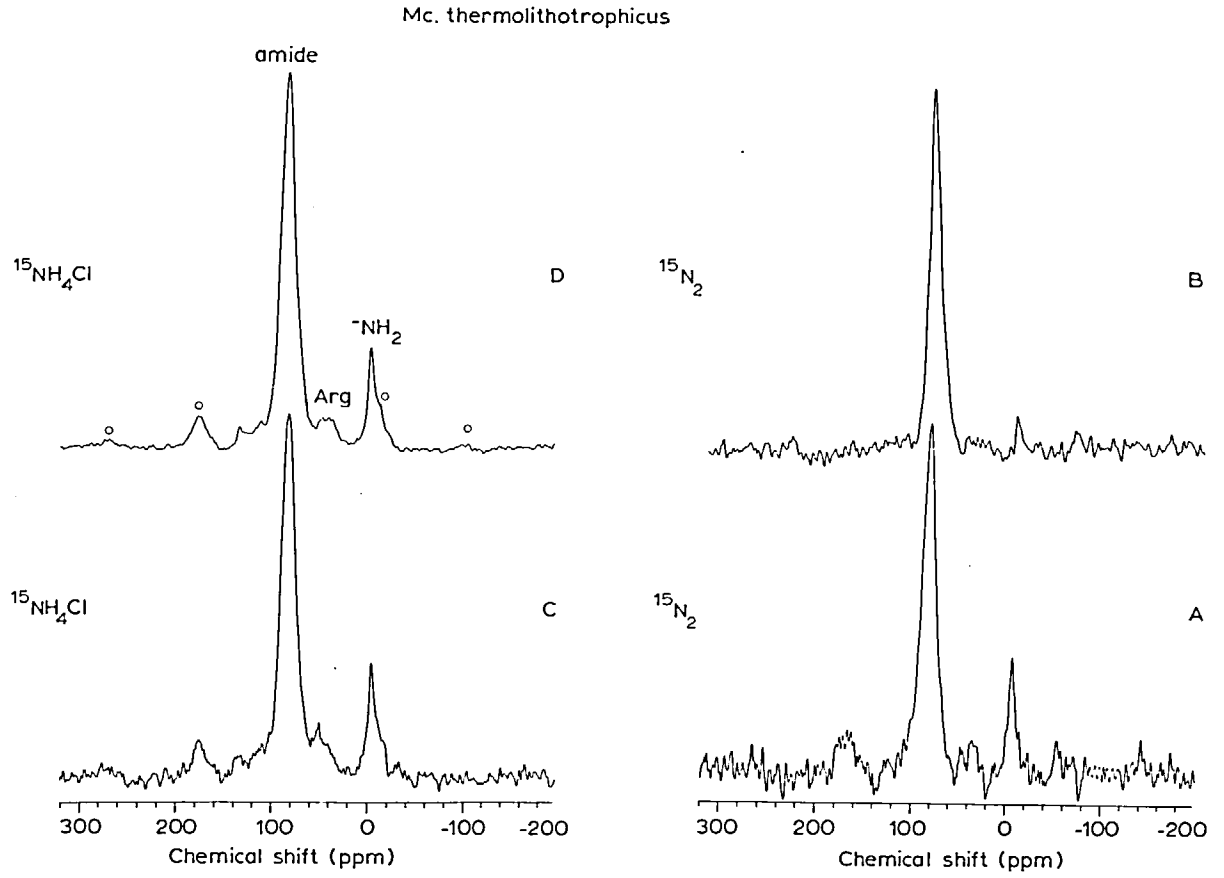


Fig. 5. CP-MASS ^{15}N-NMR (30.41 MHz) spectra of solid debris after ethanol extraction of *Mc. thermolithotrophicus* grown on $^{15}N_2$ for (A) 0.5 generation time (22.0 mg with 16000 transients) and (B) 3.0 generation times (13.0 mg with 14400 transients), and $^{15}NH_4Cl$ for (C) 0.5 and (D) 3 generation times (19.2 mg with 12000 transients and 27.7 mg with 14992 transients, respectively). Resonances are referenced to $^{15}NH_4Cl$ and identifications are indicated. The open circles mark rotational sidebands of the amide resonance.

natural abundance background (0.37%). Cells grown for approx. 0.5 generation time on $^{15}N_2$ (approx. 50% enrichment with $^{15}N_2$) in the growth medium showed a lower ^{15}N content which was only about 3-fold above the natural abundance background. If growth time and enrichment were the same as for the NH_4^+-grown cells, we would expect 14-fold above the natural abundance to be reached, because the $^{15}N_2$ in the medium was diluted by about 50%. If cells are grown on $^{15}N_2$ for 3 generation times, the ^{15}N content increases dramatically to approx. 50-fold above the natural abundance. This more closely approaches the 76-fold increase observed with 3 generation times on $^{15}NH_4^+$. Similar ^{15}N-CP MASS spectra are shown in Fig. 6 for *Msp. hungatei* grown on $^{15}N_2$ (A) and $^{15}NH_4Cl$ (B), and *Mb. bryantii* grown on $^{15}N_2$ (C) or $^{15}NH_4Cl$ (D). Again, many more transients were required to observe a signal from the debris of cells grown on $^{15}N_2$; due to the longer generation times when grown on N_2, compared to *Mc. thermolithotrophicus*, cells were not labeled for more than about 0.3 generation time, nonetheless, as shown in Table III, the normalized S/N ratios indicate $^{15}N_2$ uptake. The results for the three methanogens are summarized in a different way in Table IV. The ^{15}N enrichment over background for these methanogens grown on $^{15}NH_4Cl$ was comparable to that observed with *Mc. thermolithotrophicus* (approx. 30-fold). The ^{15}N enrichment

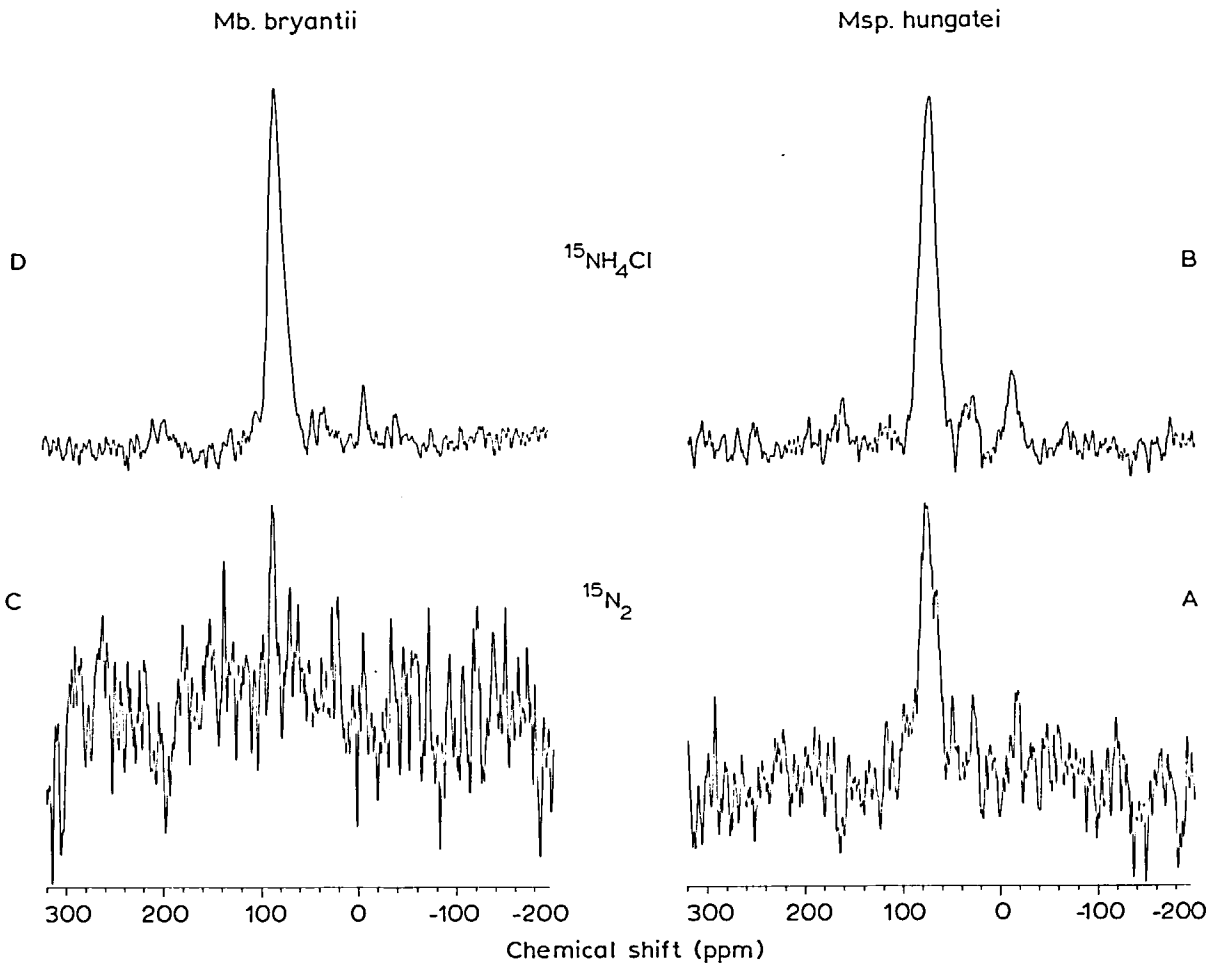


Fig. 6. CP-MASS ^{15}N-NMR (30.41 MHz) spectra of solid debris from *Msp. hungatei* cells grown for (A) 0.3 generation time on $^{15}N_2$ and (B) 0.5 generation time on $^{15}NH_4Cl$ (13.6 mg with 66824 transients, and 13.4 mg with 4000 transients, respectively), and from *Mb. bryantii* cells grown for (C) 0.3 generation time on $^{15}N_2$ and (D) 0.5 generation time on $^{15}NH_4Cl$ (14.0 mg with 28000 transients and 14.7 mg with 4880 transients, respectively).

(expressed as a $^{15}N_2$ incorporation: $^{14}NH_4^+$ incorporation ratio) for growth on $^{15}N_2$ for 0.3 generations (approx. 2 to 3-fold) was also similar to the result for *Mc. thermolithotrophicus*.

Discussion

Nitrogen fixation in methanogenic cultures was first described for *Mb. omelianskii* [4]. Since this was later shown to be a mixed culture [5], further speculations on methanogen capabilities to fix dinitrogen ceased. Since diazotrophy had been shown in a few methanogenic archaebacteria, it was thought that *Mb. bryantii*, the pure methanogenic strain derived from *Mb. omelianskii*, would be a likely candidate for diazotrophy. With our finding that *Msp. hungatei* GP1 is also a diazotroph, we can count dinitrogen fixation activities in species from four families of the methanogens [2]; also, two other families (*Methanothermacae* and *Methanoplanacae*) have genera with a gene that hybridizes to the *nif H* gene of *Anabaena* [13,33].

The whole cell K_m value for N_2 of *Mc. thermolithotrophicus* (0.11 atm N_2) was comparable to those calculated for eubacterial diazotrophs. A compilation of such data [34] presents K_m values for N_2 of 0.01–0.09 atm for whole cells and of 0.04–0.17 atm N_2 for cell-free extracts.

To prove that dinitrogen fixation was occurring in the tested methanogenic cultures, we used several different approaches. First, we demonstrated growth of *Mb. bryantii* and *Msp. hungatei* with dinitrogen gas as the sole nitrogen source and no growth in the absence of N_2 and NH_4^+. The fact that growth was slower and cell yields were lower (Table I) is consistent with dinitrogen fixation, since the reduction of gaseous N_2 to ammonium for subsequent incorporation into amino acids requires 20 and 30 ATP per molecule of N_2 reduced for *K. pneumoniae* and *Clostridium pasteurianum*, respectively [35,36]. Consequently, more methanogenesis (the energy-yielding reaction) is required, under diazotrophic conditions, for any amount of growth, than is required for growth in the presence of ammonium (1.6–4.3-times as much, as shown in Table I). Recent work with *Mb. thermoautotrophicum* [37] has presented data inconsistent with this concept; such experiments were conducted with continuous cultures, unlike the other data reported in Table I, but it is unclear why the yields are not comparable.

The second line of evidence of diazotrophy is the capacity of the diazotrophic cultures to reduce acetylene to ethylene in the absence of fixed nitrogen [29]. The dosage of acetylene added to the methanogenic cultures is critical, since it is an inhibitor of methanogenesis [30]. In the presence of acetylene concentrations that do not totally inhibit methanogenesis, we observed increasing amounts of ethylene over time in the absence of added ammonium.

The third line of evidence is that $^{15}N_2$ is incorporated into growing cells, as measured by NMR analysis. The latter shows an interesting trend for all three methanogens. While the distribution of ^{15}N label looks similar for cells grown on $^{15}N_2$ compared to growth on $^{15}NH_4Cl$, the level of enrichment, after short incubation periods, is considerably less for growth on $^{15}N_2$ compared to growth on $^{15}NH_4Cl$. Taking into account the lower growth and enrichment of the ^{15}N source in the media, labeling was approx. 10–70% of that on NH_4^+ (see normalized signal-to-noise data, Table III) in the case of *Mc. thermolithotrophicus*, it was dependent on the number of generations of the culture. Lower incorporation could represent a decrease in synthesis of nitrogen-containing molecules – i.e., cell mass has increased in C/H/O/P content compared to N components because N is limiting. Since both proteins and nucleic acids require nitrogen, such a massive decrease in overall nitrogen content is unlikely. Another explanation is that the cells use various catabolic pathways to recycle $^{14}NH_4^+$ which competes with $^{15}NH_4^+$ produced by reduction of $^{15}N_2$. One such catabolic activity has, in fact, been detected in *Mb. thermoautotrophicum* for arginine degradation [25]. While this may cause some dilution of the ^{15}N label, it is unlikely to account for all the difference between $^{15}N_2$ and $^{15}NH_4Cl$ assimilation. Perhaps the most likely explanation is that when the $^{14}N_2$ is removed and $^{15}N_2$ is added, a significant amount of non-gaseous, reduced ^{14}N (e.g., NH_4^+ or R-NH_3^+) is still present to effectively dilute $^{15}NH_4^+$ produced from $^{15}N_2$. The cell would rather use the ^{14}N species than expend more energy and reduce $^{15}N_2$ to $^{15}NH_4^+$. Precedence for such a large pool under N_2-fixing conditions exists with *Anabaena azollae*, where ammonium ions accounted for 66% of the N-pool [38]. In support of this explanation is the observation in our work of an ^{15}N-enriched amino species in the intact cell sample of *Mc. thermolithotrophicus.*

While all these data prove nitrogen fixation, it was still possible that a contaminant might be doing the actual dinitrogen reduction. However, attempts to maintain axenic cultures were rigorous; there was no microscopic evidence for contamination and our methanogenic strains did not grow in heterotrophic complex media in the absence of H_2/CO_2 nor in media suitable for sulfate reducers. We pursued a further line of verification based on the fact that BES selectively inhibits methanogenesis [39], stopping ATP synthesis and, therefore, dinitrogen fixation. This selectivity had, until recently, only been demonstrated in ecological studies [40], whereby the addition of BES stopped methanogenesis, permitting the accumulation of eubacterial fermentation products (acetate, H_2, ethanol). We have recently shown that BES had no effect on the growth of pure cultures of eubacteria grown either with complex nitrogen sources or N_2 [17]. In the work we present here, acetylene reduction by non-methanogen N_2 fixing

cultures was, likewise, unaffected. However, methanogens exposed to the inhibitor did not grow or reduce acetylene. Thus, since BES inhibition is entirely specific for methanogens, this provides strong evidence for N_2 fixation by the methanogens.

The antiquity of nitrogen fixation is not clear [41–43]. The evolutionary implications of diazotrophy in methanogens and the structural relatedness of methanogen and eubacterial nitrogenases are currently the subject of genetic and biochemical studies [10–14]. Thus, the discovery of the phenomenon in the methanogenic archaebacteria may lead to a better understanding of this topic; current data suggest that the phylogenetic relationships between methanogens and eubacteria are similar whether based on 16S rRNA or *nif* gene comparisons [44].

Recently, it has been demonstrated by Bishop and co-workers that *Azotobacter vinelandii* contains more than one system for nitrogen fixation, one of which requires vanadium and both of which only occur in the absence of molybdenum [45–47]. Robson et al. have also recently reported a vanadium nitrogenase in *Azotobacter chroococcum* [48]. The vanadium enzyme components produce low levels of ethane as well as ethylene from acetylene reduction [49]. All our work has been conducted in the presence of molybdenum, and with no added vanadium; we would thus not expect these enzymes to be present, but it is possible that a molybdenum- and vanadium-independent enzyme could account for the activities. We do not observe ethane production concurrent with ethylene appearance under our experimental conditions. However, due to the inhibition of methanogens by moderate levels of acetylene, the low acetylene levels used result in low ethylene levels (Table II) via nitrogenase activity; since the above-mentioned ethane production by the vanadium enzyme occurs at 1–2% the rate of ethylene production, very little ethane would be expected. Thus, our experiments would not detect these low ethane levels, and, we cannot conclude that ethane is not produced.

Ecologically, nitrogen fixation may be of significance to methanogens in several environments. In high carbon, low fixed-nitrogen environments, such as some landfills, the use of N_2 may be helpful to the methanogens growing there. Also, the environment of some poorly fertilized rice paddies may be nitrogen-poor, and N_2- fixation by any of several bacteria there may be significant not only to the microorganisms, but perhaps also to the plants. The finding of assimilatory nitrate reduction [28] along with nitrogen fixation shows the versatility and adaptability of methanogens to numerous different environments.

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