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Nitrate as a Sole Nitrogen Source for *Methanococcus thermolithotrophicus* and Its Effect on Growth of Several Methanogenic Bacteria

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Abstract. *Methanococcus (Mc.) thermolithotrophicus* can use nitrate as the sole source of nitrogen, but four other species of methanogens cannot. The growth rate was similar on both nitrate and ammonium, but yields were 20–25% lower on nitrate. *Mc. thermolithotrophicus, Methanobacterium thermoautotrophicum*, and *Methanobrevibacterium smithii* were not inhibited by 20 m*M* nitrate, but *Methanospirillum hungatei* was inhibited 35%, and *Methanosarcina barkeri* was completely inhibited by 20 m*M* nitrate. When *Mc. thermolithotrophicus* was growing with nitrate as the sole source of nitrogen, growth was dependent on either molybdenum or tungsten, and the presence of both gave the best growth response; vanadium or chromium did not replace the requirement for these metals. Growth on ammonium could not be strictly demonstrated to require either of these metals, but both molybdenum and tungsten stimulated growth.

Methanogenic bacteria are one of the major divisions of the archaeobacteria [13, 18]. They are strict anaerobes found in a wide variety of ecological niches, including marine and freshwater sediments, geothermal springs, hydrothermal vents, anaerobic waste digesters, and intestinal tracts of animals [18]. They derive their energy by generation of methane from a limited number of simple substrates including H_2-CO_2, acetate, formate, methanol, and methylamines [13].

All methanogenic bacteria are known to use ammonia as the sole source of nitrogen for their growth. As well, it was recently shown that several methanogen species are able to fix molecular nitrogen [4, 7, 8, 19, 24]. Other nitrogen sources, mostly organic compounds, have also been examined as nitrogen sources for some methanogens. *Methanobacterium bryantii* and *Methanobrevibacter ruminantium* were unable to use complex mixtures of amino acids and peptides tested as nitrogen sources for growth [12]. *Methanococcus voltae* was able to transport amino acids but could not utilize them as sole nitrogen sources [30, 31]. In contrast, *Methanococcus maripaludis*, *Methanococcus deltae*, and 16 other autotrophic *Methanococcus* isolates were found to assimilate several amino acids as supplementary carbon sources and utilized alanine as a sole nitrogen source for growth [31]. *Methanococcus voltae* was also unable to use nitrate, nitrite, urea, taurine, or methylamine as a source of nitrogen [30]. Purines and pyrimidines were shown to serve as sole nitrogen sources for growth of *Methanococcus vannielii* [16], but not for *Methanococcus voltae* [30].

Another inorganic source of nitrogen for cell growth that can be reductively assimilated by some bacteria is nitrate. In this study we examined nitrate as an alternative to NH_4^+ or N_2 for growth of several methanogen species, particularly those that were previously shown to use N_2. Since nitrate was previously reported to be inhibitory to methanogenesis in experiments with ecological samples [1, 3, 23, 33, 34] and pure cultures [3, 22], we also investigated its effect on pure cultures of several methanogens, with ammonium serving as the nitrogen source for cell growth. [Preliminary results of portions of this work were presented at the First Symposium on Biotech-

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Materials and Methods

Organisms. *Methanobacterium* (*Mb.*) *thermoautotrophicum* Marburg [10] was obtained from G. Fuchs. *Methanococcus* (*Mc.*) *thermolithotrophicus* [17] was a gift of K.O. Stetter. *Methanospirillum* (*Msp.*) *hungatei* GP1 [25] and *Methanobacterium bryantii* M.O.H. [11] were obtained from G.D. Sprott. *Methanobrevibacter* (*Mbr.*) *smithii* Hdm-30A [6] was isolated in our laboratory. *Methanosarcina* (*Ms.*) *barkeri* 227 (21) was provided by S.H. Zinder.

Medium and growth conditions. The methanogens were grown in serum tubes (no. 2048-00150; Bellco Glass, Inc., Vineland, New Jersey) or serum bottles (no. 223950, Wheaton Scientific) according to the anaerobic techniques described previously [2, 14]. All strains were grown on H_2-CO_2 as carbon and energy source, with other media components as indicated below.

Mb. thermoautotrophicum and *Mc. thermolithotrophicus* were grown at 65°C in the media described previously [14], but 1 μM each of sodium selenate and sodium tungstate were also included, and NH_4Cl was used at a concentration of 8.4 mM in the medium for routine growth of *Methanococcus*. For experiments on the effect of molybdenum, tungsten, and vanadium on utilization of nitrate or ammonium by *Mc. thermolithotrophicus*, medium lacking these elements was used and additions were made as indicated in the Results section from sterile, anaerobic stock solutions of sodium salts of molybdenum and tungsten, and vanadyl sulfate and chromium chloride. All glassware and stoppers for these experiments were washed as described previously [5] to minimize trace metal contamination. *Msp. hungatei* was grown at 37°C in the medium described previously [14]. *Mb. bryantii* and *Ms. barkeri* were grown at 37°C in the media described previously [15]. *Mbr. smithii* was grown at 37°C in the medium described previously [6]. Nitrate additions were made from anaerobic, sterile stock solutions of $NaNO_3$. Cell growth was monitored by measurement of absorbance at 600 nm in a Perkin-Elmer Lambda 3A spectrophotometer (Perkin-Elmer Corp., Norwalk, Connecticut) or a Spectronic 20 (Bausch and Lomb, Inc., Rochester, New York) spectrophotometer. Protein was determined by the Bradford assay [9]. Cultures were incubated in a gyratory shaker at 150 rpm, unless stated otherwise. All data are the averages of duplicate tubes or bottles.

Results

Mc. thermolithotrophicus, *Mb. thermoautotrophicum*, *Msp. hungatei*, *Mb. bryantii*, and *Ms. barkeri* were examined for their ability to utilize nitrate in place of ammonium as the sole source of nitrogen for growth. Cells grown in medium with 2.0 mM ammonium were transferred repeatedly into medium containing 5.0 mM nitrate, but no NH_4^+ or other potential nitrogen sources. Continued growth under such conditions was observed only with *Mc. thermolithotrophicus*. After several transfers of the culture in this medium, further growth studies were conducted.

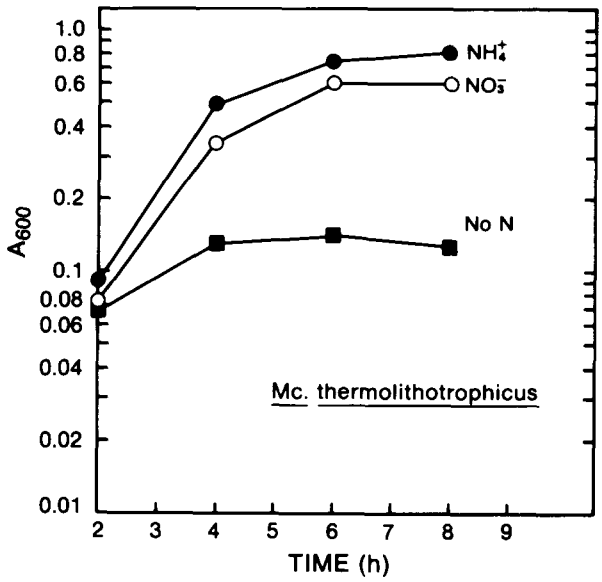

Fig. 1. Growth of *Methanococcus thermolithotrophicus* in medium with nitrate or ammonium as the sole nitrogen source. ●, 8.4 mM ammonium; ○, 5.3 mM nitrate; ■, no nitrogen source added but with 0.51 mM nitrate carryover from the inoculum which was grown in nitrate medium. The cultures were incubated without shaking for the first 2 h.

Figure 1 shows a comparison of growth of the nitrate utilizing *Mc. thermolithotrophicus* culture when inoculated into medium with NH_4^+ or NO_3^- as the nitrogen source. The growth rates were similar with either compound as the nitrogen source, but the final cell density was characteristically lower (20–25% less) during growth in the nitrate medium. The concentration of NO_3^- needed for optimal growth of the organism was also determined. Figure 2 shows cell growth with varying concentrations of nitrate in the medium. Optimal growth occurred at $\geqq$5 mM, and concentrations of up to 100 mM had no further effect. Remarkably, growth occurred at concentrations as high as 200 mM; concentrations of 150–200 mM caused a lag, but the cultures attained final cell densities similar to that obtained with 5 mM nitrate (Fig. 3).

In order to rule out the observed utilization of nitrate as due to a contaminant, *Mc. thermolithotrophicus* was plated out repeatedly, and the purity was further tested by inoculation into heterotrophic media and media containing antibiotics, as described previously [13]. No growth occurred in the absence of H_2-CO_2 when cells were transferred into hetero-

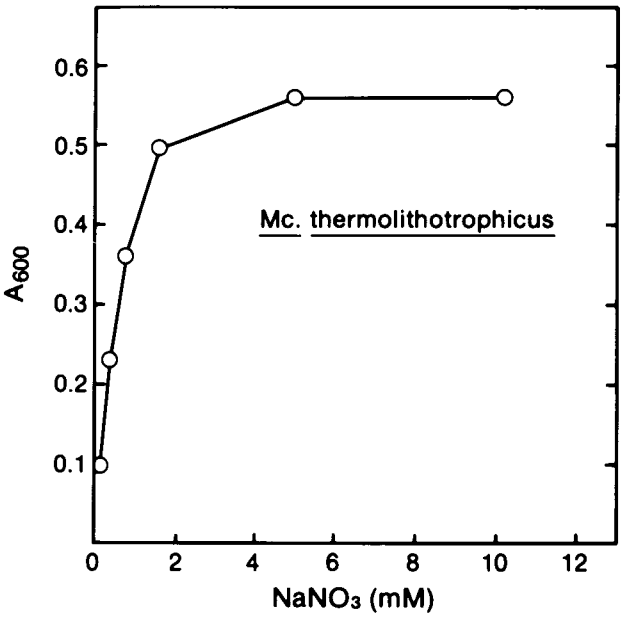

Fig. 2. Effect of various concentrations of nitrate as a nitrogen source for growth of *Methanococcus thermolithotrophicus*.

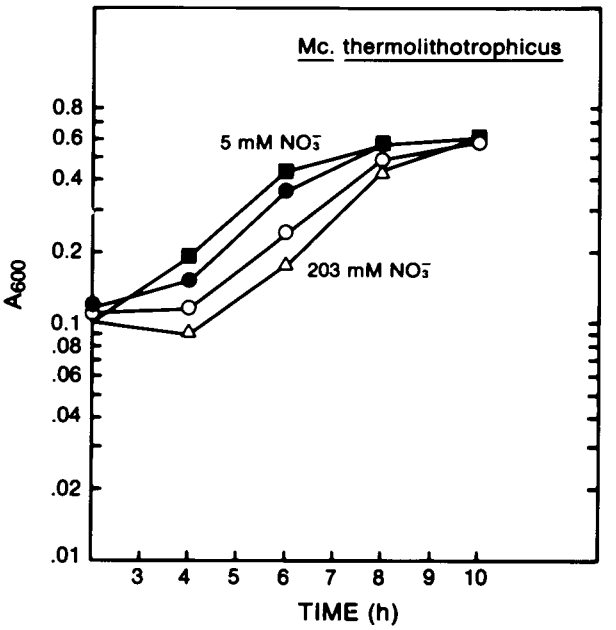

Fig. 3. Growth of *Methanococcus thermolithotrophicus* in medium containing high levels of nitrate for nitrogen source. ■, control with 5 m*M*; ●, 102 m*M*; ○, 150 m*M*; △, 203 m*M*. The cultures were grown without shaking for the initial 2 h.

trophic media or media permitting growth of sulfate reducers. Growth with nitrate or NH_4^+ as nitrogen source was not impaired by streptomycin and vancomycin, which are antibiotics known to kill eubacteria but not archaeobacteria. As well, repeated microscopic examination of the nitrate or ammonium cultures revealed no contamination.

Since it is known that nitrate reductases are molybdoproteins, we also examined the effect of molybdenum, tungsten, vanadium, and chromium on growth of *Mc. thermolithotrophicus* with NO_3^- as nitrogen source. When cells were transferred repeatedly into medium lacking molybdenum and tungsten, growth failed completely at carryover concentrations of 1.0 n*M* tungsten and 1.9 n*M* molybdenum. Figure 4A illustrates growth observed in the presence or absence of these trace elements. Growth occurred when molybdenum and tungsten were added to the medium either in combination or separately, with greater growth taking place in the presence of tungsten. Addition of vanadium or chromium did not allow growth of cells in the nitrate medium. Growth with NH_4^+ as the nitrogen source did not cease upon repeated transfer of cells into medium without molybdenum and tungsten; however, growth was reduced to about 35% of normal when carryover concentrations reached 0.16 n*M* molybdenum and 0.09 n*M* tungsten. As shown in Fig. 4B, addition of 1.0 μM molybdenum stimulated growth by about 25%, whereas the presence of 0.5 μM tungsten allowed full growth.

The effect of nitrate on growth of some of the methanogens, including *Mc. thermolithotrophicus*, was also studied. Varying concentrations of nitrate were included in the medium for growth of cells with ammonium as the nitrogen source. Growth of *Mb. thermoautotrophicum*, *Mbr. smithii*, and *Mc. thermolithogrophicus* was not affected by nitrate concentrations up to 20 m*M*; as stated above, *Mc. thermolithotrophicus* was also able to grow with nitrate as the nitrogen source at concentrations up to 200 m*M*. On the other hand, nitrate had an inhibitory effect on growth of *Ms. barkeri* and *Msp. hungatei*. *Methanosarcina* was most affected, with slight inhibition occurring at concentrations as low as 2.0 m*M* and nearly complete inhibition occurring at 20 m*M* of nitrate (Fig. 5). Growth of *Methanospirillum* cultures was lowered by about 35% with addition of 20 m*M* $NaNO_3$ compared with the control cultures with 20 m*M* NaCl added (Fig. 6).

Discussion

Although some methanogens use N_2 in place of ammonium as sole inorganic source of nitrogen [4, 7,

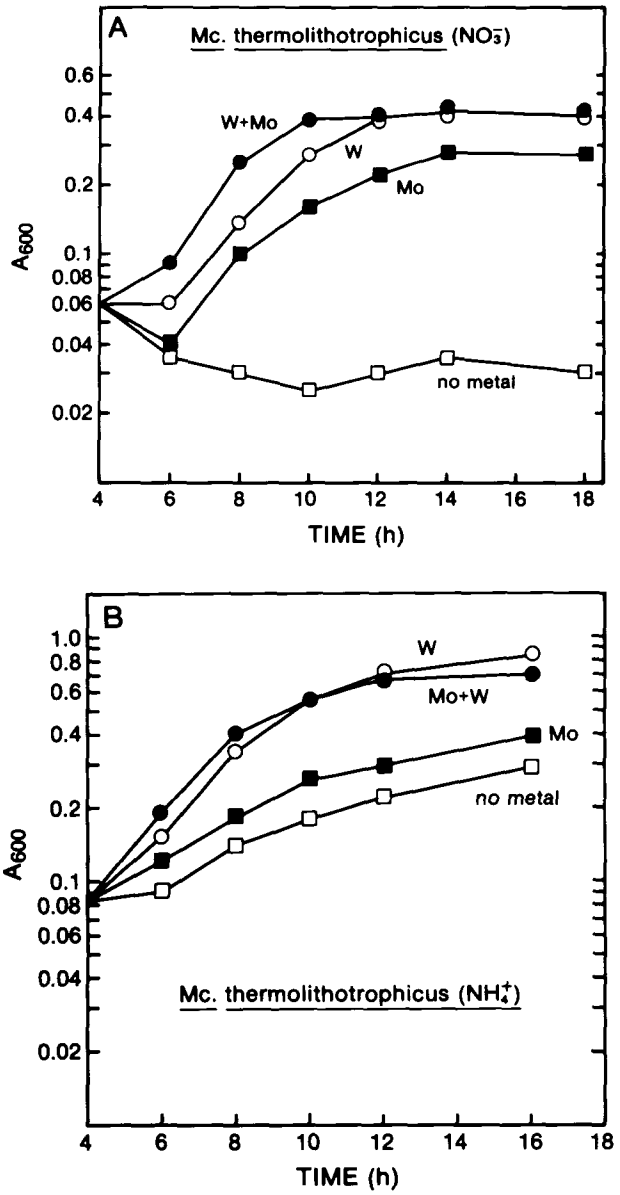

Fig. 4. Effect of molybdenum (Mo) or tungsten (W) on growth of *Methanococcus thermolithotrophicus* in medium with nitrate (panel A) or ammonium (panel B) as the nitrogen source. □, no added molybdenum or tungsten; ■, 1.0 μM Mo; ●, 0.5 μM each of Mo and W; ○, 0.5 μM W. The cultures were incubated without shaking for the first 4 h.

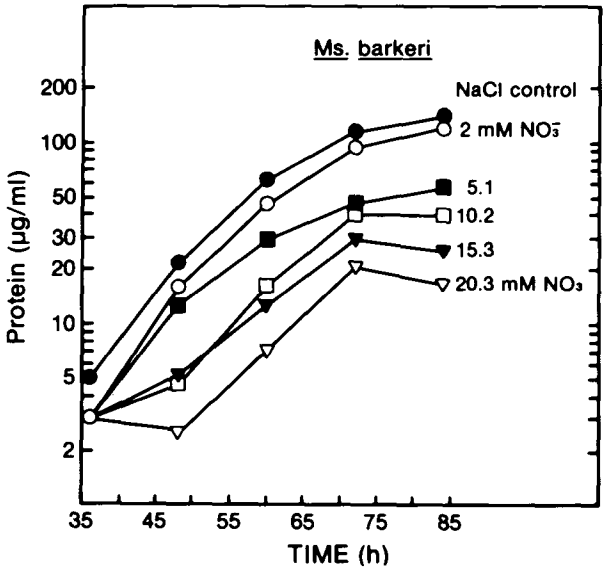

Fig. 5. Effect of nitrate on growth (measured as protein) of *Methanosarcina barkeri* 227 in medium with ammonium as the nitrogen source. ○, 2 mM; ■, 5.1 mM; □, 10.2 mM; ▼, 15.3 mM; ▽, 20.3 mM; ●, contral culture with 20.3 mM NaCl. Cultures were incubated without shaking for the first 36 h.

8, 19, 24], very little work has been done on nitrate as an alternative or its effects on pure cultures of methanogens. Assimilation of nitrate by methanogenic bacteria would be of ecological significance in some systems in which NO_3^- is available, and the scarcity of reduced nitrogen sources may be a growth limiting factor; for example, ocean water contains about 5–100 times as much nitrate as it does ammonium [26].

In previous studies *Mc. voltae* [30] and *Mb. thermoautotrophicum* ΔH (L. Daniels, M.S. thesis, University of Wisconsin, Madison, 1974) did not use NO_3^- as a nitrogen source. Our study shows that, of five methanogen species examined, only *Mc. thermolithotrophicus* used NO_3^- as the sole source of nitrogen for growth; it was also the only marine isolate in our study, and it will thus be of interest to examine other marine species for their utilization of nitrate. As well, soils exposed frequently to nitrate-containing fertilizers may contain nitrate-using methanogens. The results also show that the nitrate assimilation capacity of this organism is dependent on molybdenum or tungsten, with better growth observed in the presence of tungsten. Growth in molybdenum- and tungsten-deficient medium failed with NO_3^- as the nitrogen source, whereas limited growth occurred with NH_4^+ as the nitrogen source during repeated transfer of cells into medium lacking these elements. Contaminating levels of molybdenum from syringes used for transfer of cells or from glassware, stoppers, and media components may be sufficient to allow the limited growth observed with NH_4^+ as the nitrogen source. Previous reports on levels of molybdenum needed for growth of methanogens in NH_4^+ media range from no requirement to

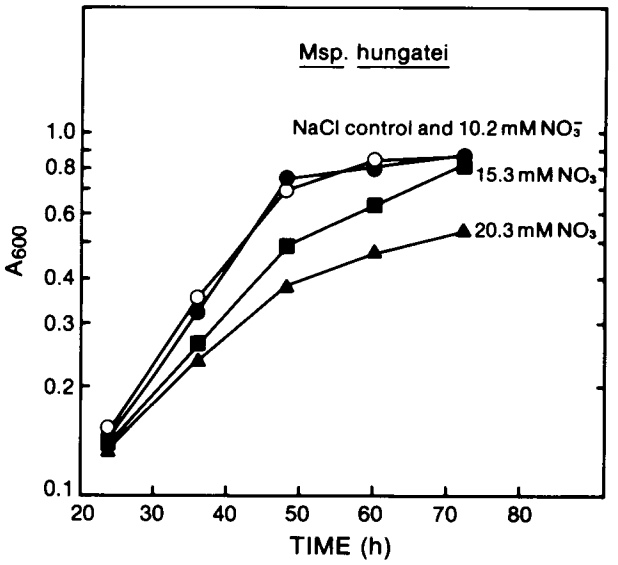

Fig. 6. Effect of nitrate on growth of *Methanospirillum hungatei* in medium with ammonium as the nitrogen source. ○, 10.2 m*M*; ■, 15.3 m*M*; ▲, 20.3 m*M*; ●, control with 20.3 m*M* NaCl. The cultures were grown without shaking for the initial 24 h.

0.5 μM (8, 27–29, 32). Furthermore, previous studies show growth of some methanogens in NH_4^+ media to be stimulated by tungsten [32, 35], a finding similar to that observed here.

It is known that nitrate reductases isolated from various bacteria are molybdoproteins and that tungsten and vanadium result in their inactivation during growth since they are competitive inhibitors of molybdenum uptake [20]. The effect of tungsten on nitrate utilization by *Mc. thermolithotrophicus* observed here suggests that it may instead play a positive role in the function of nitrate reductase from this organism.

In studies with mixed cultures from ecological samples, several workers have observed inhibition of methanogenic activity by nitrate. Levels ranging from 0.1 μM to 10 m*M* have been reported to inhibit methanogenesis, but the mechanism of inhibition was not well understood in most of these studies [1, 3, 23, 33, 34]. One study suggests inhibition by N_2O as the possible mechanism, since nitrous oxide is known to inhibit pure cultures of methanogenic bacteria [3], and the inhibitory effect on methanogenesis occurred concurrently with N_2O production in the sediments that contained nitrate [23].

Inhibition of pure cultures of some methanogens was also reported previously. A 30 and 100% inhibition of an unspecified strain of *Mb. thermoautotrophicum* and *Mb. formicicum*, respectively, was observed with the addition of 9.3 m*M* nitrate into cultures [3]. However, *Mb. thermoautotrophicum* strain ΔH was found not to be inhibited by 30 m*M* nitrate (L. Daniels, M.S. thesis, University of Wisconsin, Madison, 1974). Another methanogen, *Methanosphaera stadtmaniae*, was completely inhibited when exposed to nitrate at a concentration of 33 m*M* [22]. In our study, drastic inhibition was observed only in the case of *Methanosarcina barkeri*, and a 35% inhibition of *Methanospirillum hungatei* occurred at nitrate concentrations of 20 m*M*. Nitrate did not inhibit the other methanogens at a concentration of 20 m*M*, and surprisingly *Mc. thermolithotrophicus* could grow with nitrate as the nitrogen source at concentrations as high as 200 m*M*. These studies suggest various methanogens are affected very differently by nitrate; the mechanism of inhibition of certain methanogen species should be further studied. It is possible that in some of the mixed-culture studies inhibition arises owing to the production of N_2O from nitrate by nonmethanogenic bacteria.

The finding of assimilatory nitrate reduction by *Mc. thermolithotrophicus* suggests that yet other untested methanogen species may possess this property. As well, enzymatic studies should be carried out to further characterize the utilization of nitrate by this organism, especially given the unusual role of tungsten.

ACKNOWLEDGMENTS

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