Arch Microbiol (1985) 142:148–151

Archives of
Microbiology

Trace metal and vitamin requirements of *Methanococcoides methylutens* grown with trimethylamine

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Abstract. Trace organic nutrient and metal requirements for the growth of *Methanococcoides methylutens* strain TMA-10 were determined in defined medium that contained trimethylamine as the substrate. Biotin was the only organic supplement required in place of yeast extract, Trypticase or a mixture of 8 B-vitamins. Fe and Ni were required for growth and low concentrations of Fe^{2+} (< 5 μM) and Ni^{2+} (< 0.25 μM) provided limited growth. In the absence of added Co the growth rate was reduced by 94% and growth was limited at concentrations below 0.1 μM. Stimulation of growth by Se, Mo, B, Al, Zn, Mn or Cu could not be demonstrated.

Key words: Cobalt – Iron – Nickel – Biotin – *Methanococcoides methylutens* – Trace elements – Vitamins – Methanogens

Comprehensive studies on the trace metal and vitamin requirements of methanogenic bacteria have been reported for *Methanobacterium thermoautotrophicum* (Schönheit et al. 1979; Taylor and Pirt 1977), *Methanococcus voltae* (Whitman et al. 1982) and *Methanosarcina barkeri* (Scherer and Sahm 1981). The vitamin content of *M. thermoautotrophicum* and *M. voltae* have been reported (Leigh 1983), and the elemental compositions of 10 methanogenic species were determined by inductively-coupled plasma emission spectrometry (Scherer et al. 1983). *M. barkeri* is the only species in these studies that uses acetate, methanol and methylamines as substrates for growth. Recently, methanogenic bacteria have been described that use only methanol or methylated amines for growth (König and Stetter 1982; Sowers and Ferry 1983; Zhilina 1983). One of these species, *Methanococcoides methylutens*, was isolated from a marine canyon and some nutritional requirements have been reported (Sowers and Ferry 1983). We describe here the effect of trace metals and organic supplements on the growth of *M. methylutens* when grown in defined medium with trimethylamine.

Materials and methods

Culture conditions. Methanococcoides methylutens strain TMA-10 (= DSM 2657, = ATCC 33938) was maintained on agar slants (Sowers and Ferry 1983). Sterile media, described below, were prepared under an oxygen-free N_2-CO_2 (4:1) atmosphere by a modification of the Hungate technique (Balch and Wolfe 1976). Growth experiments were done with culture tubes (16 by 150 mm; Bellco Glass Inc., Vineland, NJ, USA) that contained 10 ml of medium and were sealed with butyl rubber septa. Cultures were incubated at 30° C and growth was measured by determining absorbance at 550 nm. All results are reported as the mean value of triplicate cultures.

Vitamin-free medium. The procedure for the organic growth factor experiments has been described (Sowers et al. 1984). Water for all organic growth factor experiments was prepared by passing demineralized water through a column of activated charcoal. All glassware was washed with concentrated sulfuric acid, rinsed with charcoal-treated water and heated at 450° C overnight. The medium contained the following constituents in charcoal-treated water (percent weight/vol): NaCl, 2.34; $MgSO_4$, 0.63; Na_2CO_3, 0.5; NH_4Cl, 0.5; trimethylamine-HCl, 0.3; KCl, 0.08; Na_2HPO_4, 0.06; $CaCl \cdot 2\,H_2O$, 0.014; $Na_2S \cdot 9\,H_2O$, 0.025; cysteine-HCl · H_2O, 0.025; and resazurin, 0.0001. In addition 1% (vol/vol) of trace metal solution (Wolin et al. 1963) was added and the pH of the medium was adjusted to 7.2 with 6 N HCl before dispensing. Cysteine was deleted from the medium when testing for stimulation by Casamino Acids. The growth factors were added to each tube of medium (0.1 ml) from 100-fold stock solutions before autoclaving. The final concentrations of growth factors tested were 0.1% (wt/vol) yeast extract, Tryptcase, Casamino acids and 1% (vol/vol) vitamin mixture (Wolin et al. 1963) and short chain fatty acid mixture that included 0.5 ml/l of each of the following components: formic acid, acetic acid, valeric acid, 2-methylbutyric acid, isobutyric acid, isovaleric acid.

Metal-free medium. Trace metal requirements were determined using the procedure of Whitman et al. (1982) with modifications described below. All glassware was acid washed and butyl rubber stoppers and polypropylene pipet tips were treated with $NaHCO_3$/EDTA solution to minimize trace metal contamination (Whitman et al. 1982). Gas cannula needles were covered with Teflon tubing to prevent metal contamination. A Chelex-100 column (1.5 × 18 cm, Bio-Rad Laboratories, Richmond, CA, USA) was pretreated to generate the sodium form (Davey et al. 1970). The flow rate was adjusted to 5 ml/min and the column was equilibrated with sodium acetate (0.5 M, pH 5.1), then rinsed with 5 bed volumes of demineralized H_2O. This pH

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was chosen in order to adsorb Mo in addition to Co, Cu, Fe, Ni and Zn (Riley and Taylor 1968). Six liters of demineralized H_2O were passed through the column and collected for rinsing and medium dilution. The medium was prepared in two parts. Part I contained the following components in demineralized H_2O (percent wt/vol): NaCl, 4.68; $MgSO_4$, 1.26; NH_4Cl, 1.0; trimethylamine-HCl, 0.6; KCl, 0.6; Na_2HPO_4, 0.12; $CaCl_2 \cdot 2\ H_2O$, 0.028; cysteine-HCl $\cdot$ H_2O, 0.05; biotin, 0.004. The pH was adjusted to 5.1 with 1 N HCl before passing through the Chelex-100 column. The first 100 ml were discarded and the remaining medium was collected in a flask continuously purged with N_2-CO_2. Part II contained the following components in demineralized H_2O (percent wt/vol): Na_2CO_3, 2.5; $Na_2S \cdot 9\ H_2O$, 0.125. The pH was adjusted to 8.0 with 1 N HCl, passed through a Chelex-100 column equilibrated with Na_2HPO_4 buffer (0.5 M; pH 8.0), and collected as described above. Part I (500 ml), part II (200 ml) and boiled Chelex-treated water (300 ml) were anaerobically mixed together in a 2 l flask and dispensed into culture tubes that contained the metal salts to be tested. Cells were harvested by centrifugation and washed three times in Chelex-treated medium. Cells were then suspended in a volume of Chelex-treated medium required to obtain an absorbance of 0.01 with at 1% inoculum.

Chemical sources. Yeast extract was from Difco Laboratories (Detroit, MI, USA). Trypticase was from BBL Microbiology Systems (Cockeysville, MD, USA). Vitamin-free, salt-free Casamino Acids was from ICN Nutritional Biochemicals (Cleveland, OH, USA). All vitamins were from Sigma Chemical Co. (St. Louis, MO, USA). Trimethylamine-HCl and the ultra-pure (99.999%) metal salts $CoSO_4 \cdot 6\ H_2O$, $NiCl_2 \cdot 6\ H_2O$, and $(NH_4)_2Fe(SO_2)_2 \cdot 6\ H_2O$ were from Aldrich Chemical Co. (Milwaukee, WI, USA). All other chemicals were of reagent grade.

Results

No significant growth occurred in vitamin-free mineral medium after three sequential transfers unless a B-vitamin mixture or biotin alone was added (Fig. 1). The addition of biotin with or without the complete vitamin mixture resulted in the same amount of growth and the individual deletion of any one vitamin, except biotin, had no significant influence on growth. An equivalent amount of growth also occurred when yeast extract or Trypticase replaced biotin (data not shown). However, no growth occurred when either vitamin-free Casamino Acids or a volatile fatty acid mixture was added to vitamin-free mineral medium and these supplements were not stimulatory when added to medium that also contained biotin. The results indicate that biotin is the only organic growth supplement required by this strain of *Methanococcoides methylutens*.

The trace metal requirements of *M. methylutens* were determined in medium that was treated with Chelex-100 ion-exchange resin to remove divalent metal cations. Each metal was added at various concentrations in the presence of fixed amounts of the other two metals as indicated in Fig. 2. Final culture densities were limited by concentrations below 5 µM Fe, 0.25 µM Ni and 0.10 µM Co. No growth occurred in medium without added Fe. A final absorbance of 0.3 was consistently obtained throughout serial transfers in medium

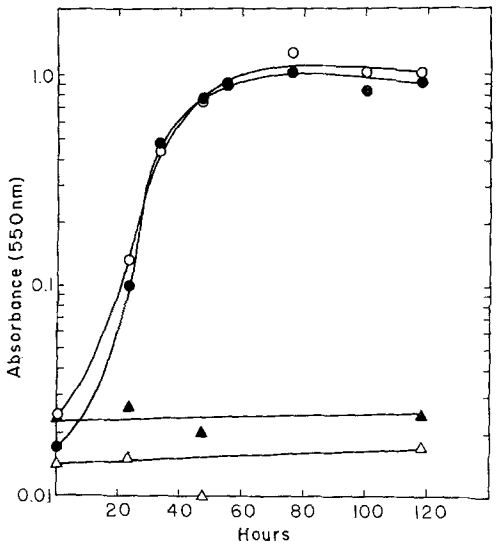

Fig. 1. Effect of biotin and seven other vitamins on growth of *Methanococcoides methylutens* with trimethylamine. Additions include complete vitamin mixture with (●) and without (▲) biotin, biotin only (○), and no vitamins (△). The final concentration of biotin was 0.02 mg/l

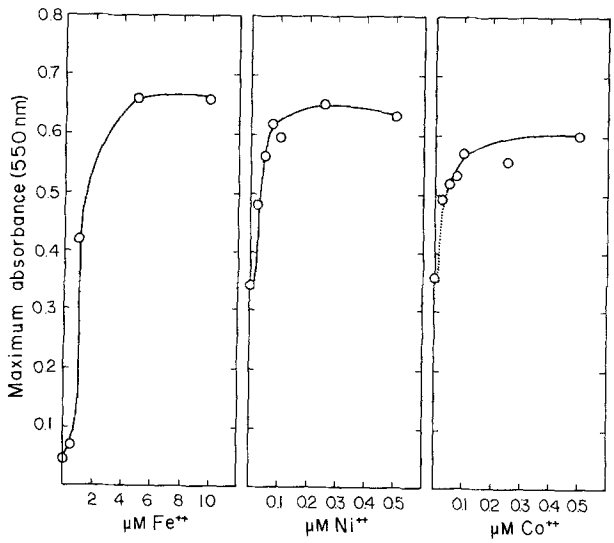

Fig. 2. Effect of Ni, Fe and Co on the growth of *Methanococcoides methylutens* with trimethylamine. Each metal salt was added at the indicated concentrations with optimum concentrations of the other two metals. The optimum concentrations in µmol/l are: Fe, 4.0; Co, 0.5; and Ni, 0.5. The values represent maximum absorbance at the end of growth with the concentrations of metals indicated. The values on the dotted line of the Co curve were obtained after 1 week rather than 2 to 3 days because of a slower growth rate

without added Ni. In medium without added Co, the specific growth rate decreased from 0.19 h^{-1}, the growth rate at optimum Co concentrations, to 0.011 h^{-1}. This lower growth rate was reproducible in subsequent transfers. Other metal salts were tested for their ability to stimulate growth in medium that contained optimum concentrations of Fe, Ni and Co. The following metal salts failed to stimulate growth when individually added at the indicated final concentrations in µmol/l: $MnSO_4$, 30; Na_2MoO_4, 10; Na_2O_3Se, 10; $Zn(C_2H_3O_2)_2$, 6; H_3BO_3, 2; $CuSO_4$, 0.4;

$AlKO_8S_2$, 0.2. Tungsten, an antagonist of molybdoenzymes, was added to medium to further test for an Mo requirement, but there was no inhibition at concentrations as high as 1 mM Na_2WO_4.

Discussion

The methanogenic archaeobacteria synthesize several unique cofactors including coenzymes M, F_{420} and F_{430} (McBride and Wolfe 1971; Eirich et al. 1978; Keltjens et al. 1983), but FAD (Lancaster 1981) and vitamins (Leigh 1983) ubiquitous among the eubacteria have also been reported in methanogenic bacteria. Results from this study indicate that *Methanococcoides methylutens* requires an exogenous source of biotin for growth. The addition of biotin to mineral medium is stimulatory to the growth of *Methanobacterium bryantii* (Bryant et al. 1971), and is required by *Methanothrix soehngenii* (S. H. Zinder, personal communication). Leigh (1983) has reported that *Methanobacterium thermoautotrophicum* contains biotin although the concentration is approximately one order of magnitude below that found in eubacteria. Among the *Methanosarcina* only *Methanosarcina barkeri* strain Fusaro requires riboflavin (Scherer and Sahm 1981); *Methanosarcina* sp. strain TM-1 requires p-aminobenzoic acid (S. H. Zinder, personal communication); and *Methanosarcina acetivorans* stain C2A has no vitamin requirements (Sowers et al. 1984). *Methanolobus tindarius*, which is phenotypically similar to *M. methylutens* is reported to have no vitamin requirements although B-vitamins are stimulatory (König and Stetter 1982). Since the vitamin requirements among methanogenic species are diverse (Mah and Smith 1981) and the requirements of several strains in each species have not been determined, it is possible that the vitamin deficiencies may be auxotrophic characteristics at the strain level. Such characteristics, if at the strain level, could be useful markers for genetic analyses.

The metal requirements of *M. methylutens* were similar to those reported for other methanogenic bacteria. Previous reports indicate that Fe is required for growth of several genera (Patel et al. 1976, 1978; Schönheit et al. 1979; Whiteman et al. 1982) and stimulates methane production from acetate in fermentors (van den Berg et al. 1980; Hoban and van den Berg 1979; Murray and van den Berg 1981). The growth-limiting concentration of Fe (< 5 mM) for *M. methylutens* was within the range reported for *Methanobacterium thermoautotrophicum* (Schönheit et al. 1979) and *Methanococcus voltae* (Whitman et al. 1982). Fe may be required for the synthesis of cytochromes which have been reported in all acetotrophic and methylotrophic species tested (Kühn et al. 1983; Kühn and Gottschalk 1983) including *M. methylutens* (Jüssofie 1984; J. Ferry, unpublished results). Iron-containing superoxide dismutase (Kirby et al. 1981), ferredoxin (Hatchikian et al. 1982) and non-heme iron-sulfur proteins (Lancaster 1980; Scherer and Sauer 1982) have also been reported in methylotrophic species. High potential iron-sulfur signals appear in electron paramagnetic resonance spectra of *M. methylutens* whole cells (M. Barber, personal communication).

Growth could not be completely inhibited when Ni was not added to Chelex-treated medium. However, growth stopped at an absorbance of 0.3 when Ni was not added suggesting that low concentrations of Ni remaining in the medium may support this small amount of growth. Based on a ratio of final absorbances at various concentrations it was estimated that there was less than 0.025 μM Ni remaining in the Chelex-treated medium. Other investigators have reported low levels of growth in medium without added Ni (Schönheit et al. 1979; Whitman et al. 1982). The growth limiting concentration of Ni for *M. methylutens* was in the same range reported for other methanogenic bacteria (Schönheit et al. 1979; Whitman et al. 1982). Ni is a component of hydrogenase (Graf and Thauer 1981) and of Factor F_{430} (Diekert et al. 1981). Ni is also a component of the carbon monoxide dehydrogenase in *M. barkeri* (Krzycki and Zeikus 1984).

The concentration of Co that limited growth of *M. methylutens* was 10-fold higher than the limiting concentration reported for *M. thermoautotrophicum* (Schönheit et al. 1979). Although the absence of Co did not completely inhibit *M. methylutens*, the growth rate was reduced 94% compared with a reduction of 50% for *Methanococcus voltae* (Whitman et al. 1982). Co may be required for corrinoids which have been found in all methanogenic bacteria tested (Krzycki and Zeikus 1980) and have been identified in the methanol methyltransferase of *M. barkeri* (van der Meijden et al. 1983).

Mo is required for growth of *M. thermoautotrophicum* (Schönheit et al. 1979) and stimulates growth of *M. barkeri* (Scherer and Sahm 1981). Scherer et al. (1983) have shown that Mo accumulates in *M. barkeri* which suggestes that it is an essential element. However, a requirement for Mo by *M. methylutens* could not be demonstrated. The addition of Mo to medium did not stimulate the growth of *M. methylutens* and the addition of W, an antagonist of molybdoenzymes, did not affect growth. Although Se stimulates growth of other methanogenic bacteria (Jones and Stadtman 1977; Jones et al. 1983a, 1983b; Scherer and Sahm 1981; Whitman et al. 1982) it did not stimulate growth of *M. methylutens* under the conditions described. Other metals have been shown to accumulate in the cells of methanogenic species (Scherer et al. 1983; Whitman et al. 1982) including Zn and Cu but as in reports on other species a physiological requirement for Zn and Cu could not be demonstrated for *M. methylutens.*

Acknowledgement. This investigation was supported by grant 5092-260-0710 from the Basic Science Division of the Gas Research Institute.

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Received December 17, 1984/Accepted March 5, 1985