Arch. Microbiol. 123, 105–107 (1979)

Archives of
Microbiology

Nickel, Cobalt, and Molybdenum Requirement for Growth of *Methanobacterium thermoautotrophicum*

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Abstract. Growth of *Methanobacterium thermoautotrophicum* on H_2 and CO_2 as sole energy and carbon sources was found to be dependent on Ni, Co, and Mo. At low concentrations of Ni (< 100 nM), Co (< 10 nM) and Mo (< 10 nM) the amount of cells formed was roughly proportional to the amount of transition metal added to the medium; for the formation of 1 g cells (dry weight) approximately 150 nmol $NiCl_2$, 20 nmol $CoCl_2$ and 20 nmol Na_2MoO_4 were required. A dependence of growth on Cu, Mn, Zn, Ca, Al, and B could not be demonstrated. Conditions are described under which the bacterium grew exponentially with a doubling time of 1.8 h up to a cell density of 2 g cells (dry weight)/l.

Key words: Nickel – Cobalt – Molybdenum – Iron – *Methanobacterium thermoautotrophicum* – Trace elements.

Methanobacterium thermoautotrophicum is a strict anaerobic bacterium which grows on H_2 and CO_2 as sole energy and carbon sources; NH_3 and H_2S are used as nitrogen- and sulfur-sources, respectively (Zeikus and Wolfe, 1972). Growth of the bacterium has been shown to be dependent on iron (Taylor and Pirt, 1977). A requirement for other transition metals has, until now, not been demonstrated.

In this communication it is schown that growth of *M. thermoautotrophicum* is dependent on nickel, cobalt and molybdenum. Nickel salts were previously not included in the medium of methanogenic bacteria. Papers on nutrient requirements of methanogenic bacteria are by Bryant et al. (1971), Jones and Stadtman (1977), Patel et al. (1978), Patel and Roth (1977), Taylor et al. (1974), Wellinger and Wuhrmann (1977), Zeikus (1977), and Zehnder and Wuhrmann (1977).

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Materials and Methods

The H_2/CO_2 gas mixture (80%/20%), H_2S (> 99.0%) and CO_2 (99.995%) were from Messer Griesheim (Düsseldorf). Nitrilotriacetate, NaCl, NH_4Cl, KH_2PO_4, $MgCl_2 \times 6H_2O$, $CaCl_2 \times 2H_2O$, $MnCl_2 \times 4H_2O$, $FeCl_2 \times 4H_2O$, $ZnCl_2 \times 6H_2O$, $AlCl_3$, H_3BO_3, $CoCl_2 \times 6H_2O$, $Na_2MoO_4 \times 2H_2O$, $CuCl_2 \times 2H_2O$, $NiCl_2 \times 6H_2O$, Na_2CO_3 and NH_3 (25%) were from E. Merck (Darmstadt). All chemicals used were reagent grade (p.a.). Resazurine was from Serva (Heidelberg).

M. thermoautotrophicum (strain Marburg) (Fuchs et al., 1978) was grown at 65° C in a 500 ml glass fermenter containing 250 ml of medium (see below). After inoculation (10% inoculum) the culture was continuously stirred with a teflon coated stirring paddle at 1,100 rpm and gassed with 80% H_2/20% CO_2/0.2% H_2S at a rate of 300 ml/min via a micro filter candle (porosity 3), (Schott, Mainz) (Fuchs et al., 1979). Growth was followed by measuring the absorbance difference at 578 nm. Samples of the culture were diluted 1:4 with water and measured in a cuvette ($d = 1$ cm) in a Zeiss PL 4 spectrophotometer against the 1:4 diluted medium blank. A $\Delta A_{578} = 1$ corresponds to a cell concentration of 0.4 g cells (dry weight)/l. The pH of the culture was kept constant at 6.75 by the addition of 0.62 ml 2 N $(NH_4)_2CO_3$ each time the ΔA_{578} of the culture increased by 1. This was necessary because NH_3 assimilation leads to an acidification of the medium.

The medium was prepared with quartz distilled water and had the following composition: NH_4Cl, 40 mM; KH_2PO_4, 10 mM; nitrilotriacetate. 150 µM; $MgCl_2$, 200 µM; $FeCl_2$, 25 µM; $CoCl_2$, 1 µM; Na_2MoO_4, 1 µM; $NiCl_2$, 1 µM; and resazurine, 20 µM. Where indicated $NiCl_2$, $CoCl_2$, Na_2MoO_4, or $FeCl_2$ were omitted. The pH of the medium was adjusted to 6.75 by the addition of 2 ml 1 M Na_2CO_3. After the addition of the Na_2CO_3 solution the medium was gassed with 80% H_2/20% CO_2/0.2% H_2S for 15 min at a rate of 150 ml/min and was then inoculated.

Results

Figure 1 shows that growth of *M. thermoautotrophicum* on H_2 and CO_2 as sole energy and carbon sources was dependent on nickel. In the absence of nickel the bacterium grew only very slowly and stationary phase was reached at a cell concentration of 0.4 g (dry weight) per l ($\Delta A_{578} = 1$). Both the growth rate and the cell concentration at the end of growth increased, when the medium was supplemented with $NiCl_2$ (Fig. 1A).

0302-8933/79/0123/0105/$01.00

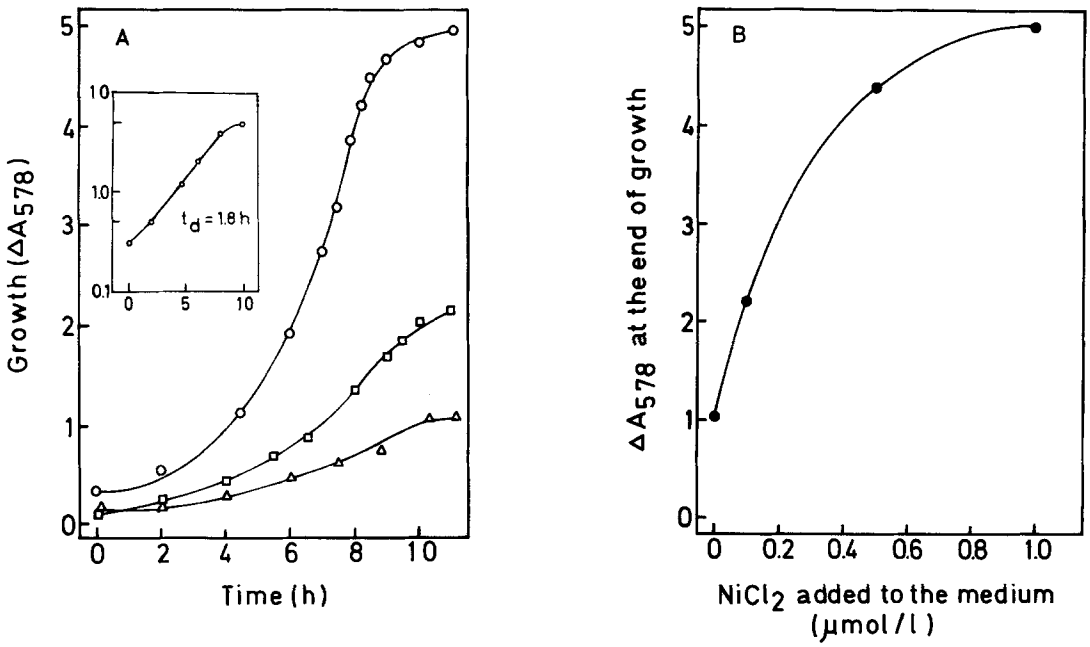

Fig. 1A and B. Growth of *Methanobacterium thermoautotrophicum* in the absence (△) and in the presence of added $NiCl_2$ (□ = 0.085 μmol/l; ○ = 1 μmol/l). The inset shows a semilogarithmic plot of growth in the presence of 1 μM $NiCl_2$. **A** ΔA_{578} of 1 corresponds to 0.4 g cells (dry weight)/l. **B** Cell concentration of *M. thermoautotrophicum* at the end of growth as a function of the amount of $NiCl_2$ added to the medium

Maximal stimulation was observed at a $NiCl_2$ concentration of 1 μM (Fig. 1 B). At this concentration the bacterium grew exponentially with a doubling time of 1.8 h up to a cell density of 2 g cells (dry weight) per l. Growth was limited under these conditions by the uptake of H_2 from the gasphase rather than by the non-availability of nickel. At low concentrations of $NiCl_2$ (< 100 nM) the amount of cells formed was roughly proportional to the amount of nickel added to the medium (Fig. 1 B); from the slope it was calculated that approximately 150 nmol of nickel were required for the formation of 1 g cells (dry weight).

Growth of *M. thermoautotrophicum* in the absence of added nickel was probably due to trace nickel present as contaminant in the medium. A dependance of growth on nickel could only be demonstrated, when no parts of the fermenter in contact with the medium was built from stainless steel. Stainless steel, e.g. of syringe needles (Henke – Sass-Wolf, Stuttgart) contains approximately 10 % nickel. In the presence of H_2S enough nickel is dissolved from the needles to maintain growth of the bacterium on a medium not supplemented with nickel.

When *M. thermoautotrophicum* was grown on media not supplemented with either $CoCl_2$, Na_2MoO_4 or $FeCl_2$ growth was only poor. At low concentrations of $CoCl_2$(< 10 nM), Na_2MoO_4 (< 10 nM) and $FeCl_2$ (< 5 μM) the amount of cells formed at the end of growth was roughly proportional to the amount of transition metal added. From the concentration dependence it was calculated that 20 nmol of cobalt, 20 nmol of molybdenum, and 10 μmol iron were required for the synthesis of 1 g cells (dry weight). It is important to note that the requirement for nickel was higher than for cobalt and molybdenum.

$CaCl_2$, $MnCl_2$, $CuCl_2$, $ZnCl_2$, $AlCl_3$ and H_3BO_3 are generally added to the growth medium of *M. thermoautotrophicum* (Zeikus and Wolfe, 1972). A requirement for these components could not be demonstrated.

Discussion

The finding that growth of *Methanobacterium thermoautotrophicum* on H_2 and CO_2 as sole energy and carbon sources is dependent on nickel is of interest, since this element is generally not essential for growth of bacteria. The only other chemotrophic bacterium known to require nickel for cell proliferation is *Alcaligenes eutrophus* when growing chemolithotrophically on H_2, O_2 and CO_2 as sole energy and carbon sources (Bartha nad Ordal, 1965; Repaske and Repaske, 1976; Tabillion and Kaltwasser, 1977). Recently it was found that the synthesis of carbon monoxide dehydrogenase in *Clostridium pasteurianum* is dependent on nickel (Diekert et al., 1979). Nickel has been reported to be a component of jack bean urease (Dixon et al., 1975, 1976; Fishbein et al., 1976; Polacco, 1977). Van Baalen and O'Donnell (1978) isolated a

nickel dependent blue-green alga. Evidence is also available that nickel has an important role in the metabolism of vertebrates (Nomoto et al., 1971; Nielson and Ollerich, 1974). A discrete function of nickel in *Alcaligenes eutrophus*, cyanobacteria, and vertebrates has, so far, not been identified.

Cobalt is generally required by bacteria for the synthesis of corrinoid compounds. The transition metal is, however, also a component of e.g. transcarboxylase (Nothrop and Wood, 1969). The dependence of growth of *M. thermoautotrophicum* on cobalt does therefore not necessarily indicate the presence of corrinoids in this organism.

CO_2-reductase of *Clostridium pasteurianum* has been shown to be a molybdo-protein (Scherer and Thauer, 1978). The enzyme catalyzes the reduction of CO_2 to formate. The active species of CO_2 reduced to formate has been shown to be CO_2 rather than HCO_3^- Thauer et al., 1975). In methanogenic bacteria CO_2 rather than HCO_3^- is reduced to CH_4 via carrier bound formate (Wolfe, 1979; Fuchs et al., 1979). The finding that relatively high amounts of molybdenum are required for growth of *M. thermoautotrophicum* suggests that the enzyme mediating the reduction of CO_2 in *M. thermoautotrophicum* may also be a molybdo-protein. The involvement of molybdenum as prosthetic group of enzymes has recently been reviewed by Bray and Swann (1972).

Acknowledgements. This work was supported by grants from the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

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Received May 30, 1979