Nickel Requirement and Factor F₄₃₀ Content of Methanogenic Bacteria

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Received 1 June 1981/Accepted 15 July 1981

*M. thermoaotrophicum has been reported to require nickel for growth and to contain high concentrations of a nickel tetrapyrrole designated factor F₄₃₀. In this communication it is shown that all methanogenic bacteria investigated incorporated nickel during growth and also synthesized factor F₄₃₀. This was also true for Methanobrevibacter smithii, which is dependent on acetate as a carbon source, and for Methanosarcina barkeri growing on acetate or methanol as energy sources. Other bacteria, including Acetobacterium woodii and Clostridium thermoaceticum, contained no factor F₄₃₀. It is further shown that two yellow nickel-containing degradation products were formed from factor F₄₃₀ when heated at pH 7. This finding explains why several forms of factor F₄₃₀ were found in methanogenic bacteria when a heat step was employed in the purification procedure.

Growth of Methanobacterium thermoautotrophicum has been shown to be dependent on nickel (23). The transition metal was found to be a component of factor F₄₃₀ (7, 9, 27), a yellow compound present in M. thermoautotrophicum (17). Ellefson and Wolfe (W. L. Ellefson and R. S. Wolfe, J. Biol. Chem., in press) recently obtained evidence that factor F₄₃₀ may be the prosthetic group of methyl coenzyme M (CoM) reductase. The complete structure of factor F₄₃₀ has not yet been elucidated. Labeling studies with [¹⁴C]succinate and [⁸-¹⁴C]aminolevulinic acid indicate that factor F₄₃₀ has a nickel tetrapyrrole structure (5, 6).

The only other organism in which factor F₄₃₀ has been found is Methanobacterium bryantii (27), a member of the same genus as M. thermoautotrophicum (1). Two nickel-containing yellow compounds were isolated from this methanogen which were designated factors F₄₃₈ and -b. Whether less related methanogenic bacteria also contain nickel tetrapyrroles similar to or identical with factor F₄₃₈ or -b has not yet been investigated.

In the following communication, it is shown that all methanogens examined contained factor F₄₃₀: Methanobrevibacter smithii, Methanococcus vannielii, Methanospirillum hungatii, and Methanosarcina barkeri. From these organisms, two are of special interest: (i) M. smithii, because it lacks the ability to grow autotrophically, and (ii) M. barkeri, because this methanogen can grow organotrophically on acetate, methanol, or methyl amines. All other methanogens grow on H₂ plus CO₂ or formate as the sole energy source (1, 18, 28).

MATERIALS AND METHODS

The 80% H₂-20% CO₂ gas mixture (H₂ > 99.999%; CO₂ > 99.995%) and H₂S (>99.0%) was from Messer Griesheim (Düsseldorf). QAE-Sephadex A-25 was from Pharmacia Fine Chemicals (Uppsala); Bio-Gel P-6 (100–200 mesh) was from Bio-Rad Laboratories (München). Thin-layer cellulose plates or silica gel plates were from E. Merck (Darmstadt). [⁵⁷⁷Ni]nickel II chloride (11.8 mCi/mg of nickel) was from Amer- sham Buchler (Braunschweig), and Aqualuma scintill- ator was from Baker Chemicals (Deventer). Pyridine-2-carbaldehyde-2-chinolyl-hydrazone (purum) was from Fluka (Buchs).

Acetobacterium woodii strain WB1 (DSM1030), Methanococcus vannielii strain SB (DSM1224), Methanospirillum hungatii strain JF1 (DSM864), Methanobrevibacter smithii strain FS (DSM861), Methanobacterium thermoautotrophicum strain ΔH (DSM1053), Methanosarcina barkeri strain Fusaro (DSM804), Clostridium thermoaceticum (DSM251), and Escherichia coli neotype strain (DSM30083) were obtained from the Deutsche Sammlung von Mikroorganismen (Göttingen). M. thermoautotrophicum strain Marburg is the strain isolated by Fuchs et al. (16).

Cultivation of organisms. Unless stated otherwise, the organisms were mass cultured with the general procedure described by Balch and Wolfe (3). M. thermoautotrophicum strains ΔH and Marburg were grown at 65°C, and M. smithii was grown at 37°C by the procedure of Schönheit et al. (24). Cells of M. barkeri were kindly supplied by P. Scherer (KFA Jülich), who grew the cells at 37°C either on methanol or on acetate as the energy source (21, 22). A. woodii was grown on H₂ plus CO₂ (2), C. thermoaceticum was
grown on glucose medium (11), and E. coli was grown aerobically on a medium containing (per liter of water): lactose, 18 g; KH₂PO₄, 13.6 g; (NH₄)₂SO₄, 2 g; MgSO₄·7H₂O, 0.2 g; FeSO₄·7H₂O, 5 mg; thiamine, 0.5 mg (pH 7). Where indicated, the medium was supplemented with 25 mM NaCl.

Growth was followed by measuring the increase in absorbance at 578 nm (A₅₇₈) with medium as a blank or by determining the increase in cell mass with cellulose acetate filters (0.45-μm pore size). At the end of exponential growth, the cells were harvested by centrifugation at 16,000 × g for 30 min and washed twice with 50 mM potassium phosphate (pH 7), except for M. vanneili, which lysed under these conditions and was therefore resuspended in the same buffer without washing. The cells were stored at −70°C until analysis of the factor F₄₃₀ content.

Nickel uptake during growth. Nickel uptake of the cells was followed with ⁶⁵Ni. Samples (1 ml) were withdrawn from the cultures, transferred to 1.5-ml Eppendorf capped tubes, and centrifuged at 10,000 × g for 2 min. From the supernatant 0.5-ml samples were added to Aqualuma scintillator and counted in a Beckman LS 7500 liquid scintillation spectrometer with ⁶⁵Ni as the internal standard. The cell pellet was washed twice with 50 mM phosphate buffer (pH 7.0) (with the exception of M. vanneili) and resuspended in 1 ml of the same buffer; 0.5-ml samples of the suspension were counted in 5 ml of Aqualuma. The amount of nickel taken up by the cells was calculated from the specific radioactivity of nickel in the medium and the radioactivity incorporated per milligram (dry weight) of cells.

Determination of nickel. The nickel concentrations of the media were determined with pyridine-2-carbaldehyde-2-chinolylhydrazone (19). Samples containing 1 to 5 μg of nickel were added to 2 ml of 10% (wt/wt) sodium citrate. After the addition of 0.2 ml of thiglycolic acid, the sample was shaken. One milliliter of a pyridine-2-carbaldehyde-2-chinolylhydrazone solution (10 mg in 20 ml of 2.5 mM HCl) and 2 ml of a 5 M NH₄NO₃ solution (pH 10.0) were mixed with the sample. Then 1 ml of benzene was added. The mixture was agitated for 1 min on a Vortex mixer, thus extracting the reagent-nickel complex. The upper (benzene) phase was removed and transferred to a cuvette (diameter, 1 cm). The A₄₃₀ was measured in a Zeiss spectrophotometer PMQ-3 and related to a standard curve. As iron disturbs the test, the nickel concentration in the culture medium was determined before the addition of iron.

Quantitative determination of factor F₄₃₀. One to two grams of wet cells of known dry weight were suspended in 10 ml of 10 mM potassium phosphate (pH 7). The cells were either disrupted by passing the suspension through a French pressure cell at 20,000 psi (137,000 kPa) (M. vanneili, M. hungatii, M. smithii, and C. thermoautotrophicum) or made permeable by adding sodium deoxycholate to a final concentration of 0.5% (wt/vol) and then incubating for 30 min at 0°C (M. thermoautotrophicum, M. barkeri, A. woodii, and E. coli). Then 0.5 M HClO₄ was added with continuous stirring until a pH of 2 was attained. After stirring for a further 45 min, precipitated material was removed by centrifugation for 30 min at 27,000 × g, and the pellet obtained was extracted repeatedly with 10 mM HClO₄ until the extract no longer absorbed at 430 nm. The supernatants containing factor F₄₃₀ were combined, and the pH was adjusted to 9.5 with 1 M KOH; they were then diluted 1:4 with water and applied to a QAE-Sephadex A-25 column (diameter, 1 cm; length, 4 cm) previously equilibrated with 50 mM glycine-KOH buffer (pH 9.5). Factor F₄₃₀ was eluted with the same buffer containing 0.3 M NaCl (9). The fractions containing factor F₄₃₀ were pooled, and the A₄₃₀ was measured. The concentration of factor F₄₃₀ was calculated by using an extinction coefficient of 22,500 cm⁻¹ · liter-mol of Ni⁻¹ (7, 9).

When purified factor F₄₃₀ was added to cells of known F₄₃₀ content and was then isolated by the procedure described above, more than 95% was recovered. Also, the amount of factor F₄₃₀ obtained was proportional to the amount of cells analyzed. Therefore, the method used to quantitate the factor F₄₃₀ content can be considered to give reliable results.

For further purification of factor F₄₃₀, the pooled fractions were diluted fivefold with water and reapplied to a QAE-Sephadex A-25 column previously equilibrated with 50 mM tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.5). The column was first washed with 10 ml of 1 mM HCl, and factor F₄₃₀ was then eluted with 2.5 mM HCl. The fractions containing factor F₄₃₀ were pooled, diluted 2.5-fold, and applied to a further QAE-Sephadex column pre-equilibrated with 1 mM HCl. The column was washed with 10 ml of 1 mM HCl, and factor F₄₃₀ was eluted with 3 mM HCl. The fractions containing factor F₄₃₀ were subsequently pooled and lyophilized. The A₄₃₀/A₃₇ₐ ratio of the purified factor was near 1.15.

Determination of the nickel content of factor F₄₃₀. The nickel content of factor F₄₃₀ was determined after isolation of the factor from cells grown in the presence of 5 μM ⁶⁵Ni of known specific radioactivity. The radioactivity and absorbance of the purified factor solution was determined. From these data ϵ₄₃₀ (cm⁻¹ · liter-mol of Ni⁻¹) was calculated, which is a value proportional to the nickel content per mole of factor.

Chromatographic analysis of factor F₄₃₀. The chromatographic behavior of factor F₄₅₁, -III, -II (= factor F₄₃₀) was analyzed on thin-layer cellulose plates and on silica gel plates (20 by 20 cm) using ethyl acetate-pyridine-water-glacial acetic acid (5:5:3:1) as a solvent. The R₁ values for factor F₄₃₀ (= F₄₅₁) and F₄₅₁ were 0.61 on cellulose and 0.29 on silica gel. For factor F₄₃₀, R₁ values were 0.49 on cellulose and 0.24 on silica gel. The electrophoretic mobility of the factor(s) was determined by thin-layer electrophoresis on cellulose plates (10 by 10 cm) at 600 V and 18°C with a buffer containing 20 ml of pyridine, 9.5 ml of glacial acetic acid, and 970 ml of water (pH 5.2). Factors F₄₅₁, -II, and -III were separated by chromatography on Bio-Gel P-6 at pH 3 (see Fig. 2). Two milliliters of a factor-containing solution in 1 mM HCl was applied to a Bio-Gel P-6 column (diameter, 1.2 cm; length, 50 cm) which was equilibrated with 1 mM HCl. The factors were eluted with 1 mM HCl (5).

RESULTS

Nickel requirement. The medium described by Balch et al. (1) for growth of most methano-
Methanogenic bacteria contains 0.2% yeast extract and 0.2% Trypticase, but is not supplemented with nickel salts. The methanogenic bacteria tested grew readily on this medium. If the concentration of yeast extract was significantly lowered, good growth was only observed upon addition of nickel chloride (1 μM) to the medium (Fig. 1).

*M. smithii* was grown in a 500-ml glass fermentor on the medium recommended for this bacterium, with the only modification that the yeast extract concentration was lowered to 0.005%. This amount proved essential for growth. In the absence of added nickel, *M. smithii* grew slowly and to a cell concentration of only 0.3 g/liter. When 1 μM NiCl₂ was added to the medium, both the growth rate and the final cell concentration drastically increased. No other transition metal could substitute for nickel in this respect.

Yeast extract contains considerable amounts of nickel. Also, the mineral salts and trace elements used to make the medium are contaminated with low amounts of nickel (23). The nickel concentration in the medium prepared by the method of Balch et al. (1) was found to be between 0.1 and 0.5 μM even though nickel was not added. The extent of nickel contamination was dependent on whether the medium was in contact with stainless steel (needles), from which nickel readily dissolves. The high background level of nickel in the medium is the reason why nickel requirement for growth of methanogenic bacteria has long been overlooked.

The maximal amount of nickel required for growth of the different methanogens was estimated by growing the bacteria on media containing 5 μM NiCl₂ of known specific radioactivity and by measuring the amount of Ni⁶⁶ taken up by the growing cells (Table 1). The minimal amount required is probably considerably lower since bacteria tend to store trace elements when these are present in excess.

**Factor F₄₃₀ content.** All methanogenic bacteria investigated contained factor F₄₃₀ (Table 1). The highest concentrations were found in *M. thermoautotrophicum* and *M. barkeri*, and the lowest was found in *M. vannielii*. The factor F₄₃₀ content of the cells was dependent on the nickel concentration in the medium, the growth rate, and the growth phase. Cells grown with limiting amounts of nickel contained considerably less factor F₄₃₀ than those grown with excess nickel. However, no growth conditions were found in which the cells did not contain significant amounts of factor F₄₃₀ (50 nmol/g in cultures containing 90 nmol of nickel per liter).

The factors isolated from the different methanogenic bacteria were identical with respect to nickel content (ε₄₃₀ = 22,500 ± 2,000 cm⁻¹ liter-mol of Ni²⁻⁻), ultraviolet and visible light spectrum (A₄₃₀/A₃₇₅, 1.15), electrophoretic mobility, and chromatographic behavior on thin-layer plates or Bio-Gel P-6 columns. There is thus no indication that factor F₄₃₀ differs in structure from organism to organism.

Acetogenic bacteria have been reported to be dependent on nickel (8). Evidence is available

![Graph](image)

**Fig. 1. Growth of M. smithii in the presence (●) and absence (○) of added nickel (1 μM).** The bacterium was grown at 37°C in a 500-ml glass fermentor filled with 250 ml of medium and gassed with 80% H₂, 20% CO₂, and 0.2% H₂S at a rate of 200 ml/min. The medium was the one recommended by Balch et al. (1), except that it contained only 0.005% yeast extract. A ΔA₅₇₈ of 1 corresponded to a cell concentration of 300 mg (dry weight) of cells per liter.

**Table 1. Nickel and factor F₄₃₀ content of bacteria grown in the presence of 5 μM nickel**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Ni sto</th>
<th>F₄₃₀ sto</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanogenic bacteria</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. thermoautotrophicum</em></td>
<td>1,100</td>
<td>800</td>
</tr>
<tr>
<td>(Marburg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. thermoautotrophicum</em></td>
<td>ND*</td>
<td>643</td>
</tr>
<tr>
<td>(ΔΗ)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. smithii</em></td>
<td>680</td>
<td>307</td>
</tr>
<tr>
<td><em>M. vannielii</em></td>
<td>290</td>
<td>227</td>
</tr>
<tr>
<td><em>M. hungatii</em></td>
<td>581</td>
<td>492</td>
</tr>
<tr>
<td><em>M. barkeri</em> (methanol</td>
<td>ND</td>
<td>800</td>
</tr>
<tr>
<td>grown)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. barkeri</em> (acetate grown)</td>
<td>ND</td>
<td>800</td>
</tr>
<tr>
<td>Acetogenic bacteria</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. thermoaceticum</em></td>
<td>250</td>
<td>&lt;10</td>
</tr>
<tr>
<td><em>A. woodii</em></td>
<td>400</td>
<td>&lt;10</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>ND</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

* Nanomoles per gram (dry weight) of cells.
* ND, Not determined.
that the nickel is involved in the reduction of methyl tetrahydrofolate to acetate, a reaction analogous to the reduction of methyl CoM to methane (11, 14). It was therefore of interest to determine whether acetogenic bacteria contain factor F450. A. woodii and C. thermoaceticum were analyzed. With our method a factor similar to factor F450 was not found. A nickel compound with considerably different chromatographic properties would, however, have escaped detection.

When the methanogenic bacteria were grown under conditions of nickel limitation, only 50 to 70% (depending on the bacterium) of the nickel taken up by the cells was used to synthesize factor F450. The rest was incorporated into the protein fraction where most of it could be released as free Ni²⁺ ions when the pH values were lower than 3 through acidification. Thus, not all of the nickel taken up by the cells was used for the synthesis of the nickel tetrapyrrole.

Nickel-containing degradation products of factor F450. When all steps in the purification were performed at temperatures below 10°C only one yellow compound (designated F450) was obtained from M. thermoautotrophicum in an amount of 800 nmol per g of cells. The purification procedure described by Gunsalus and Wolfe (17), which includes a heat step (30 min, 100°C, pH 7), resulted in three nickel-containing yellow compounds in a total amount of 750 nmol/g. The three factors could be separated by chromatography on Bio-Gel P-6 in 1 mM HCl (Fig. 2). They all had an absorbance maximum at 430 nm and therefore were designated F450I, -II, and -III in the order of their elution from the P-6 column. Factor F450II had spectral and chromatographic properties identical with those of the factor obtained when the heat step was circumvented; factor F450II is therefore considered to be identical with factor F450. Factors F450I and -III are degradation products formed from F450II during the heat step. This was shown by anaerobically heating purified factor F450II to 100°C in tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.8) (Fig. 3). The concentration of factor F450II rapidly decreased, that of factor F450I increased, whereas that of factor F450III first increased and then decreased again. When purified factor F450I was heated to 100°C for 60 min, it was quantitatively converted to F450I. Factor F450III is thus an intermediate in the formation of factor F450I from F450II.

Factors F450I and -II had the same chromatographic properties as described for factors F450A and -b, respectively. Factors F450A and -b are the two forms of factor F450 isolated from M. bryantii by Whitman and Wolfe (27), who heated the

![Figure 2. Chromatography on Bio-Gel P-6 of factor(s) F450 extracted from M. thermoautotrophicum (A) with HClO₄ at 0°C and (B) with water at 100°C for 30 min under anaerobic conditions. The factors were eluted from the Bio-Gel P-6 column with 1 mM HCl, and the ⁶⁵Ni concentration (Δ) and the A₄₃₀ (○) of 2-ml fractions were measured. The specific radioactivity of ⁶⁵Ni in the culture medium was 3.9 × 10⁴ dpm/μmol.](http://jb.asm.org/)

**DISCUSSION**

All methanogenic bacteria investigated were found to contain factor F450. One or several members of each of the four families of methanogenic bacteria (1) were examined. It is therefore very probable that all species of methanogenic bacteria contain this nickel tetrapyrrole.

Methanogenic bacteria have a unique energy metabolism (1), and the autotrophic species also have a unique pathway of autotrophic CO₂ fixation (15). The finding that the heterotrophic M. smithii contains F450 indicates that the nickel compound is probably involved in methane formation rather than in carbon assimilation. M. smithii assimilates acetate plus CO₂ via pathways known not to be dependent on factor F450.
The finding that methanogens contain the factor with methanol, acetate, or H₂ plus CO₂ as the energy source points to the methyl CoM reductase reaction. The pathways of methane formation from methanol, acetate, and CO₂ probably have only the methyl CoM reduction in common. Indeed, Ellefson and Wolfe (in press) recently obtained evidence that factor F₄₃₀ is the prosthetic group of methyl CoM reductase. Of further interest is the finding of Keltjens and Vogels (J. T. Keltjens and G. D. Vogels, in Proceedings of the 3rd International Symposium on Microbial Growth on C₁ compounds, in press) that some preparations of factor F₄₃₀ contain covalently bound CoM or methyl CoM. Up to 12% of the cell protein of methanogens can be methyl CoM reductase (Ellefson and Wolfe, in press); this could explain why the factor F₄₃₀ content of the cells is relatively high.

Most of the factor F₄₃₀ is tightly bound to the protein fraction of the methanogenic bacteria. The factor can be dissociated from the protein fraction by heating at pH 7 or by acidification. It was shown that factor F₄₃₀ is converted to two yellow nickel-containing compounds when heated to 100°C at pH 7 (Fig. 3). This finding explains why several forms of factor F₄₃₀ are obtained when a heat step is included in the purification procedure (5, 27). When the heat step is omitted only one form of factor F₄₃₀ is obtained. This finding should facilitate the elucidation of the structure of the nickel-containing compound since now one factor rather than three has to be considered; this factor is obtainable in higher amounts and is easier to purify. The possibility of forming distinct degradation products that can be isolated and characterized may also help in unravelling the structure.

Nickel was found to be required by the methanogens not only for the synthesis of factor F₄₃₀, but also for incorporation into the protein fraction, from which most of it could be released as free Ni²⁺ after acidification. The function of the nickel protein(s) is not known. There are some indications that hydrogenase of methanogenic bacteria could be a nickel protein (13). Most methanogens contain a carbon monoxide dehydrogenase (4) which in clostridia proved to be a nickel enzyme (10, 14). Urease, too, is a nickel enzyme (12, 20, 25). There is, however, no evidence that methanogenic bacteria contain urease. M. thermoautotrophicum (Marburg) was found not to grow on urea as the sole nitrogen source (unpublished results). For a review on the biological role of nickel see Thauer et al. (26).

ACKNOWLEDGMENTS

This work was supported by a grant from the Deutsche Forschungsgemeinschaft, Bonn-Bad Godesberg, and by the Fonda der Chemischen Industrie, Frankfurt am Main.

We thank P. Scherer, KFA Jülich, for providing us with methanol- and acetate-grown cells of M. barkeri.

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