Importance of Cobalt for Individual Trophic Groups in an Anaerobic Methanol-Degrading Consortium

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Methanol is an important anaerobic substrate in industrial wastewater treatment and the natural environment. Previous studies indicate that cobalt greatly stimulates methane formation during anaerobic treatment of methanolic wastewaters. To evaluate the effect of cobalt in a mixed culture, a sludge with low background levels of cobalt was cultivated in an upflow anaerobic sludge blanket reactor. Specific inhibitors in batch assays were then utilized to study the effect of cobalt on the growth rate and activity of different microorganisms involved in the anaerobic degradation of methanol. Only methylotrophic methanogens and acetogens were stimulated by cobalt additions, while the other trophic groups utilizing downstream intermediates, \( \text{H}_2-\text{CO}_2 \) or acetate, were largely unaffected. The optimal concentration of cobalt for the growth and activity of methanol-utilizing methanogens and acetogens was 0.05 mg liter\(^{-1}\). The higher requirement of cobalt is presumably due to the previously reported production of unique corrinoid-containing enzymes (or coenzymes) by direct utilizers of methanol. This distinctly high requirement of cobalt by methylotrophs should be considered during methanolic wastewater treatment. Methylotroph methanogens presented a 60-fold-higher affinity for methanol than acetogens. This result in combination with the fact that acetogens grow slightly faster than methanogens under optimal cobalt conditions indicates that acetogens can outcompete methanogens only when reactor methanol and cobalt concentrations are high, provided enough inorganic carbon is available.

Acetate and \( \text{H}_2-\text{CO}_2 \) intermediates during anaerobic degradation of organic matter are recognized as important methanogenic substrates. Consequently, considerable research has been dedicated to the behavior of acetoclastic and hydrogenotrophic methanogens during anaerobic wastewater treatment. Less studied is the role of methylotrophic methanogens which are involved in the metabolism of \( \text{C}_1 \) substrates in wastewaters, wastes, and the natural environment.

Methanol is utilized in several chemical industries, such as in the production of formaldehyde (41) and esters (47). Methanol is also generated in condensation processes at pulp mills (25, 31) and coal gasification installations (28). In kraft pulp mill evaporator condensates, methanol is the main organic pollutant, with concentrations ranging from 1.5 to 24.5 g liter\(^{-1}\) (26). Methanol is also present in natural anaerobic environments, such as anoxic lake sediments and rumen fluid (33, 35). Methanol is released from the anaerobic decomposition of pectin (34) or, similarly, the equivalent of methanol is metabolized from methoxylated phenols (2, 7, 13, 22, 30). In marine sediments, methanol may also be a methane precursor, although methylamines are more important methanogenic substrates (18). The same microorganisms involved in the anaerobic degradation of methanol are also involved in the anaerobic degradation of halomethane compounds (3, 8, 24, 27, 38). Halogenated \( \text{C}_1 \) compounds are present in the environment as natural (48) or xenobiotic (16) substances.

Methanol, a simple \( \text{C}_1 \) compound, potentially supports a complex food chain composed of a variety of possible trophic groups. Table 1 presents the main reactions involved in the anaerobic degradation of methanol and summarizes their stoichiometry and Gibbs free-energy changes under standard conditions. Several species of methanogens (17) and acetogens (22) are able to directly utilize methanol as a carbon and energy source for growth. Nitrate- and sulfate-reducing bacteria which can utilize methanol are also known (4, 23, 29). Acetogens produce acetate or \( \text{H}_2-\text{CO}_2 \) from methanol (5). Thus, methanol can indirectly support hydrogenotrophic (14, 17, 22) and acetoclastic organisms (17, 46) when acetate or \( \text{H}_2-\text{CO}_2 \) is generated.

Previously, we have studied the effect of trace elements on the anaerobic degradation of methanol by anaerobic sludge (10). Cobalt was the only trace element tested which greatly enhanced methanogenesis from methanol. Cobalt had no remarkable influence on methanogenesis when acetate was used as the substrate. Aside from methanogenesis, acetogenesis of methanol was also stimulated by cobalt, which can result in reactor upsets due to a pH drop from the accumulation of acetate (20, 21). In continuous experiments, less acetate was formed in a cobalt-deprived reactor than in a cobalt-supplemented reactor. These results suggested that cobalt levels could be used to prevent acetate formation from methanol. Of particular interest is the possibility that the cobalt concentration could be used as a parameter for controlling substrate flow during anaerobic treatment of methanolic wastewaters. Since cobalt may play a crucial role in the anaerobic degradation of methanol, the objective of this study was to determine which trophic groups in a natural mixed culture respond to cobalt addition. For this purpose, a methanol-degrading consortium with low background levels of cobalt was cultivated in a continuous anaerobic reactor deprived of cobalt supplementation (10). Methanogenic and acetogenic activity batch assays utilizing specific inhibitors were used to study the composition of the population. The


**TABLE 1. Reported reactions and estimated Gibb's free energy changes possibly involved in the anaerobic degradation of methanol and basis for determining activities and apparent growth rate**

<table>
<thead>
<tr>
<th>Reaction*</th>
<th>(\Delta G^\circ) (kJ/reaction)</th>
<th>(H_2) sink required</th>
<th>Competitive reaction no.</th>
<th>Inhibitor-competitive pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. (4\text{CH}_3\text{OH} \rightarrow 3\text{CH}_4 + \text{HCO}_3^- + H^+ + \text{H}_2\text{O})</td>
<td>-314.6</td>
<td>3, 4</td>
<td>Vancomycin</td>
<td></td>
</tr>
<tr>
<td>2. (\text{CH}_2\text{OH} + H_2 \rightarrow \text{CH}_4 + \text{H}_2\text{O})</td>
<td>-112.5</td>
<td>3, 4 (5, 6)</td>
<td>Vancomycin</td>
<td></td>
</tr>
<tr>
<td>3. (4\text{CH}_3\text{OH} + 2\text{HCO}_3^- \rightarrow 3\text{CH}_3\text{COO}^- + H^+ + 4\text{H}_2\text{O})</td>
<td>-221.6</td>
<td>1, 2, 4</td>
<td>BESA</td>
<td></td>
</tr>
<tr>
<td>4. (\text{CH}_3\text{OH} + 2\text{H}_2\text{O} \rightarrow 3\text{H}_2 + \text{HCO}_3^- + H^+)</td>
<td>23.0</td>
<td>1, 2, 3</td>
<td>BESA</td>
<td></td>
</tr>
<tr>
<td>Via (\text{H}_2\text{CO}_3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. (2\text{HCO}_3^- + 3\text{H}_2 \rightarrow \text{CH}_2\text{COO}^- + 4\text{H}_2\text{O})</td>
<td>-104.6</td>
<td>6</td>
<td>BESA</td>
<td></td>
</tr>
<tr>
<td>6. (\text{HCO}_3^- + 4\text{H}_2 + H^+ \rightarrow \text{CH}_4 + 3\text{H}_2\text{O})</td>
<td>-135.6</td>
<td>5</td>
<td>Vancomycin</td>
<td></td>
</tr>
<tr>
<td>Via acetate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. (\text{CH}_3\text{COO}^- + 4\text{H}_2 \rightarrow 2\text{HCO}_3^- + 4\text{H}_2 + H^+)</td>
<td>104.6</td>
<td>+</td>
<td>8</td>
<td>BESA</td>
</tr>
<tr>
<td>8. (\text{CH}_3\text{COO}^- + \text{H}_2\text{O} \rightarrow \text{CH}_4 + \text{HCO}_3^-)</td>
<td>-31.0</td>
<td></td>
<td>7</td>
<td>Vancomycin or none</td>
</tr>
</tbody>
</table>

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*From references 22, 42, 49, and 51.
*b Reaction 6 not stopped.
*c BESA stops reaction 4 indirectly because of its effect on methanogens (destroys \(H_2\) sink).
*d Not tested in this study since no \(H_2\) sink was applied.
+ \(H_2\) sink is required.
/ Reaction 3 not stopped.

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The effect of cobalt on each of the trophic groups was also evaluated by measuring their growth rate.

**MATERIALS AND METHODS**

**Biomass.** All experiments were carried out with freshly collected methanogenic granular sludge taken from a laboratory-scale upflow anaerobic sludge blanket reactor. The sludge was cultivated on methanol as the only substrate in a mineral medium deprived of cobalt for 1 year (10). The sludge was rinsed with demineralized water before being used in the batch assays.

The contents of total suspended solids and the volatile suspended solids (VSS) of the sludge were 7.7 and 7.0%, respectively, as determined by standard methods (1). The total cobalt concentration of the sludge after 4 months of cultivation was 3.5 mg of Co kg of total suspended solids (10), four times lower than the seed sludge and more than 100 times lower than a control sludge cultivated on methanol in cobalt-sufficient medium.

**Culture medium, inhibitors, and chemicals.** Unless otherwise stated, the inorganic macronutrients used in all experiments contained the following: (in milligrams per liter of basal medium) \(\text{NH}_4\text{Cl}, 280; \text{K}_2\text{HPO}_4, 318; \text{MgSO}_4, 7\text{H}_2\text{O}, 100; \text{CaCl}_2, 2\text{H}_2\text{O}, 10; \text{FeCl}_3, 4\text{H}_2\text{O}, 2; \text{H}_2\text{BO}_3, 0.050; \text{ZnCl}_2, 0.050; \text{MnCl}_2, 4\text{H}_2\text{O}, 0.5; \text{CuCl}_2, 2\text{H}_2\text{O}, 0.038; \text{NH}_4\text{Mo}_3\text{O}_12\cdot 4\text{H}_2\text{O}, 0.050; \text{AlCl}_3, 6\text{H}_2\text{O}, 0.090; \text{NiCl}_2, 6\text{H}_2\text{O}, 0.142; \text{Na}_2\text{SeO}_3, 5\text{H}_2\text{O}, 0.164; \text{CoCl}_3, 6\text{H}_2\text{O}, 2; \text{EDTA}, 1; \text{and resazurin}, 0.2; and (in milliliters per liter) 36% \text{HCl}, 0.001. The medium was made up in demineralized water. To ensure pH stability, 6.72 g of NaHCO\(_3\) (80 mM) was added per liter of the basal medium in combination with 30% CO\(_2\) in the headspace. When the substrate was methanol plus pure hydrogen in the headspace, phosphate buffers were utilized instead (11). The pH values were set around 7.2. When required, the inhibitors vancomycin and 2-bromoethanesulfonic acid (BESA) were utilized at concentrations of 100 mg liter\(^{-1}\) and 6.33 g liter\(^{-1}\) (30 mM), respectively.

All chemicals were of analytical grade and were supplied by E. Merck AG, Darmstadt, Germany, except for resazurin, which was purchased from Fluka Chemie AG, Buchs, Switzerland, and vancomycin and BESA, which were obtained from Sigma Chemical Co., St. Louis, Mo., and Janssen, Tilburg, The Netherlands, respectively. The gases were supplied by Hoekloos, Schiedam, The Netherlands.

**Analyses.** The pH was determined potentiometrically. Methanol and volatile fatty acids (VFA) were determined by gas chromatography by using a flame ionization detector (10). Samples for measuring hydrogen (500 \(\mu\)l) and methane (100 \(\mu\)l) were obtained by using gas-tight syringes equipped with Pressure-Lok valves (Dynatech, Baton Rouge, La.). Methane was analyzed by using a flame ionization gas chromatograph model 438/6 (Packard-Becker, Delft, The Netherlands). The gas chromatograph was equipped with a steel column (2 m by 2 mm) packed with Porapak Q (80/100 mesh; Millipore Corp., Bedford, Mass.). The temperatures of the column, injector port, and the flame ionization detector were 60, 200, and 220°C, respectively. Nitrogen was used as a carrier gas at a flow rate of 20 ml min\(^{-1}\). Hydrogen was detected by thermal conductivity in a model 5890 gas chromatograph (Hewlett-Packard, Palo Alto, Calif.). The gas chromatograph was equipped with a steel column (1.5 m by 2 mm) packed with molecular sieve 0.5 mm (60/80 mesh; Chrompack, Bergen op Zoom, The Netherlands). Argon was used as a carrier gas (45 ml min\(^{-1}\)). The column, injection port, and thermal conductivity detector temperatures were 40, 110, and 125°C, respectively.

**Experimental setup.** Specific inhibitors were utilized to study the composition of the population by activities and apparent growth rate measurements. Figure 1 and Table 1 represent the general strategy utilized for blocking the competitive reactions and the substrates utilized for evaluating the activity or growth of individual pathways for the anaerobic degradation of methanol. To minimize cobalt contamination, all glassware was washed with 5 M HNO\(_3\) solution and rinsed with demineralized water. All experiments were conducted by using macro- and micronutrients at the concentrations outlined previously for the basal medium, except for cobalt. This micronutrient was either omitted, added at the concentration of the basal medium, or added at increasing concentrations. As substrates, either 44 mM methanol or 33 mM acetate was added to provide a concentration of around 2.1 g of chemical oxygen demand (COD) per liter of liquid. H\(_2\)-CO\(_2\) was also used as a substrate by supplying a gas mixture of H\(_2\)-CO\(_2\) (60:40) at a pressure of 152 kPa, which was equivalent to 2.1 g of COD per liter of liquid. The
liquid and bottle volumes that were used depended on the substrate and inhibitor utilized: a 50-ml working volume in a 315-ml serum bottle was used for pathways 1 and 2; 200 ml in a 570-ml serum bottle was used for pathways 3, 4, 7, and 8; and 120 ml in a 570-ml serum bottle was used for pathways 5 and 6. The biomass concentration varied from 0.05 to 0.35 g of VSS liter⁻¹, depending on the assay. In all experiments, a control with methanol and complete basal medium was included (200 ml in 570-ml serum bottles). The bottles were sealed with butyl rubber septa (Rubber B.V., Hilversum, The Netherlands). The headspace was flushed with an excess of oxygen-free N₂-CO₂ (70:30), pure H₂, or H₂-CO₂ as described above. Incubations were done in a temperature-controlled room at 30°C ± 2°C, in an orbital-motion shaker (Gerhardt, Bonn, Germany) at 120 strokes per min. Liquid and gas samples were taken periodically to analyze substrate consumption and product formation. The flasks were shaken vigorously before sampling. All results are reported as the mean value of triplicate cultures. The standard deviations were less than 5% of the triplicate mean values. The final pH values of the cultures were determined at the end of each series and were less than 0.35 pH unit below the initial pH of 7.2.  

**Determination of specific activities, apparent specific growth rate (μ), and substrate affinity (K_s).** The specific methanogenic and acetogenic activities were determined from the linear increase of products (CH₄ and VFA) in the beginning of the experiment, when no lag phase was observed. In this period, net growth compared with the initial population size was considered negligible; when necessary, higher sludge concentrations were utilized. For determination of growth rates, sludge concentrations that were low enough to ensure an exponential increase in product formation were utilized. Assuming that product formation and growth remained coupled, the apparent specific growth rates were calculated from the slope of the linear portion of the plot of the natural logarithm of the product yield versus time by performing a least-squares analysis. The apparent substrate (methanol) affinity coefficients for methanogens and acetogens were estimated from a Lineweaver-Burk plot by comparing the growth rates as a function of the substrate concentration at different times during substrate depletion.

**RESULTS**

The effect of BESA and cobalt on methanol utilization by the cobalt-deprived enrichment culture for a typical experiment is illustrated in Fig. 2. Methane production occurred without a lag phase (Fig. 2A), independent of cobalt addition. However, the addition of cobalt greatly stimulated the rate of methane production from methanol, which was totally consumed in less than 4 days in contrast to 14 days required for total substrate consumption in cobalt-deprived bottles. The period at the start of the assay (between 0 and 2 days) was used to determine the microbial activities. In this period, the rate of methane formation was constant, and at least 70% of the substrate applied was converted to methane (Fig. 2A), indicating a large initial population of methylotrophic methanogens. BESA, at a concentration of 30 mM, halted methanogenesis from methanol (Fig. 2B). Instead, VFA were formed in an exponentially increasing pattern, which indicated the growth of acetogenic microorganisms during the assay, although the initial activity was low. Acetate was the main VFA produced and represented more than 98% of the VFA formed. Cobalt greatly stimulated acetogenesis from methanol. When vancomycin and BESA were applied together, methanol was not degraded (data not shown).

The effect of inhibitors and cobalt on the product yield for several substrates, after 1 week of incubation, is presented in Table 2. BESA effectively blocked the methane production
TABLE 2. Product formation, substrate remaining, and COD recovery (after 1 week)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Inhibitor</th>
<th>Coa</th>
<th>Product formed (mg of COD · liter⁻¹)b</th>
<th>Substrate remaining (mg of COD · liter⁻¹)</th>
<th>Recovery (%)c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>CH₄</td>
<td>VFAtotal</td>
<td>C₂</td>
</tr>
<tr>
<td>H₂-CO₂</td>
<td>-</td>
<td>-</td>
<td>600</td>
<td>1,473</td>
<td>1,402</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>222</td>
<td>1,832</td>
<td>1,764</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>-</td>
<td>-</td>
<td>1,685</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>+</td>
<td>+</td>
<td>1,697</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>BESA</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>2,096</td>
<td>2,006</td>
</tr>
<tr>
<td>BESA</td>
<td>+</td>
<td>+</td>
<td>3</td>
<td>2,046</td>
<td>1,972</td>
</tr>
<tr>
<td>Methanol</td>
<td>-</td>
<td>-</td>
<td>834</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>1,797</td>
<td>28</td>
<td>28</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>-</td>
<td>-</td>
<td>1,759</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>+</td>
<td>+</td>
<td>1,759</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>BESA</td>
<td>-</td>
<td>-</td>
<td>5</td>
<td>39</td>
<td>39</td>
</tr>
<tr>
<td>BESA</td>
<td>+</td>
<td>+</td>
<td>14</td>
<td>669</td>
<td>658</td>
</tr>
<tr>
<td>Acetate</td>
<td>-</td>
<td>-</td>
<td>46</td>
<td>NDd</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>48</td>
<td>NDd</td>
<td></td>
</tr>
<tr>
<td>BESA</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>NDd</td>
<td></td>
</tr>
<tr>
<td>BESA</td>
<td>+</td>
<td>+</td>
<td>2</td>
<td>NDd</td>
<td></td>
</tr>
</tbody>
</table>

a, without Co addition; +, 0.5 mg of Co · liter⁻¹ added.
b H₂ as a product from methanol of acetic was lower than 0.2 mg of COD · liter⁻¹ (0.013 mM), VFAtotal = acetate (C₂) + propionate (C₃) + butyrate (C₄).c Recovery = 100 (CH₄ + VFAtotal + substrate remaining)/substrate applied. COD = (Σ COD gas phase + COD liquid phase) per liter of liquid phase. Initial substrate concentrations were 2.0, 2.2, and 2.1 g of COD · liter⁻¹ for acetate, H₂-CO₂, and methanol, respectively. One mole each of methanol, methane, acetate, propionate, and butyrate is 48, 64, 64, 112, and 160 g of COD, respectively.
d nd, not detectable.
ed ND, not determined.

from all substrates utilized. When methanol and H₂-CO₂ were used as substrates, the main product formed in the absence of methanogenesis was acetate. Vancomycin blocked acetogenesis, with methane being the main product from methanol and H₂-CO₂. Hydrogen was always detected, as a product from methanol or acetate, but only in trace amounts. When no inhibitors were applied, methane was the main product from methanol, whereas acetate was the main product from H₂-CO₂. From methanol plus H₂, methane was the main product, similar to the results with methanol alone (data not shown).

Table 3 presents the effect of cobalt on the initial specific activities for methanogens and acetogens in the mixed culture. The activity measurements provide reliable information about the predominant population present in the sludge. The only major populations were those involved in the conversion of methanol to methane. Cobalt stimulated their activity. Activity assays with specific inhibitors indicate that methane was being formed directly from methanol and not

TABLE 3. Effect of cobalt on the initial specific activities of methanogens and acetogens in the mixed population of the cobalt-deprived enrichment culture

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Cobalta</th>
<th>Methanogens (mg of CH₄ · COD · g of VSS⁻¹ · day⁻¹)</th>
<th>Acetogens (mg of VFAtotal · g of VSS⁻¹ · day⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>-</td>
<td>297.0</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2,577.0</td>
<td>8.4</td>
</tr>
<tr>
<td>Acetate</td>
<td>-</td>
<td>12.2</td>
<td>NDd</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>13.0</td>
<td>NDd</td>
</tr>
<tr>
<td>H₂-CO₂</td>
<td>-</td>
<td>41.2</td>
<td>11.0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>46.4</td>
<td>17.3</td>
</tr>
</tbody>
</table>

a, without Co addition; +, 0.5 mg of Co · liter⁻¹ added.
b ND, not determined.
via acetate formation nor H₂ (Fig. 3). Figure 3 also illustrates that cobalt remarkably stimulated the activity of methylotrophic methanogens by more than eightfold.

Activity assays do not adequately indicate the presence of nondominant populations that could quickly grow and take over when conditions are favorable. Such conditions are created when the competitor is switched off by specific inhibitors. The effect of cobalt on the growth rate for each individual population is illustrated in Fig. 4. Cobalt enhanced the growth rate of methanol-consuming methanogens and acetogens by factors of 3 and 4, respectively. Methanogenesis and acetogenesis of the other substrates besides methanol were practically unaffected by supplementation of cobalt. In some cases, mild inhibition occurred. These results clearly show that cobalt had only a strong stimulating effect on the trophic groups that were directly utilizing methanol. Among the substrates utilized, a remarkably high growth rate was observed for acetogens utilizing H₂-CO₂. A noteworthy growth rate of methanogens on H₂-CO₂ was also evident. The lowest growth rate was found for aceticlastic methanogens, and the supplementation with cobalt apparently lowered their rate slightly.

Since it was shown that growth and activity of methylotrophic anaerobes were greatly stimulated by the cobalt addition, the optimum cobalt concentration required for growth of acetogens and methanogens was determined (Fig. 5). Optimum concentrations for the growth of acetogens and methanogens in both cases were around 0.05 mg of Co liter⁻¹, and both of these populations had similar growth rates, although that of acetogens was slightly higher at high cobalt levels. Growth in the absence of cobalt addition is attributed to low levels of cobalt in the inoculum or as contaminants in chemicals in the mineral medium. For the methanogens, specific methanogenic activity was also determined as a function of cobalt addition (data not shown). Vancomycin addition had no effect on the activity results since the initial concentration of acetogens in the sludge and thus substrate competition were low. The pattern of cobalt stimulation was similar to that found for growth.

The apparent substrate affinity coefficients based on a Lineweaver-Burk plot were 0.25 and 16 mM methanol (12 and 770 mg of COD liter⁻¹) for methylotrophic methanogens and acetogens, respectively. These determinations were conducted when cobalt was supplied at the concentration present in the standard basal medium.

**DISCUSSION**

**Influence of cobalt.** Cobalt is the central ion in corrinoids present in all methanogens and acetogens (40). A number of cobrinoid-dependent reactions are known to take place in the intermediary metabolism of substrates by methanogens and acetogens (49). In methanogens, corrinoids are involved in methyl transfer from methanol to methyl coenzyme M (43, 45), the common precursor of methane from all substrates. In acetogens, corrinoids participate in the formation of acetyl coenzyme A, the precursor intermediate of acetate and cell synthesis (49). However, the content of corrinoids in anaerobic bacteria varies greatly among species and substrates utilized but is always higher when cells are grown on methanol (19). The initial step of methanol conversion in methanol-consuming anaerobes, such as the methanogen *Methanosarcina Barkeri* and the acetogen *Euobacterium limosum*, proceeds in a similar way and is catalyzed by an additional corrinoid-containing enzyme known as methyltransferase (43, 44). Recently, an induced corrinoid-containing protein was reported to occur only in methanol-grown cells of an aceticogenic bacterium *Sporomusa ovata* (39).

Thus, the high requirement for cobalt found in our studies is presumably due to the production of unique corrinoid-containing enzymes or coenzymes that are only present in methylotrophs.

The addition of cobalt stimulated the activity and growth of both methanogens and acetogens, which directly utilized methanol in the cobalt-deprived enrichment culture. The
optimum cobalt concentration in our methylotrophic mixed culture is in the range reported for the marine-isolated methanogen *Methanococccoides methylutens* grown on trimethylamines (37). A much lower requirement for cobalt has been reported for the thermophilic hydrogenotroph methanogen *Methanobacterium thermoautotrophicum* (36). In our consortium, methanogenesis of acetate and H$_2$-CO$_2$ as well as acetogenesis of H$_2$-CO$_2$ was not seriously affected by cobalt addition, despite low cobalt levels. It is impossible to ascertain whether cobalt is an essential nutrient for the latter microorganisms under the experimental conditions utilized because the amount required could be so low that contamination (of chemicals or utensils) and the background level in the seed inoculum could have been adequate. Nonetheless, this study clearly demonstrates that methylotrophic methanogens and acetogens have a distinctly higher requirement for cobalt, which should be considered during wastewater treatment.

Ecology of anaerobic methanol degradation. Although methanol is a simple compound with only one carbon, it can support a very complex food chain under anaerobic conditions. The methylotrophic food web is outlined in Fig. 1. Methane can be directly produced from methanol by the action of methylotrophic methanogens or indirectly by action of acetogens in conjunction with aceticlastic or hydrogenotrophic methanogens. Acetate is also directly formed from methanol, but its formation is limited by the availability of inorganic carbon. If no exogenous inorganic carbon (e.g., HCO$_3^-$) is added, the maximum acetate production will be equal to 33% of the consumed methanol, when methanogenesis occurs. The complexity of this food web is even greater when inorganic electron acceptors such as sulfate are also available (14, 32). Syntrophy and competition among organisms may occur in such environments (6, 32). The formation of H$_2$-CO$_2$ from methanol is dependent on the removal of H$_2$ by hydrogenotrophic anaerobes (5, 14) since hydrogen accumulation would be thermodynamically unfavorable (32, 42). Thus, it is necessary that the syntrophic organism keeps the hydrogen partial pressure extremely low (42). Competition for methanol may occur between methylotrophic acetogens and methanogens, and also competition for H$_2$-CO$_2$ between hydrogenotrophs is possible (13). H$_2$ is also produced in minor amounts by direct methanogenesis of methanol (32).

Direct methylotrophic methanogenesis was the main pathway in our enrichment culture. The activities of methanogens were almost the same in the presence or absence of vancomycin, indicating that methylotrophic acetogens were not participating in the degradation of methanol. In addition, almost no aceticlastic methanogenic or hydrogenotrophic activities were observed. These populations would be expected if methanol was degraded via H$_2$ or acetate, respectively.

Dominance of methylotrophic methanogens. Several factors might have contributed to the dominance of methylotrophic methanogens over their acetogenic competitors in this food web with an excess of inorganic carbon. These could include competition for cobalt, the size of the initial population, and competition for methanol. In relation to cobalt, both populations had similar optimum concentrations of cobalt for growth, and their growth rates were almost the same. The growth rate of acetogens was slightly higher at optimal cobalt concentrations. The cobalt optimum can only be determined when the competitor is switched off by specific inhibitors. In coculture, however, the methanogens may have been better scavengers for cobalt. Additionally, an initial advantage in population size would ensure that most of the cobalt would be captured by the methanogens by virtue of sheer numbers. The initial sludge used to seed the cobalt-deprived enrichment culture did in fact have methylotrophic methanogenic activity, while little acetogenic activity was evident. Finally, we have shown that methylotrophic methanogens have approximately 60-fold-higher affinity for methanol than the acetogens have. Thus, assuming that in wastewater treatment the effluent concentration should be low, methanogens would likely be more competitive for methanol than would acetogens. The effective methanol concentration inside the bioreactor could be very critical in determining whether methanogens or acetogens will predominate. During the cultivation of the cobalt-deprived enrichment culture, the average reactor methanol concentration was 50 mM (10). At this concentration, the growth rate of methanogens exceeds that of acetogens by a factor of 1.4 under cobalt-limiting conditions and by a factor of 1.1 under cobalt-sufficient conditions (Fig. 6). Cobalt limitation enhances the competitive edge but by no means is the decisive factor because both methylotrophic methanogens and acetogens have similar response to cobalt supplementation. Therefore, cobalt concentration and methanol concentration are two factors which are important in the competition between the two populations, provided that sufficient inorganic carbon is available.

The absence of any significant population of aceticlastic methanogens, H$_2$-CO$_2$-utilizing methanogens, and acetogens in the cobalt-deprived sludge can easily be explained by the predominance of methylotrophic methanogens. These methanogens consumed all of the substrate so that products from methylotrophic acetogens were not present in significant quantities to support other members of the food chain. This might be the reason why *Methanosarcina* species were preferentially enriched instead of acetogens when methanol was used as the sole organic carbon and energy source (2, 50). Thus, acetogens can dominate only when special conditions are created by the specific methanogenic inhibitor BESA or by specific toxicity of methanogens such as that caused by dichloromethane (12) and chloroform (15). Also, toxicity due to undissociated acetic acid, resulting from the accumulation of acetic acid exceeding the buffer capacity, selectively inhibited the methanogens and caused the predominance of acetogens (9).

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REFERENCES


