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Choline and \(N,N\)-Dimethylethanolamine as Direct Substrates for Methanogens

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Choline (\(N,N,N\)-trimethylethanolamine), which is widely distributed in membrane lipids and is a component of sediment biota, has been shown to be utilized anaerobically by mixed prokaryote cultures to produce methane but not by pure cultures of methanogens. Here, we show that five recently isolated \textit{Methanococcoides} strains from a range of sediments (Aarhus Bay, Denmark; Severn Estuary mudflats at Portishead, United Kingdom; Darwin Mud Volcano, Gulf of Cadiz; Napoli mud volcano, eastern Mediterranean) can directly utilize choline for methanogenesis producing ethanolamine, which is not further metabolized. Dimethyl- and monomethylethanolamine are metabolic intermediates that temporarily accumulate. Consistent with this, dimethylethanolamine was shown to be another new growth substrate but monomethylethanolamine was not. The specific methanogen inhibitor 2-bromoethanesulfonate (BES) inhibited methane production from choline. When choline and trimethylamine are provided together, diauxic growth occurs, with trimethylamine being utilized first, and then after a lag (\(\sim\)7 days) choline is metabolized. Three type strains of \textit{Methanococcoides} (\textit{M. methylutens}, \textit{M. burtonii}, and \textit{M. alaskense}), in contrast, did not utilize choline. However, two of them (\textit{M. methylutens} and \textit{M. burtonii}) did metabolize dimethylethanolamine. These results extend the known substrates that can be directly utilized by some methanogens, giving them the advantage that they would not be reliant on bacterial syntrophs for their substrate supply.

In sediments, terminal oxidation processes show a vertical sequence which follows the decreasing redox potentials of the respective electron acceptors. Aerobic respiration is present at the sediment surface, followed by nitrate and metal reduction, sulfate reduction, and methanogenesis (19). A more positive redox potential allows a more efficient use of the electron donor and consequently a lower threshold concentration for electron donors (7). For example, sulfate reducers can efficiently use hydrogen or acetate at lower concentration and therefore can easily outcompete methanogens (15, 18). There are, however, exceptions to this vertical zonation. Methanogenic archaea have been detected in significant numbers in the upper sediment layers by cultivation-based (8) and molecular methods (26,27,34) as well as directly by in situ methanogenic activity measurements (6, 14, 20, 22). The occurrence and activity of methanogens in these layers despite the presence of relatively high sulfate concentrations has been explained by the use of noncompetitive substrates consumed exclusively by methanogens (13, 21, 35). These are typically \(C_1\) compounds, such as methanol and methylated nitrogen (methylamine, dimethylamine, and trimethylamine), or sulfur compounds (methanethiol and dimethyl sulfide). Other \(N\)-methylated amines bearing a larger side chain, such as ethanolamine in \(N\)-methylethanolamine and choline (\(N,N,N\)-trimethylethanolamine), have not been shown to support growth of methanogen pure cultures (9, 11, 29); in contrast, they can be degraded by sulfate reducers (2, 9, 30). This is supported by environmental studies which showed a stimulation of methanogenic activity upon choline or glycine betaine addition but also a simultaneous increase in sulfate reduction, suggesting a two-step degradation pathway involving a sulfate reducer (or fermenter) releasing trimethylamine that serves as a substrate for methanogenesis (9, 11, 13).

In the present study, we demonstrate the methanogenic utilization of choline and \(N,N\)-dimethylethanolamine by pure cultures of methanogens affiliated with the genus \textit{Methanococcoides}.

The potential implications of this novel methanogenic pathway will be discussed.

**MATERIALS AND METHODS**

**Source of organisms.** Five new \textit{Methanococcoides} strains (Table 1) plus the three type strains of the genus were investigated in the present study. The three type strains were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany): \textit{Methanococcoides methylutens} DSM 2657\(^T\), \textit{M. burtonii} DSM 6242\(^T\), and \textit{M. alaskense} DSM 17273\(^T\). The other strains were newly isolated on methanol (Table 1) using deep-agar shake tubes (23) or dilution-to-extinction series. All cultures were incubated at 25°C.

**Cultivation and media.** For enrichments, isolation, strain maintenance, and initial experiments, a bicarbonate-buffered and FeS-reduced artificial seawater medium was used (28) which contained (in g · liter\(^{-1}\)):

- \(\text{NaCl (24.3)}\)
- \(\text{MgCl}_2 \cdot 6\text{H}_2\text{O (10)}\)
- \(\text{CaCl}_2 \cdot 2\text{H}_2\text{O (1.5)}\)
- \(\text{KCl (0.66)}\)
- \(\text{KBr (0.1)}\)
- \(\text{H}_3\text{BO}_3 (0.025)\)
- \(\text{SrCl}_2 \cdot 6\text{H}_2\text{O (0.04)}\)
- \(\text{K}_2\text{H}_2\text{PO}_4 (0.0054)\)
- \(\text{NaF (0.003)}\)

The medium was supplemented with 1 ml · liter\(^{-1}\) unchelated trace element solution SL10 and 0.2 ml · liter\(^{-1}\) of a selenite and tungstate solution (33). After autoclaving, the medium was cooled under a \(\text{N}_2\)-\(\text{CO}_2\) mixture (80/20, vol/vol). To the cold medium 10 ml of a solution of 10 vitamins (3) and 30 ml · liter\(^{-1}\) of a 1 M NaHCO\(_3\) solution were added from sterile stocks. Finally, the medium was reduced by addition of \(\text{Na}_2\text{S}\) and acid FeCl\(_3\) solutions to final concentrations of 1.5 and 0.5 mmol · liter\(^{-1}\), respectively. The pH of reduced medium was adjusted to 7.2 to 7.4 with sterile HCl or Na\(_2\)CO\(_3\) if necessary. For enrichment and isolation, 10 mmol methylamine per liter was added. Sterile substrate stocks were produced by dissolving the compound in \(\text{N}_2\) gased...
Choline Utilization by Methanococoides spp.

TABLE 1 Methanococoides strains isolated in this study that were able to utilize choline and/or N,N-dimethylethanolamine as substrates

<table>
<thead>
<tr>
<th>Strain</th>
<th>Location</th>
<th>Sediment depth (cm)</th>
<th>Closest 16S rRNA gene sequence match</th>
<th>Alignment length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM1</td>
<td>Aarhus Bay, Denmark</td>
<td>40–80</td>
<td>M. methylutens DSM2657T (99)</td>
<td>1,443</td>
</tr>
<tr>
<td>DM1</td>
<td>Darwin mud volcano, Western Moroccan mud volcano field, Gulf of Cadiz</td>
<td>10–12</td>
<td>M. methylutens DSM2657T (99)</td>
<td>1,306</td>
</tr>
<tr>
<td>NM1</td>
<td>Napoli mud volcano, Olimpi mud volcano field, eastern Mediterranean</td>
<td>0–5</td>
<td>M. methylutens DSM2657T (98)</td>
<td>1,443</td>
</tr>
<tr>
<td>PM1</td>
<td>Woodhill Bay, Portishead, United Kingdom</td>
<td>10–15</td>
<td>M. methylutens DSM2657T (99)</td>
<td>1,371</td>
</tr>
<tr>
<td>PM2</td>
<td>Woodhill Bay, Portishead, United Kingdom</td>
<td>30–35</td>
<td>M. methylutens DSM2657T (99)</td>
<td>1,357</td>
</tr>
</tbody>
</table>

RESULTS

Utilization of methylamines. Like the three type strains of Methanococoides species, the five new strains grew well with monomethylamine (MMA), dimethylamine (DMA), and trimethylamine (TMA) as the substrate. Growth rates ranged from 0.63 day⁻¹ for strain DM1 to 1.18 day⁻¹ for strain TM1 with MMA. Growth with TMA was faster than with MMA (1.39 day⁻¹ with TMA and 0.75 day⁻¹ with MMA for strain AM1).

Methane production generally correlated well with the number of methyl groups available. For example, strains AM1 and NM1 produced about three times more methane from TMA (2.32 and 2.06 mol of methane per mol of substrate consumed) than from MMA (0.72 and 0.62 mol of methane per mol of substrate consumed). Strain AM1 produced approximately three times higher protein yields with TMA (2.69 mg protein per mol of TMA) than with MMA (0.84 mg protein per mol of MMA). During growth with DMA, MMA was generally detected as an intermediate but at relatively low concentrations. Similarly, with TMA, both DMA and MMA were produced as metabolic intermediates before being metabolized to methane and ammonium (Fig. 1).

Choline and N,N-dimethylethanolamine as the substrates for methanogenesis. Methylamine-grown cultures were used to inoculate media with choline, N,N-dimethylethanolamine (DMEA), N-monomethylethanolamine (MMEA), or ethanolamine as the substrate, and the formation of methane was monitored over time. The different strains showed significant differ-
ences with respect to lag phase and methane production rate. In the first choline-amended cultures of strain AM1, methane formation was detected after 1 to 2 weeks but was already detected after 2 to 4 days in subsequent subcultures. Strain AM1 consumed choline within 10 to 15 days. Strain DM1, in contrast, required between 200 and 260 days for the consumption of 4.95 mM choline, producing 4.7 mmol ethanolamine per liter. Strains NM1, PM1, and PM2 produced only very low levels of methane. After 220 days, strain NM1 had demethylated only 2.1 of 9.9 mmol liter⁻¹ of choline into ethanolamine. After 500 days, strains PM1 and PM2 had consumed 10 mmol liter⁻¹ choline and produced 2.7 and 3.1 mmol liter⁻¹ MMEA but not any ethanolamine (DMEA was not analyzed). Cell-free controls did not show any methane formation, and neither did the type strains of the three Methanococcoides spp. even after more than 300 days of incubation.

DMEA was utilized by all five strains that produced methane from choline, although methanogenesis was slightly slower. For example, in cultures of strain AM1, methane was detected first after 10 days (in contrast to 2 to 4 days with choline). Surprisingly, DMEA also was consumed by M. methylutens and M. burtonii but not by M. alaskense. None of the strains grew or formed methane with N-methylethanolamine or ethanolamine as sole substrates.

**Growth with choline by Methanococcoides sp. strain AM1.** Of the choline-utilizing strains, Methanococcoides sp. strain AM1 was the fastest and, hence, was studied further. When strain AM1 was grown with choline, there was transient formation of DMEA and MMEA. However, concentrations of both remained relatively low, while concentrations of ethanolamine increased first with choline and then with DMEA and MMEA consumption (Fig. 2). This indicates that only a fraction of the choline was partially demethylated, in contrast to trimethylamine, which was apparently first completely converted into DMA and MMA before finally being demethylated to ammonium. After choline removal, first DMEA and then MMEA concentrations decreased. While methane formation from DMEA occurred at a rate comparable to that of choline, MMEA turnover was much slower (day 11 onwards in Fig. 2). Interestingly, with MMEA as the sole substrate no methane was formed, even after 300 days of incubation. The maximum growth rate with choline as a substrate was 0.74 day⁻¹. The formation of methane from choline was inhibited by 2-bromoethanesulfonate (BES).

In cultures grown with MMA, DMA, TMA, or choline, the amount of methane produced was slightly lower than the theoretical value (Table 2). This can partly be explained by the assimilation of carbon into cell biomass, although it may be possible that some methane was lost during sampling. The highest growth yield as g dry weight per methyl group consumed was obtained with choline, followed by dimethylamine, trimethylamine, and methylamine.

**Impact of trimethylamine on methanogenesis from choline by Methanococcoides sp. strain AM1.** Two experiments were conducted to find out which substrate is preferred by strain AM1, choline or trimethylamine. In the first experiment, choline and trimethylamine were both added at the start of the experiment. Strain AM1 showed diauxic growth by consuming trimethylamine.
amine completely before starting to use choline as a substrate for methanogenesis (Fig. 3), with methane formation from TMA being significantly faster than from choline.

In a second experiment, trimethylamine was added to cultures already growing with choline. Surprisingly, there was no obvious change in growth rate (as estimated from methane concentration) after TMA addition (Fig. 4), suggesting simultaneous use of both substrates. However, as can be seen from the presence of intermediates (Fig. 4B), MMEA was formed before TMA was added, but the concentration remained constant at about 0.05 mmol liter\(^{-1}\) after TMA addition while MMA increased. After TMA was consumed MMEA increased again, indicating that choline was not consumed in the presence of TMA.

### DISCUSSION

**Choline and N,N-dimethylethanolamine as substrates for methanogenic pure cultures.** We have demonstrated the use of choline and DMEA as direct substrates for methanogens in new isolates belonging to the genus *Methanococcoides*. Previously, methanogenesis from choline was only described in cocultures of either methanogens and sulfate-reducing *Desulfovibrio* spp. (9) or mixed cultures of fermenters, sulfate reducers, and methanogens for complete mineralization (13). In both cases, the syntrophic partners metabolized choline to trimethylamine, which could be used as a substrate by the methanogen. *Desulfovibrio* spp. oxidized the carbon backbone of the choline to acetate and used the gained electrons for sulfate reduction. No PCR product was obtained from the methanogen cultures using *Bacteria*-specific primers, which excludes the presence of any bacterial syntrophic contaminants in the cultures. In addition, the end products of the methanogenic degradation of choline were methane, carbon dioxide, and ethanolamine, and potential fermentation products (e.g., methylamines and acetate) were not detected. This demonstrates that choline was directly demethylated. The measured consumption and production of products also fits the expected stoichiometries (Table 3). Finally, the specific methanogen inhibitor 2-bromoethanesulfonate (BES) inhibited growth and methane production from choline.

### TABLE 2 Metabolic products and growth yield of strain AM1 grown with methylamine, dimethylamine, trimethylamine, and choline

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Product formed [mM]</th>
<th>Protein formed (mg liter(^{-1}))</th>
<th>Growth yield (g dry wt [mol methyl group](^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Substrate added (mM)</td>
<td>Ammonium</td>
<td>Ethanolamine</td>
</tr>
<tr>
<td>Methylamine</td>
<td>5.3</td>
<td>5.3</td>
<td>3.3</td>
</tr>
<tr>
<td>Dimethylamine</td>
<td>4.7</td>
<td>4.8</td>
<td>6.2</td>
</tr>
<tr>
<td>Trimethylamine</td>
<td>4.7</td>
<td>4.7</td>
<td>9.7</td>
</tr>
<tr>
<td>Choline</td>
<td>4.7</td>
<td>4.7</td>
<td>10.1</td>
</tr>
</tbody>
</table>

*Protein formed was converted into dry mass assuming that protein represents 50% of the dry weight (1).*
TABLE 3 Equations and free energies of reaction for the methanogenic degradation of choline, \( N,N \)-dimethylethanolamine, and \( N \)-methylethanolamine to ethanolamine and for methanogenesis from trimethylamine

<table>
<thead>
<tr>
<th>Equation</th>
<th>Reaction</th>
<th>( \Delta G^\circ ) (kJ/reaction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>( 4(\text{CH}_3)2\text{N}^+ \text{CH}_2\text{CH}_3\text{OH} + 6\text{H}_2\text{O} \rightarrow 4\text{H}_2\text{NCH}_2\text{CH}_3\text{OH} + 9\text{CH}_4 + 3\text{CO}_2 + 4\text{H}^+ )</td>
<td>-567.6</td>
</tr>
<tr>
<td>2</td>
<td>( 2(\text{CH}_3)2\text{NCH}_2\text{CH}_3\text{OH} + 2\text{H}_2\text{O} \rightarrow 2\text{H}_2\text{NCH}_2\text{CH}_3\text{OH} + 3\text{CH}_4 + \text{CO}_2 )</td>
<td>-140.8</td>
</tr>
<tr>
<td>3</td>
<td>( 4(\text{CH}_3)2\text{NHCH}_2\text{CH}_3\text{OH} + 2\text{H}_2\text{O} \rightarrow 4\text{H}_2\text{NCH}_2\text{CH}_3\text{OH} + 3\text{CH}_4 + \text{CO}_2 )</td>
<td>-95.2</td>
</tr>
<tr>
<td>4</td>
<td>( 4(\text{CH}_3)2\text{N} + 6\text{H}_2\text{O} \rightarrow 4\text{NH}_2\text{CH}_2\text{CH}_3 + 9\text{CH}_4 + 3\text{CO}_2 )</td>
<td>-278.1</td>
</tr>
</tbody>
</table>

\( \ast \) Equation 1, choline; equation 2, \( N,N \)-dimethylethanolamine; equation 3, \( N \)-methylethanolamine; equation 4, trimethylamine. \( \Delta G^\circ \) values for the single compounds were taken from Jankowski et al. (12). \( \Delta G^\circ \) for choline (+51.0 kJ mol\(^{-1}\)), DMEA (−88.6 kJ mol\(^{-1}\)), MMEA (−131.5 kJ mol\(^{-1}\)), and ethanolamine (−151.7 kJ mol\(^{-1}\)) were estimated using the group contribution method described by Jankowski et al. (12).

At present we can only speculate how widespread the capacity to use choline and \( N,N \)-dimethylethanolamine as the substrates is among methanogens in general, but as five of our recent *Methanoarcocoides* isolates (\( n = 15 \)) utilized choline and 10 used DMEA, which was also used by two of three *Methanococcales* type strains, these metabolic capabilities may be quite widespread among *Methanoarcocoides* spp. Both compounds have been unsuccessfully tested as the substrates for a limited number of methanogens before (e.g., *Methanosarcina* and *Methanococcales* spp.) (9, 29). However, as in our experiments, lag phases in some of the tests were very long (up to 60 days for *M. burtonii* on DMEA) and in some cases growth was very slow (strain DM1) or not detected (strains NM1, PM1 and PM2), thus it may be that previous tests were terminated too early to show activity. In the present study, all cultures were incubated for at least 1 year to make sure that no activity was missed. However, in future studies both compounds should be tested as potential substrates.

Strain AM1 grows well with choline and DMEA but not with MMEA if it is the sole substrate. This is surprising, as the MMEA transiently formed during growth on choline was consumed (Fig. 2). This might be explained by the relatively low energy yield per mol of MMEA compared to that of DMEA and choline (Table 3). However, if the energy yield is normalized per methyl group, the value for MMEA is similar to that for TMA (−23.8 kJ per mol for MMEA and TMA, respectively). Another possibility is that the cells do not possess a sensor or an uptake system for MMEA, and this compound is unspecifically transported by a choline or DMEA transporter. This would explain the low rate of methane formation and why the presence of MMEA did not induce the production of the required enzymes for methane formation from MMEA. Similarly, MMEA could be demethylated by previously produced methyltransferases for choline and/or DMEA, with MMEA either not having or not being able to induce a specific methyltransferase. Such unspecified uptake and demethylation may be the cause of the slow consumption of choline by strains NM1, PM1, and PM2.

**Choline as a substrate for methanogens in the environment?**

These results clearly show that choline can be a direct substrate for some methanogens, and as choline is widely distributed in membrane lipids and is a component of sediment biota, it could be a significant new methanogenic substrate in the environment, along with its metabolite DMEA. However, whether *Methanoarcocoides* spp. can compete for choline in the environment needs to be tested. While strain AM1 grows relatively fast, strain DM1, for example, needed more than 200 days to degrade 5 mM choline but grows much faster with TMA. Strain DM1 would hardly be able to compete for choline with fast-growing fermenters (10) and sulfate reducers such as *Desulfovibrio* spp. (24) but would easily grow in a syntrophic relationship with TMA as the substrate. Experiments with intertidal sediments suggest choline is degraded syntrophically (13), as association with BES to inhibit methanogenesis resulted in about 80% of added choline appearing as TMA. However, when sulfate reduction was inhibited by the addition of molybdate, the maximum TMA concentration observed was less than 40% of that of the added choline. This indicates that TMA was rapidly used up by methanogens while accumulating, but it might also be due to some of the choline being degraded by methanogens directly. Any direct methanogenic choline utilization would have resulted in accumulation of ethanolamine, but this compound was not analyzed (13). Ethanolamine can be fermented by some *Clostridium* spp. (4) and used by some sulfate-reducing bacteria (e.g., *Desulfofrigus*; H. Sass, unpublished data). Therefore, choline could be demethylated to form ethanolamine by methanogens with subsequent utilization by fermenters or sulfate reducers. Further studies of methylated ethanolamines are needed to determine the following: whether they can be used by methanogens other than *Methanoarcocoides*, the ability of methanogens to compete for these compounds with other anaerobic prokaryotes, and their degradation pathways in a range of anaerobic habitats.

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