Glycine Betaine as a Direct Substrate for Methanogens (Methanococcoides spp.)

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Glycine betaine (N,N,N-trimethylglycine) is one of the most common compatible solutes in nature and is found in all three domains of life (1–3). In addition to its role in osmoadaptation, glycine betaine has been suggested to play a role in microbial cryoprotection and barotolerance (4, 5). Considering that intracellular glycine betaine concentrations can be some hundreds of millimoles per liter, depending on the salinity of the medium (6), it is clear that it must be very abundant in saline environments. For example, in hypersaline mats, total glycine betaine contents of up to 0.1 mmol per gram (dry weight) of sediment have been found (7).

In anoxic sediments, the addition of glycine betaine leads to methanogenic activity, but also to a simultaneous stimulation of sulfate reduction (8). However, the transient formation of similar amounts of trimethylamine (TMA) and acetate indicates that the reduction of betaine, as found in members of the genera Clostridium and Halanaerobacter (9, 10), is the first step in degradation. While acetate is utilized mainly by sulfate reducers, trimethylamine is a well-known noncompetitive substrate for methanogens (8, 11), allowing them to thrive within the sulfate reduction zone. This degradation pattern involving three different metabolic groups is quite complex, and it could be argued that it would be advantageous for the methanogens if they could demethylate glycine betaine directly, similar to direct choline (N,N,N-trimethyl-ethanolamine) utilization, which has recently been documented (12). Although a number of methanogens have been tested, no glycine betaine consumption by methanogens has been reported so far (13–15).

In the present study, we demonstrate the partial demethylation of glycine betaine (N,N,N-trimethylglycine) to N,N-dimethylglycine (DMG) by members of the genus Methanococcoides. The potential implications of this novel methanogenic pathway are discussed.

MATERIALS AND METHODS

Sources of organisms. In total, nine Methanococcoides strains were investigated. They included the three type strains Methanococcoides methylutens DSM 2657T, M. burtonii DSM 6242T, and M. alaskense DSM 17273T, obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) (Braunschweig, Germany), and five new Methanococcoides strains (AM1, DM1, NM1, PM1, and PM2) obtained from a range of marine habitats (12). Their 16S rRNA genes (GenBank numbers HE862406 to HE862410) share >99% similarity with that of M. methylutens DSM 2657T. One additional strain, MKM1, was isolated from an enrichment inoculated with sediment from the Meknes mud volcano of the Gulf of Cadiz with methylamine (MMA) as the substrate, using agar shake tubes (16). All cultures were incubated at 25°C.

Cultivation and media. A bicarbonate-buffered and FeS-reduced artificial seawater medium (12, 17) was used for isolation, strain maintenance, and physiological experiments. The pH of the reduced medium was adjusted to 7.2 to 7.4 with sterile HCl or Na2CO3, if necessary. For enrichment and isolation, 10 mmol methylamine per liter was added.

For growth experiments, 150-ml serum bottles filled with 30 ml medium under an N2-CO2 (80/20 [vol/vol]) headspace and with 5 mmol of substrate per liter were used. Growth was monitored by the increase in headspace methane, and the specific growth rate (μ) was calculated from plots of the total accumulated methane against time (12, 18, 19). The growth yield was estimated from the increase in protein content analyzed by the method of Bradford (20).

Analytical techniques. Headspace gas was measured by gas chromatography (PerkinElmer/Arnel Clarus [Sheldon, CT] 500 Natural Gas Analyzer), and the methane contents in the headspace and medium were calculated as described previously (12). Anions (including the organic acids acetate, lactate, and formate) were analyzed on a Dionex ICS-2000 Ion Chromatography System equipped with an AS50 autosampler (Dionex, Camberley, United Kingdom) (21).

Prior to ion chromatographic analysis, 1 ml of culture was centrifuged (15 min at 16,000 × g at 10°C), and the supernatant was diluted (1:10 [vol/vol]) in ultrapure water (>18.2 MΩ; Milli-Q system; Millipore).
Cations (including ammonium, methylamines, betaine, and dimethylglycine) were analyzed using ion chromatography with nonsuppressed conductivity detection (22) on a Dionex ICS-2000 Ion Chromatograph equipped with a D56 heated conductivity cell (45°C) and an AS50 autosampler (Dionex, Camberley, United Kingdom). Chromatographic separation was conducted on an Ionpac CS16 column at 50°C using methanesulfonic acid eluent (3 mmol · liter⁻¹) and acetonitrile (10%) at a flow rate of 1.30 ml min⁻¹.

RESULTS

Utilization of N-methylated glycines by Methanococcoides spp.

All Methanococcoides strains tested grew well with mono-, di-, and trimethylamine, and fresh methylamine-grown cultures were used to inoculate media with glycine betaine (N,N,N-trimethylglycine), DMG, or N-monomethylglycine (sarcosine) as the substrate. While none of the strains formed methane from DMG or sarcosine, three strains (NM1, PM2, and MKM1) produced methane from glycine betaine within 1 to 2 weeks. These positive results were confirmed by subcultivation on the same substrate. Negative cultures were inoculated with the error bars indicating 1 standard deviation. □, methane; ◦, glycine betaine; ○, N,N-dimethylglycine; △, days.

FIG 1 Metabolism of glycine betaine by Methanococcoides sp. NM1. All values are the averages of three replicates, with the error bars indicating 1 standard deviation. □, methane; ◦, glycine betaine; ○, N,N-dimethylglycine; △, days.

TABLE 1 Metabolic products and growth yields of Methanococcoides sp. strain NM1 grown on methylamine, dimethylamine, trimethylamine, and glycine betaine

<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>Ammonium</td>
<td>DMG</td>
<td>Methane</td>
</tr>
<tr>
<td>Methylamine</td>
<td>5.4</td>
<td>5.4</td>
<td>3.1</td>
</tr>
<tr>
<td>Dimethylamine</td>
<td>5.1</td>
<td>5.1</td>
<td>6.7</td>
</tr>
<tr>
<td>Trimethylamine</td>
<td>4.9</td>
<td>4.9</td>
<td>10.1</td>
</tr>
<tr>
<td>Betaine</td>
<td>5.4</td>
<td>5.2</td>
<td>3.6</td>
</tr>
</tbody>
</table>

All data are averages of triplicate cultures. The protein formed was converted into dry mass assuming that protein represents 50% of the dry weight (34).
lization (Fig. 2). As in previous studies (12, 13), TMA was first partially demethylated to DMA and MMA. However, although TMA was utilized first, there was some simultaneous decrease in glycine betaine in the presence of TMA. The highest rate of glycine betaine consumption occurred immediately after TMA was depleted, and this was simultaneous with DMA consumption. Strain NM1 utilized MMA only when glycine betaine and DMA were almost depleted. This pattern differs significantly from that found for *Methanococcales* sp. strain AM1 in the presence of choline and TMA, where a significant lag occurred between the consumption of TMA and its intermediates and the start of choline utilization (12).

**Glycine betaine content in cells of *Methanococcales* sp. strain NM1.** At the end of the growth experiment shown in Fig. 1, 1.5 ml of culture was washed in artificial seawater, and the cell pellet was resuspended in 1.5 ml of deionized water to lyse the cells. Cation analysis of three parallel cultures revealed the presence of N,N-dimethylglycine (35 ± 140 μmol · liter⁻¹), Na⁺ (34 ± 10 mmol · liter⁻¹), and K⁺ (0.69 ± 0.29 mmol · liter⁻¹), but no glycine betaine, methylamines, or ammonium, in the cell pellets. In contrast, cells grown with trimethylamine (10 mmol · liter⁻¹) contained significant concentrations of ammonium (53 μmol · liter⁻¹), MMA (294 μmol · liter⁻¹), DMA (41 μmol · liter⁻¹), Na⁺ (5.3 mmol liter⁻¹), and K⁺ (5.3 mmol liter⁻¹), but no detectable glycine betaine or DMG.

**DISCUSSION**

**Glycine betaine, a new substrate for methanogenic pure cultures.** In this study, we have shown the direct use of glycine betaine by pure cultures of methanogens. Previously, methanogenic degradation of glycine betaine was thought to require syntrophic interaction with a fermenter (or sulfate reducer) producing trimethylamine, which was then used by the methanogen (8, 13). However, like choline and N,N-dimethylglycine, which have recently been reported to be novel direct substrates for methanogens (12), glycine betaine can also be directly demethylated by methanogens. The presence of a syntrophic partner in our cultures can be ruled out, as no intermediates, TMA or acetate, were detected, which would have accumulated if glycine betaine was degraded by coculture.

At present, we can only speculate about how widespread the capacity to use glycine betaine is among methanogens. Like choline and N,N-dimethylglycine, glycine betaine is an N-methylated amine bearing a C₂ side chain and belongs to a group of compounds that was thought not to support the growth of methanogenic pure cultures. Therefore, only a limited number of pure cultures belonging to the genera *Methanococcales*, *Methanosarcina*, *Methanolohalophilus*, and *Methanomicrococcus* (13–15, 23, 24) have been tested with glycine betaine or choline. However, choline and glycine betaine are not the only C₂ methylated amines utilized by methanogens. *Methanosarcina barkeri* was shown to grow with N-ethylmethylamine, but not with choline, glycine betaine, or N,N-diethylmethylamine (13). However, since N-ethylmethylamine was considered of little biological significance, later studies neglected this substrate. Glycine betaine, in contrast, is a common osmolyte in saline environments (1, 3), and choline and N,N-dimethylglycine are headgroups of phospholipids present in anoxic sediments (25). Considering that three of the nine strains tested used glycine betaine and 5 out of 15 *Methanococcales* spp. have been recently shown to utilize choline or N,N-dimethylglycine (12), it is clear that methanogens are more versatile than previously thought. Therefore, this physiological diversity, particularly with respect to N-methylated amines bearing a larger side chain, has been largely overlooked.

Whether glycine betaine is a direct substrate for methanogens in the marine environment needs to be investigated, although it is unlikely that they can compete with sulfate reducers for the substrate. Several sulfate reducers can utilize glycine betaine as an electron donor (26, 27), and it was shown that in intertidal sediments, sulfate reduction was strongly stimulated by the addition of glycine betaine (8). In sulfate-free layers, however, being able to use glycine betaine directly would make the methanogens independent of syntrophic interaction with fermenters, some of which may not release trimethylamine that could then be used by the methanogens and therefore would restrict methanogenesis. For example, in the presence of glycine betaine when methanogens were inhibited in intertidal sediments by the addition of 2-bromoethanesulfonate (BES), less than 60% of the theoretically possible TMA was formed (8). This indicates that either not all of the betaine is degraded via trimethylamine or that some of the TMA is used by other processes, such as homoacetogenesis.

**Incomplete degradation of glycine betaine.** All three strains utilizing glycine betaine only partially demethylated their substrates to N,N-dimethylglycine. This may be surprising, particularly considering that the *Methanococcales* spp. using choline demethylated their substrates completely to ethanolamine (12). However, a range of organisms also produce DMG from glycine betaine, including several *Desulfbacterium* spp. and *Acetobacterium* spp. (26, 28). In addition, *Eubacterium limosum* converts glycine betaine and CO₂ into DMG, acetate, and butyrate (29), while some homoacetogens, like *Sporomusa* spp., ferment glycine betaine into acetate, trimethylamine, and DMG (30).

The demethylation of glycine betaine to DMG or glycine produces −180.4 and −248.2 kJ per mol of glycine betaine, respectively (Table 2). This means that the first methyl group yields more than five times more energy than the other two. This high energy yield may also explain the relatively high
growth yield observed for growth on glycine betaine (Table 1). However, the ΔG°' for the demethylation of DMG to glycine is still −67.8 kJ per mol DMG, and considering that DMG has two methyl groups, the ΔG°' per methyl group is comparable to the value for methylamine (−43.0 kJ per mol). However, although it seems a potential waste of energy, the cultures investigated here did not utilize the DMG produced even after prolonged incubation of several weeks.

Glycine betaine as a compatible solute in Methanococcales sp. NMI. Both glycine betaine and DMG have been documented as compatible solutes in halotolerant and halophilic methanogenic archaea (31–33). However, cells of strain NMI grown in artificial seawater with trimethylamine as the substrate did not contain any detectable amounts of glycine betaine but showed a slight accumulation of K⁺ plus significant amounts of methylamine. This is similar to other methanogens, like Methanosarcina spp., that can accumulate K⁺ for osmoregulation and synthesize the amino acids α-glutamate and N⁺-acetyl-L-lysine as osmolytes but can take up glycine betaine if it is present in the medium (33). However, the uptake and accumulation of glycine betaine in Methanosarcina spp. suppresses the formation of other osmolytes, which is thought to save significant energy. Cells of strain NMI might not only save energy by taking up glycine betaine instead of synthesizing other osmolytes, they also can use glycine betaine as a metabolic substrate. Since DMG acts as a compatible solute, as well, this means that the partial demethylation of glycine betaine allows energy generation and energy saving by the metabolic end product being an osmoregulant.

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