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Glycine betaine as a direct substrate for methanogens (*Methanococcoides* spp.)

Running title: Betaine utilization by methanogens

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Abstract

Nine marine methanogenic *Methanococcoides* strains, including the type strains of *M. methylutens*, *M. burtonii* and *M. alaskense*, were tested for the utilization of *N*-methylated glycines. Three strains (NM1, PM2 and MKM1) used glycine betaine (*N*,*N*,*N*-trimethylglycine) as a substrate for methanogenesis, partially demethylating it to *N*,*N*-dimethylglycine, whereas none of the strains used *N*,*N*-dimethylglycine or sarcosine (*N*-methylglycine). Growth rates and growth yields per mol of substrate with glycine betaine (3.96 g dw per mol) were similar to those with trimethylamine (4.11 g dw per mol). However, as glycine betaine is only partially demethylated, the yield per methyl group was significantly higher than with trimethylamine. If glycine betaine and trimethylamine are provided together, trimethylamine is demethylated to dimethyl- and methylamine with limited glycine betaine utilization. After trimethylamine is depleted, dimethylamine and glycine betaine are consumed rapidly, before methylamine. Glycine betaine extends the range of substrates that can be directly utilized by some methanogens allowing them to gain energy from this substrate without the need for syntrophic partners.

Introduction

Glycine betaine (*N*,*N*,*N*-trimethylglycine) is one of the most common compatible solutes in nature and 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 hundred millimoles per litre 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 of sediment dry weight have been found (7).

In anoxic sediments the addition of glycine betaine leads to methanogenic activity but also to a simultaneous stimulation of sulphate reduction (8). However, the transient formation of similar amounts of trimethylamine and acetate indicates that the reduction of betaine, as found in members of the genera *Clostridium* and *Halanaerobacter* (9-10), is the first step during degradation. While acetate is utilized mainly by sulphate reducers, trimethylamine is a well known non-competitive substrate for methanogens (8, 11), allowing them to thrive within the sulphate 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*-trimethylethanolamine) 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 (e.g. 13-15).

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

Materials and Methods

Source of organisms

In total, nine *Methanococcoides* strains were investigated. These included the three type strains *Methanococcoides methylutens* DSM 2657^T, *M. burtonii* DSM 6242^T and *M. alaskense* DSM 17273^T obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany), and five new *Methanococcoides* strains (AM1, DM1, NM1, PM1, 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 2657^T. One additional strain, MKM1, was isolated from an enrichment inoculated with sediment from the Meknes mud volcano of the Gulf of Cadiz with methylamine as substrate using agar shake tubes (16). All cultures were incubated at 25ºC.

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 – 7.4 with sterile HCl or Na_2CO_3 if necessary. For enrichment and isolation 10 mmol methylamine per litre was added.

For growth experiments 150 mL serum bottles filled with 30 mL medium under a N_2/CO_2 (80/20, v/v) headspace and with 5 mmol of substrate per litre were used. Growth was monitored by increase in headspace methane and the specific growth rate (μ) calculated from plots of the total accumulated methane against time (12, 18-19). Growth yield was estimated from the increase in protein contents analysed by the method of Bradford (20).

Analytical techniques

Headspace gas was measured by gas chromatography (Perkin Elmer/Arnel Clarus 500 Natural Gas Analyser, Sheldon, CT) and methane contents in headspace and medium calculated as described previously (12). Anions (including the organic acids acetate, lactate and formate) were analysed on a Dionex ICS-2000 Ion Chromatography System equipped with an AS50 autosampler (Dionex, Camberley, UK) (21).

Prior to ion chromatographic analysis 1 mL of culture was centrifuged (15 min at 16000 g at 10 °C) and the supernatant diluted (1:10, v/v) in ultrapure water (>18.2 MOhm; Milli-Q system®, Millipore™). Cations (including ammonium, methylamines, betaine, and dimethylglycine) were analysed using ion chromatography with non-suppressed conductivity detection (22) on a Dionex ICS-2000 Ion Chromatograph equipped with a DS6 heated conductivity cell (45°C) and an AS50 autosampler (Dionex, Camberley, UK). Chromatographic separation was conducted on an Ionpac CS16 column at 50°C using methanesulphonic acid eluent (3 mmol·L^{-1}) and acetonitrile (10 %) at a flow rate of 1.30 mL min^{-1}.

Results

Utilisation 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), *N*,*N*-dimethylglycine (DMG) or *N*-monomethylglycine (sarcosine) as substrate. While none of the strains formed methane from DMG or sarcosine, three strains (NM1, PM2, MKM1) produced methane from glycine betaine within one to two weeks. These positive results were confirmed by subcultivation on the same substrate. Negative cultures were incubated for at least three months and regularly measured for methane production, since methanogenic cultures sometimes show very long lag phases (12).

When the three strains were grown with glycine betaine there was only a relatively small amount of methane formed, with a methane to glycine betaine ratio of around 0.7. This suggested that glycine betaine was only partly demethylated. Since the three strains showed similar lag phases and growth rates, only one strain, NM1, was investigated in more detail. Ion chromatographic analysis identified DMG as the end product of methanogenesis from glycine betaine by strain NM1 (Fig. 1). At the end of the growth experiment, residual betaine concentrations were below the detection limit (130 µmol l^{-1}). After glycine betaine was consumed cultures were further incubated for a number of weeks but showed no decrease in DMG concentrations.

The maximum growth rate of strain NM1 with glycine betaine was 0.93 ± 0.01 d^{-1} (n = 3). This is a growth rate comparable to cultures with methylamine (0.96 d^{-1}) but slightly slower than cultures with di- (1.05 d^{-1}) or trimethylamine (1.24 d^{-1}) and faster than with methanol (0.64 d^{-1}). On average, 0.97 moles of DMG and 0.67 moles of methane were formed per mole of betaine. The amount of protein formed in cultures with trimethylamine

and glycine betaine was similar. However, as glycine betaine is only partially demethylated the growth yield per methyl group is 3.96 g dw mol (methyl group)$^{-1}$ and significantly higher than with mono-, di- or trimethylamine (Table 1). Acetate, formate and other organic acids were found only at minor concentrations (< 0.04 mmol·L^{-1}).

Impact of trimethylamine on methanogenesis from glycine betaine by strain NM1

Cultures of strain NM1 with trimethylamine and glycine betaine showed no clear diauxic substrate utilization (Fig. 2). Like in previous studies (12-13), TMA was first partially demethylated to dimethylamine (DMA) and methylamine (MMA). However, although TMA was utilized first, there was some simultaneous decrease in glycine betaine in the presence of TMA. The fastest 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 *Methanococcoides* sp. 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 *Methanococcoides* sp. 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 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 (353±140 µmol·L^{-1}), Na^+ (34±10 mmol·L^{-1}) and K^+ (0.69±0.29 mmol·L^{-1}), but no glycine betaine, methylamines or ammonium in the cell pellets. In contrast, cells grown with trimethylamine (10 mmol L^{-1}) contained significant concentrations of ammonium (53

µmol·L^{-1}), MMA (294 µmol·L^{-1}), DMA (41 µmol·L^{-1}), Na^+ (5.3 mmol L^{-1}), and K^+ (5.3 mmol L^{-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 between a fermenter (or sulphate reducer) producing trimethylamine which was then used by the methanogen (8, 13). However, like choline and *N*,*N*-dimethylethanolamine that have recently been reported as 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 co-culture.

At present we can only speculate how widespread the capacity to use glycine betaine is among methanogens. Like choline and *N*,*N*-dimethylethanolamine, glycine betaine is an *N*-methylated amine bearing a C_2 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 *Methanococcoides*, *Methanosarcina*, *Methanohalophilus* and *Methanomicrococcus* (13-15, 23-24) has been tested with glycine betaine or choline. However, choline and glycine betaine are not the only C_2 methylated amine utilized by methanogens. *Methanosarcina barkeri* was shown to grow with *N*-ethyldimethylamine but not with choline, glycine betaine or *N*,*N*-diethylmethylamine (13).

However, since *N*-ethyldimethylamine 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*-dimethylethanolamine are headgroups of phospholipids present in anoxic sediments (25). Considering that three of the nine strains tested used glycine betaine and five out of fifteen *Methanococcoides* spp. have been recently shown to utilize choline or *N*,*N*-dimethylethanolamine (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 sulphate reducers for this substrate. Several sulphate 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 sulphate-free layers, however, being able to use glycine betaine directly would make the methanogens independent from 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 BES, less than 60% of theoretically possible TMA were 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 substrate to *N*,*N*-dimethylglycine. This may be surprising, particularly considering that the *Methanococcoides* spp. using choline demethylated their substrate completely to ethanolamine (12). However, a

range of organisms also produce DMG from glycine betaine, including several *Desulfobacterium* spp. and *Acetobacterium* spp. (26, 28). In addition, *Eubacterium limosum* converts glycine betaine and CO_2 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 -183.1 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_o' 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_o' 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 Methanococcoides sp. NM1?

Both, glycine betaine and DMG have been documented as compatible solutes in halotolerant and halophilic methanogenic archaea (31-33). However, cells of strain NM1 grown in artificial seawater with trimethylamine as 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^{ε}-acetyl-β-lysine as osmolytes, but can take up glycine betaine if 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

NM1 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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Figure legends

Figure 1 Metabolism of glycine betaine by *Methanococcoides* sp. NM1. All values are the average of three replicates with the error bars indicating one standard deviation. Symbols: □, methane; ●, glycine betaine; ○, *N*,*N*-dimethylglycine.

Figure 2 Successive metabolism of trimethylamine, its intermediates and glycine betaine by *Methanococcoides* sp. NM1. Both substrates were present in the medium from day 0. Note the different scale in (B) showing the concentrations of intermediates of trimethylamine consumption. Only the first ten days of the experiment shown. Cultures were monitored for another three weeks but did not show any significant concentration changes. All values are the average of three replicates with the error bars indicating one standard deviation. Symbols: □, methane; ●, glycine betaine; ○, *N,N*-dimethylglycine; ▽, trimethylamine; ▼, ammonium; ◆ dimethylamine; ◇, methylamine.

Table 1. Metabolic products and growth yields of *Methanococcoides* sp. NM1 grown on methylamine, dimethylamine, trimethylamine and glycine betaine. All data are average of triplicate cultures. Protein formed was converted into dry mass assuming that protein represents 50% of dry weight (34).

Substrate	Substrate consumed [mM]	Product formed [mM]			Protein formed [mg l^{-1}]	Growth yield [g dw (mol methyl group)$^{-1}$]
		Ammonium	DMG	Methane		
Methylamine	5.4	5.4		3.1	5.81	2.15
Dimethylamine	5.1	5.1		6.7	8.95	1.75
Trimethylamine	4.9	4.9		10.1	10.1	1.37
Betaine	5.4		5.2	3.6	10.7	3.96

Table 2. Equations and free energies of reaction for the methanogenic degradation of glycine betaine to *N*,*N*-dimethylglycine (eq. 1), glycine betaine to glycine (eq. 2), *N*,*N*-dimethylglycine to glycine (eq. 3), sarcosine to glycine (eq. 4) and methanogenesis from methylamine (eq. 5). $\Delta G_f^{o\prime}$ values for the single compounds were taken from Jankowski et al. (35, supplementary material). $\Delta G_f^{o\prime}$ for glycine betaine (-129.8 kJ mol^{-1}), *N*,*N*-dimethylglycine (-306.6 kJ mol^{-1}) and sarcosine (-331.3 kJ mol^{-1}) were estimated using the group contribution method described Jankowski et al. (35). All values are calculated for standard conditions (298 K, pH 7, 1 atm) in aqueous systems and for the predominant ions at neutral pH.

Eq.	Reaction	$\Delta G_o'$
1	$4\ (CH_3)_3N^+CH_2COO^- + 2\ H_2O \rightarrow 4\ (CH_3)_2NH^+CH_2COO^- + 3\ CH_4 + CO_2$	-721.7 kJ/reaction
2	$4\ (CH_3)_3N^+CH_2COO^- + 6\ H_2O \rightarrow 4\ H_3N^+CH_2COO^- + 9\ CH_4 + 3\ CO_2$	-992.8 kJ/reaction
3	$2\ (CH_3)_2NH^+CH_2COO^- + 2\ H_2O \rightarrow 2\ H_3N^+CH_2COO^- + 3\ CH_4 + CO_2$	-135.5 kJ/reaction
4	$4\ (CH_3)NH_2^+CH_2COO^- + 2\ H_2O \rightarrow 4\ H_3N^+CH_2COO^- + 3\ CH_4 + CO_2$	-157.7 kJ/reaction
5	$4\ (CH_3)NH_3^+ + 2\ H_2O \rightarrow 4\ NH_4^+ + 3\ CH_4 + CO_2$	-172.1 kJ/reaction