RESEARCH LETTER – Physiology & Biochemistry

The hydrogen threshold of obligately methyl-reducing methanogens

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One sentence summary: Based on their extremely low hydrogen threshold, methylotrophic methanogens (MM) should always outcompete hydrogenotrophic methanogens (HM) for hydrogen, provided that methyl groups are available in sufficient amounts.

Editor: Nathan Basiliko

ABSTRACT

Methanogenesis is the final step in the anaerobic degradation of organic matter. The most important substrates of methanogens are hydrogen plus carbon dioxide and acetate, but also the use of methanol, methylated amines, and aromatic methoxy groups appears to be more widespread than originally thought. Except for most members of the family Methanosarcinaceae, all methylotrophic methanogens require external hydrogen as reductant and therefore compete with hydrogenotrophic methanogens for this common substrate. Since methanogenesis from carbon dioxide consumes four molecules of hydrogen per molecule of methane, whereas methanogenesis from methanol requires only one, methyl-reducing methanogens should have an energetic advantage over hydrogenotrophic methanogens at low hydrogen partial pressures. However, experimental data on their hydrogen threshold is scarce and suffers from relatively high detection limits. Here, we show that the methyl-reducing methanogens Methanosphaera stadtmanae (Methanobacterales), Methanimicrococcus blatticola (Methanosarcinales), and Methanomassiliicoccus luminyensis (Methanomassiliicoccales) consume hydrogen to partial pressures < 0.1 Pa, which is almost one order of magnitude lower than the thresholds for M. stadtmanae and M. blatticola reported in the only previous study on this topic. We conclude that methylotrophic methanogens should outcompete hydrogenotrophic methanogens for hydrogen and that their activity is limited by the availability of methyl groups.

Keywords: hydrogen; threshold; methanol; Methanosphaera; Methanimicrococcus; Methanomassiliicoccus

INTRODUCTION

Increasing atmospheric levels of methane have intensified the interest in understanding the sources of this second-most important greenhouse gas (Mikaloff, Fletcher and Schaefer 2019). About half of the global methane emission is biogenic, stemming mostly from natural wetlands and sediments, and to an increasing extent from agriculture and ruminant livestock (Kirschke et al. 2013). Methanogenesis is catalyzed by methanogenic archaea, which utilize either hydrogen plus carbon dioxide or acetate, the major products of microbial fermentations, as substrates (Liu and Whitman 2008; Thauer et al. 2008). However, also methylated compounds are emerging as an important group of methanogenic substrates (Evans et al. 2019; Söllinger and Urich 2019). While the contribution of hydrogenotrophic and aceticlastic methanogenesis to methane production has been intensively studied, the importance of methylotrophic methanogenesis remains unclear (Conrad 2020).
Hydrogenotrophic methanogens reduce carbon dioxide to methane via the archaeal variant of the Wood–Ljungdahl pathway. The key intermediate is methyl-coenzyme M, whose formation by a membrane-bound methyl-THF:coenzyme M methyltransferase is highly exergonic and allows the conservation of energy in the form of an electrochemical sodium gradient (Thauer et al. 2008). By contrast, methylotrophic methanogens transfer the methyl groups of methanol or other methylated substrates directly to coenzyme M (Thauer et al. 2008; Yan and Ferry 2018). This bypasses the methyltransferase reaction and therefore requires other modes of energy conservation (Sprenger, Hackstein and Keltjens 2005; Fricke et al. 2006; Lang et al. 2015).

Also methylotrophic methanogens generally require molecular hydrogen for methanogenesis. Only the only methyl-fermenting members of the family Methanosarcinaceae, which possess a complete Wood–Ljungdahl pathway and a membrane-bound electron transport chain, can generate reducing equivalents for methanogenesis by oxidizing methyl groups to carbon dioxide (Thauer et al. 2008). Obligately methyl-reducing methanogens comprise phylogenetically and biochemically heterogeneous lineages, including the isolates Methanosphaera stadtmanae (Methanobacteriales; Miller and Wolin 1985), Methanomicrococcus blatticola (Methanosarcinales; Sprenger et al. 2000), and Methanomassiliicoccales luminyensis (Methanomassiliicoccales; Didri et al. 2012), the recently described Methanomonarchaeum thermophilum (Methanomonarchaeae; Sorokin et al. 2018) and several Candidatus strains from enrichment cultures (Borrel et al. 2012; Paul et al. 2012; Borrel et al. 2013; lino et al. 2013; Sorokin et al. 2018). Also a few hydrogenotrophic Methanobacterium species can facultatively reduce methanol (Krivushin et al. 2010; Borrel et al. 2012). Evidence for an even wider range of putatively hydrogen-dependent, methylotrophic methanogens is provided by a growing number of metagenome-assembled genomes of uncultured archaea (Evans et al. 2019; Söllinger and Urich 2019).

Under standard conditions, hydrogenotrophic methanogenesis (Equation 1) is thermodynamically more favorable than the hydrogen-dependent reduction of methanol to methane (Equation 2) (Thauer 1998).

$$4 \text{H}_2 + \text{CO}_2 \rightarrow \text{CH}_4 + 2\text{H}_2\text{O} \quad (\Delta G^{\circ} = -131 \text{kJ mol}^{-1} \text{methane}) \quad (1)$$

$$\text{H}_2 + \text{CH}_3\text{OH} \rightarrow \text{CH}_4 + \text{H}_2\text{O} \quad (\Delta G^{\circ} = -112.5 \text{kJ mol}^{-1} \text{methane}) \quad (2)$$

However, the different stoichiometries of the reactions dictate that the free energy of hydrogenotrophic methanogenesis decreases more strongly with decreasing hydrogen partial pressure, and methyl-reducing methanogens should eventually outcompete hydrogenotrophic methanogens for their common substrate. Hydrogen thresholds of hydrogenotrophic methanogens have been investigated in a number of studies. In most cases, the threshold value ranges between 2.8 and 10 Pa (Table 1). An exception is members of the genus Methanosarcina, which exhibit considerably higher threshold values for hydrogen when grown hydrogenotrophically (Table 1) and even accumulate hydrogen at steady-state levels of 8–20 Pa during fermentation of acetate, methanol or methylamines by hydrogen cycling (e.g. Lovley and Ferry 1985; Kulikarni, Mandy and Metcalf 2018). Data on obligately methyl-reducing methanogens, however, are scarce. The only hydrogen threshold values reported in the literature (1.0 Pa for Methanosphaera stadtmanae and < 0.7 Pa for Methanimicrococcus blatticola) are around the detection limit given in that study (Sprenger, Hackstein and Keltjens 2007). Therefore, we re-investigated the hydrogen thresholds of these species and of the hitherto unstudied Methanomassiliicoccales luminyensis, the only isolate of the exclusively methyl-reducing Methanomassiliicoccales, using an analytical setup that was more sensitive by almost one order of magnitude (detection limit 0.1 Pa). A hydrogenotrophic Methanobrevibacter species was included for benchmarking purposes.

**MATERIAL AND METHODS**

Methanosphaera stadtmanae (DSM 3091), Methanimicrococcus blatticola (DSM 13328), and Methanomassiliicoccales luminyensis (DSM 25720) were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). The obligately hydrogenotrophic Methanobrevibacter strain AMG-1, a member of the Methanobrevibacter arboriphilus clade (Genbank accession number MT249795; 94.5% 16S rRNA gene sequence similarity to the type strain), was isolated from the gut of the millipede Anobolus monilicornis (C. Netz, K. Lang and A. Brune, unpublished results). All strains were grown in AM5 medium (Tegtmeier et al. 2016) supplemented with (final concentration) 2-mercaptoethane sulfonate (0.001%), casamino acids (0.2%, Roth), yeast extract (0.2%; Roth), cysteine-HCl (2 mM), acetate (1 mM), dithiothreitol (1 mM), formate (0.5 mM) and methanol (50 mM). Serum bottles (120 ml) filled with 30 ml medium under a headspace of N₂ – CO₂ (80/20) were inoculated with 3 ml preculture (three replicates). After addition of hydrogen (150 Pa) to the headspace, the cultures were incubated statically at their optimum growth temperatures (37°C for M. stadtmanae and M. luminyensis, and 30°C for M. blatticola and Methanobrevibacter strain AMG-1). The amount of methanol in the medium (1.5 mmol) by far exceeded the amount of hydrogen added to the cultures (5.6 µmol per addition).

Hydrogen partial pressures were monitored once per week. Aliquots (0.2 ml) of the headspace were injected into a gas chromatograph (GC 8A, Shimadzu, Kyoto, Japan) equipped with a Molsieve column (60/80 mesh, 6 ft length, 2.1 mm inner diameter, Restek, Bad Homburg, Germany; column temperature 80°C) and a reducing gas detector for hydrogen partial pressures < 10 Pa (RGD2, Trace Analytical, Techmation, Düsseldorf, Germany). Hydrogen partial pressures > 10 Pa were measured with a gas chromatograph equipped with a thermal conductivity detector. Standard curves were generated by injecting aliquots (0.1, 0.2 and 0.4 ml) of hydrogen standards (2 ppm and 1000 ppm, in N₂; Messer, Bad Soden, Germany). All injections were carried out with a gas-tight 0.5-ml precision syringe (Grace Davison Discovery Science, Deerfield, IL, USA). The detection limit of the assay was defined as the amount of hydrogen that caused a peak that had twice the height of the baseline noise at the retention time of hydrogen when a non-reducing gas (nitrogen) was injected. To monitor growth of the precultures, methanogenesis was routinely measured using a separate gas chromatograph equipped with a flame ionization detector (Lang et al. 2015).

**RESULTS AND DISCUSSION**

All cultures consumed the hydrogen added to the headspace. The hydrogenotrophic Methanobrevibacter strain AMG-1 consumed hydrogen until a threshold value of 5.7 ± 0.7 Pa was reached. The three obligately methyl-reducing methanogens, Methanosphaera stadtmanae, Methanimicrococcus blatticola and Methanomassiliicoccales luminyensis, however, always consumed...
hydrogen to partial pressures that were below the detection limit of our assay system (0.1 Pa; Fig. 1). When hydrogen was added again, all strains immediately resumed hydrogen consumption, which indicated that the cultures remained metabolically active.

The hydrogen threshold of Methanobrevibacter strain AMG-1 is about 40% lower than the values reported for Methanobrevibacter arborphilus and Methanobrevibacter smithii (9–10 Pa, Cord-Ruwisch, Seitz and Conrad 1988), but within the range reported for other hydrogenotrophic methanogens (2.5–6.9 Pa; Table 1). The hydrogen thresholds of the three methyl-reducing species, although not determined exactly, are definitely below our detection limit (i.e. < 0.1 Pa). This value is up to one order of magnitude lower than the hydrogen thresholds reported for M. stadtmanae and M. blatticola in the only previous study on this topic. The discrepancy is explained by the higher detection limit of the assay system used by Sprenger, Hackstein and Keltjens (2007), which was at or close to the reported threshold levels.

These hydrogen thresholds are consistent with thermodynamics. Assuming standard conditions for all reactants, the thermodynamic equilibrium of hydrogenotrophic methanogenesis (Equation 1) will be reached at a hydrogen partial pressure of 0.18 Pa (Thauer et al. 2008). In methanogens without cytochromes, which have low growth yields and may gain as little as 0.3 ATP per methane (e.g. Methanobrevibacter arborphilus), energy metabolism and ATP synthesis would be in equilibrium at a hydrogen partial pressure of 1 Pa. Methanogens with cytochromes, however, are far more efficient in energy conservation (1.5 ATP per methane for Methanosarcina barkeri), and their theoretical hydrogen threshold should be more than one order of magnitude higher (Thauer et al. 2008). These considerations are in agreement with the experimental thresholds (Table 1), except for the values reported for Methanobrevibacter bryantii (Karadagli and Rittmann 2007) and Methanoculleus bourgensis (Neubeck et al. 2016), which are close to or even lower than those expected at the thermodynamic equilibrium.

Using the same theoretical framework, we found that also the much lower hydrogen threshold of methylotrophic methanogens matches theoretical expectations. Assuming standard conditions for all reactants but hydrogen, and a phosphorylation potential of 50 kJ per mol ATP (Thauer et al. 2008), methyl-reducing methanogenesis (Equation 2) would be in thermodynamic equilibrium at a hydrogen partial pressure of
1.8 × 10^{−15} \text{ Pa}. At a methanol concentration of 50 mM (experimental conditions), which decreases the free energy change of the reaction to −105.1 kJ mol^{−1}, the theoretical hydrogen threshold of methylotrophic methanogenesis ranges between 3.5 × 10^{−11} \text{ Pa} (at 0.3 ATP per methane) and 0.5 Pa (at 1.5 ATP per methane). Even at a methanol concentration of 10 μM, which decreases the free energy change of the reaction to −84.5 kJ mol^{−1}, a methylotrophic methanogen should still be able to synthesize 1 ATP per methane at a hydrogen partial pressure of 0.1 Pa. It is important to note that due to the reaction stoichiometry, the hydrogen threshold value of methyl-reducing methanogens will be affected more strongly by their actual ATP gain per mol of methane than that of hydrogenotrophic methanogens.

Although all methyl-reducing methanogens studied to date employ the same biochemistry of methane formation, their modes of heterodisulfide reduction and energy conservation differ fundamentally between members of different orders. Like other methanogens without cytochromes, Methanosphaera stadtmanae uses a soluble hydrogenase/heterodisulfide reductase complex (MvhADG/HdrABC) to regenerate the coenzymes and to produce reduced ferredoxin by electron bifurcation; the free energy of the reduced ferredoxin is harvested with an energy-coupling sodium gradient (Thauer et al. 2008). Methanomassiliicoccus blatticola, a methanogen with cytochromes, uses an electron transport chain consisting of a hydrogenase (Ehb complex) in the form of an electrochemical sodium gradient (Thauer et al. 2008). Methanomassiliicoccus blatticola, which lacks both energy-converting hydrogenases and cytochromes, employs a new metabolic model that was previously proposed for Methanomassiliicoccus luminyensis (Gottschling and Deppenmeier 2017). This agrees with the metabolic model that was previously proposed for Methanomassiliicoccales, which predicts the translocation of 3–4 protons per two molecules of methane (Lang et al. 2015). This would yield only about 0.5 ATP per methane, which is in the same range as in hydrogenotrophic methanogens and may allow an even lower hydrogen threshold than in other methyl-reducing species.

While methyl-reducing methanogens compete with hydrogenotrophic methanogens for hydrogen, they compete with methyl-fermenting methanogens for methanol and other methylated substrates. Based on the amount of methane produced, methanol fermentation (Equation 3) yields almost the same amount of free energy as methanol reduction with hydrogen (Equation 2) under standard conditions, but due to reaction stoichiometry, it is more sensitive to changes in methanol concentration.

\[
4\text{CH}_3\text{OH} \rightarrow 3\text{CH}_4 + \text{CO}_2 + 2\text{H}_2\text{O} \\
(\Delta G' = −106.5 \text{ kJ mol}^{−1} \text{ methane}) \quad (3)
\]

At the low methanol concentrations encountered in cockroach guts (10 μM; Sprenger, Hackstein and Keltjens 2007), methanol reduction (\(\Delta G' = −84 \text{ kJ/mol}\)) is considerably more exergonic than methanol fermentation (\(\Delta G' = −67 \text{ kJ/mol}\)).
all other reactants at standard conditions) and remains energetically more favorable at hydrogen partial pressures down to 100 Pa. This matches the substrate affinities for methanol of Methanimicrococcus blatticola and Methanosphaera stadtmanae ($K_h = 5–20 \mu M$), which are much higher than those of Methanosarcina barkeri ($K_h = 180–250 \mu M$), and explains why Methanimicrococcus blatticola dominates the community of methylotrophic methanogens in cockroach guts (Sprenger, Hackstein and Keltjens 2007).

It is intriguing that most methyl-reducing methanogens studied to date (see above) have been enriched or isolated from the intestinal tracts of animals. They are conspicuously abundant in the intestinal tracts of millipedes and insects, the rumen of cows and sheep, and the colon of mammals (e.g. Henderson et al. 2013; Conway de Macario and Macario 2018; Brune 2019)—environments that are characterized by relatively high hydrogen partial pressures. This suggests that at least in these environments, their ecological success may not be based on their competitiveness for hydrogen but on their ability to utilize methyl groups at concentrations that are not accessible to methyl-fermenting methanogens. The obligately methyl-reducing Methanimicrococcus blatticola colonizes the hindgut of cockroaches feeding on pectin (Sprenger, Hackstein and Keltjens 2007), and selective feeding of termites with xylan, another plant cell wall component rich in methyl groups, increases the relative abundance of uncultured, putatively methyl-reducing Methanomassiliicoccales (Miyata et al. 2007). Also, lignin-derived methoxyethylated aromatic compounds are demethylated by the hindgut microbiota of termites (Brune, Miambi and Breznak 1995). The organisms responsible for this activity are not known, but the capacity for this reaction has been demonstrated in the methyl-disproportionating Methanococcus shenglensis (Methanosarcinales), an isolate from coal beds (Mayumi et al. 2016). Another abundant substrate source for methylotrophic methanogens in intestinal environments is methylamines (Poulsen et al. 2013; Gaci et al. 2014).

Methanol and methylamines drive methanogenesis also in organic-rich marine and estuarine sediments, where methanogens are outcompeted for acetate and hydrogen by sulfate reducing bacteria (Oremland and Polcin 1982; Oremland, Marsh and Polcin 1982). Originally, the utilization of such ‘non-competitive’ substrates had been attributed to methyl-fermenting methanotrophs (e.g. Methanosarcina and Methanococcales spp.; Lyimo et al. 2009), but the hydrogen thresholds of sulfate reducers (in the range of 1 Pa; Cord-Ruwisch et al. 1988; Ozuolmez et al. 2015) would permit methyl-reducing methanogens to utilize hydrogen even in the presence of sulfate if the concentration of methyl groups is sufficient. This agrees with the recent finding that metagenome-assembled genomes (MAGs) of uncultured, putatively methyl-reducing Methanomassiliicoccales and Methanofastidiosida represent the most active methanogens in coastal mangrove sediments (Zhang et al. 2020). Further evidence for the presence of putatively methyl-reducing methanogens in other environments (Evans et al. 2015; Nobu et al. 2016; Vanwonterghem et al. 2016; Sorokin et al. 2018; Berghuis et al. 2019; Liu et al. 2020) underscores that (hydrogen-dependent) methylotrophic methanogenesis is widespread and—due to the high affinity for hydrogen—most likely limited by the availability of methyl groups.

ACKNOWLEDGMENTS

We thank Lena Mikulski and Dorothee Tegtmeier for providing Methanobrevibacter strain AMG-1.

FUNDING

This study was supported by a grant of the Deutsche Forschungsgemeinschaft (DFG) in the Collaborative Research Center SFB 987.

Conflict of interest. The authors declare no conflict of interest.

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