Effect of methanogenic substrates on anaerobic oxidation of methane and sulfate reduction by an anaerobic methanotrophic enrichment

Roel J. W. Meulepas · Christian G. Jagersma · Ahmad F. Khadem · Alfons J. M. Stams · Piet N. L. Lens

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Abstract Anaerobic oxidation of methane (AOM) coupled to sulfate reduction (SR) is assumed to be a syntrophic process, in which methanotrophic archaea produce an interspecies electron carrier (IEC), which is subsequently utilized by sulfate-reducing bacteria. In this paper, six methanogenic substrates are tested as candidate-IECs by assessing their effect on AOM and SR by an anaerobic methanotrophic enrichment. The presence of acetate, formate or hydrogen enhanced SR, but did not inhibit AOM, nor did these substrates trigger methanogenesis. Carbon monoxide also enhanced SR but slightly inhibited AOM. Methanol did not enhance SR nor did it inhibit AOM, and methanethiol inhibited both SR and AOM completely. Subsequently, it was calculated at which candidate-IEC concentrations no more Gibbs free energy can be conserved from their production from methane at the applied conditions. These concentrations were at least 1,000 times lower can the final candidate-IEC concentration in the bulk liquid. Therefore, the tested candidate-IECs could not have been produced from methane during the incubations. Hence, acetate, formate, methanol, carbon monoxide, and hydrogen can be excluded as sole IEC in AOM coupled to SR. Methanethiol did inhibit AOM and can therefore not be excluded as IEC by this study.

Keywords Anaerobic oxidation of methane · Interspecies electron carrier · Methanogenic substrates

Introduction

Anaerobic oxidation of methane (AOM) coupled to sulfate reduction (SR) according to Eq. 1, is an important process in the global carbon cycle (Hinrichs and Boetius 2002). The process was discovered during geochemical studies in marine sediments (Martens and Berner 1974; Barnes and Goldberg 1976; Reeburgh 1976).

\[ \text{CH}_4 + \text{SO}_4^{2-} \rightarrow \text{HCO}_3^- + \text{HS}^- + \text{H}_2\text{O} \quad \Delta G^\circ = -16.6 \text{ kJ.mol}^{-1} \]  

(1)

Phylogenetic analysis of AOM-SR sediments identified three novel groups of archaea putative called anaerobic methanotrophs (ANME); ANME-1, ANME-2, and ANME-3. These ANME are distantly related to cultivated methanogenic members from the orders Methanosarcinales and Methanomicrobiales (Hinrichs et al. 1999; Orphan et al. 2002; Knittel et al. 2005; Niemann et al. 2006). Orphan et al. (2001, 2002) showed that cells belonging to ANME-1 and ANME-2 assimilated carbon from methane (CH_4) during AOM. ANME probably mediate a form of reversed methano-
The standard Gibbs free energy changes were obtained from Thauer et al. (1977).

| Candidate-IEC | Potential subconversions in AOM coupled to SR | $\Delta G^\circ$ | $\Delta G^\circ$ |
|--------------|---------------------------------------------|-----------------|-----------------|
| Acetate      | $\text{CH}_4 + \text{HCO}_3^- \rightarrow \text{CH}_3\text{COO}^- + \text{H}_2\text{O}$ | +31 kJ mol$^{-1}$ CH$_4$ | $\Delta G^\circ$ production |
|              | $\text{CH}_3\text{COO}^- + \text{SO}_4^{2-} \rightarrow \text{HS}^- + 2\text{HCO}_3^-$ | -47 kJ mol$^{-1}$ SO$_4^{2-}$ | $\Delta G^\circ$ consumption |
| Formate      | $\text{CH}_4 + 3\text{HCO}_3^- \rightarrow 4\text{HCO}_2^- + \text{H}^+ + \text{H}_2\text{O}$ | +128 kJ mol$^{-1}$ CH$_4$ | $\Delta G^\circ$ production |
|              | $4\text{HCO}_2^- + \text{SO}_4^{2-} + \text{H}^+ \rightarrow \text{HS}^- + 4\text{HCO}_3^-$ | -144 kJ mol$^{-1}$ SO$_4^{2-}$ | $\Delta G^\circ$ consumption |
| Methanol     | $\text{CH}_4 + \text{H}_2\text{CO}_3^- + \frac{1}{3}\text{H}^+ + \frac{1}{3}\text{H}_2\text{O} \rightarrow \frac{4}{3}\text{CH}_3\text{OH}$ | +104 kJ mol$^{-1}$ CH$_4$ | $\Delta G^\circ$ production |
|              | $\frac{4}{3}\text{CH}_3\text{OH} + \text{SO}_4^{2-} \rightarrow \text{HS}^- + \frac{4}{3}\text{HCO}_3^- + \frac{1}{3}\text{H}^+ + \frac{4}{3}\text{H}_2\text{O}$ | -120 kJ mol$^{-1}$ SO$_4^{2-}$ | $\Delta G^\circ$ consumption |
| Carbon monoxide | $\text{CH}_4 + 3\text{HCO}_3^- + 3\text{H}^+ \rightarrow 4\text{CO}^- + \text{H}_2\text{O}$ | +196 kJ mol$^{-1}$ CH$_4$ | $\Delta G^\circ$ production |
|              | $4\text{CO}^- + \text{SO}_4^{2-} + 4\text{H}^+ \rightarrow \text{HS}^- + 4\text{HCO}_3^- + 3\text{H}^+$ | -212 kJ mol$^{-1}$ SO$_4^{2-}$ | $\Delta G^\circ$ consumption |
| Methanethiol | $\text{CH}_4 + \text{H}_2\text{CO}_3^- + \frac{4}{3}\text{H}^+ + \frac{4}{3}\text{HS}^- \rightarrow \frac{4}{3}\text{H}_2\text{CSH} + \text{H}_2\text{O}$ | +55 kJ mol$^{-1}$ CH$_4$ | $\Delta G^\circ$ production |
|              | $\frac{4}{3}\text{H}_2\text{CSH} + \text{SO}_4^{2-} \rightarrow \frac{4}{3}\text{HS}^- + \frac{4}{3}\text{HCO}_3^- + \frac{4}{3}\text{H}^+$ | -71 kJ mol$^{-1}$ SO$_4^{2-}$ | $\Delta G^\circ$ consumption |
| Hydrogen     | $\text{CH}_4 + 3\text{H}_2\text{O} \rightarrow 4\text{H}^+ + \text{HCO}_3^- + \text{H}^+$ | +136 kJ mol$^{-1}$ CH$_4$ | $\Delta G^\circ$ production |
|              | $4\text{H}^+ + \text{SO}_4^{2-} + \text{H}^+ \rightarrow \text{HS}^- + 4\text{H}_2\text{O}$ | -152 kJ mol$^{-1}$ SO$_4^{2-}$ | $\Delta G^\circ$ consumption |

The standard Gibbs free energy changes were obtained from Thauer et al. (1977).
15 °C, sparged with 4.8 L L\(^{-1}\) day\(^{-1}\) pure CH\(_4\) gas and fed with 0.14 L L\(^{-1}\) day\(^{-1}\) marine medium. The basal medium consisted of: NaCl (19.8 g L\(^{-1}\)), KCl (0.45 g L\(^{-1}\)), MgCl\(_2\)-6H\(_2\)O (4.25 g L\(^{-1}\)), NH\(_4\)Cl (0.25 g L\(^{-1}\)), CaCl\(_2\)-2H\(_2\)O (1.19 g L\(^{-1}\)), MgSO\(_4\)-7H\(_2\)O (5.10 g L\(^{-1}\)), KH\(_2\)PO\(_4\) (0.34 g L\(^{-1}\)), K\(_2\)HPO\(_4\)-3H\(_2\)O (1.25 g L\(^{-1}\)), a trace element solution (1 mL L\(^{-1}\)), a vitamin solution (1 mL L\(^{-1}\)), 0.002 to 0.6 mmol L\(^{-1}\) of members of the Deltaproteobacteria and Bacteroidetes (Jagersma et al. 2009). During AOM, carbon derived from CH\(_4\) was incorporated in both archaeal and bacterial lipids (Jagersma et al. 2009).

Incubations with candidate-IECs

Experiments were done in 35-mL serum bottles closed with butyl rubber stoppers and caps. After determining the exact weight and volume, the bottles were eluted with nitrogen gas. Subsequently, 30 mL undiluted reactor suspension was transferred from the bioreactor to the bottles by syringe. The reactor suspension contained 0.59 g volatile suspended solids per liter (a measure for the biomass content). The reactor suspension was homogenized and 1.7 mL min\(^{-1}\) gas sparging (2 L min\(^{-1}\)) was applied prior to and during sampling. Incubations were done in duplicate. However, duplicates could not be repeated due to a limited biomass production. The bottles were incubated at 15 °C and shake in an orbital shaker at 100 rpm. The gas composition, pH, and pressure were determined once or twice a day. The carbon monoxide and hydrogen fraction in the headspace, the sulfate and formate concentration, the dissolved sulfide concentration, and the concentration of fatty acids and alcohols were analyzed immediately after inoculation and after 4 days. Sampling was done at the incubation temperature (15 °C).

Analysis

The headspace composition was measured on a gas chromatograph–mass spectrometer (GC-MS) from Interscience (Breda, The Netherlands). The system was composed of a Trace GC equipped with a GS-GasPro column (30 m by 0.32 mm; J&W Scientific, Folsom, CA, USA), and a Ion-Trap MS. Helium was the carrier gas at a flow rate of 1.7 mL min\(^{-1}\). The column temperature was 30 °C. The fractions of CH\(_4\) and CO\(_2\) in the headspace were derived from the peak areas in the gas chromatograph. The fractions of 13C-labeled CH\(_4\) (13CH\(_4\)) and 13C-labeled CO\(_2\) (13CO\(_2\)) were derived from the mass spectra as done by Shigematsu et al. (2004). The headspace pressure, sulfate concentration, and pH were analyzed as described by Meulepas et al. (2009). The sulfate concentration was analyzed according to Sipma et al. (2004), and the acetate and methanol concentration were analyzed according to Weimja et al. (2000)

Calculations

The 13C-dissolved inorganic carbon (13C-DIC, 13C-labeled CO\(_2\), and 13C-labeled bicarbonate) and 12C-DIC per bottle were calculated according to the equation given by Meulepas et al. (2009)

The concentration of each candidate-IEC at which no more energy can be obtained (Δ\(_{IEC}\)production=0) from their production from CH\(_4\) (Table 1) was calculated. This is done according to Eq. 2.

\[
Δ\(_{IEC}\)production = Δ\(_{IEC}\)production + R T \ln \left( \frac{\prod_{products} n_{product}}{\prod_{substrates} n_{substrate}} \right)
\]
Results

Incubations

The $^{13}$C-DIC production is taken as a measure for $^{13}$CH$_4$ oxidation because the percentage $^{13}$C in DIC produced from other (not $^{13}$C-enriched) sources is only around 1.1%, while the $^{12}$C-DIC production (Fig. 2a) did not even exceed the $^{13}$C-DIC production (Fig. 1) in any of the incubations. The sulfide production was taken as a measure for SR, which was in all incubations coupled to the sulfate removal (Fig. 2b).

Fig. 1 $^{13}$C-DIC production in time, during 4-day batch incubations, in the absence (control) or in the presence of one candidate-IEC. The bottles contained 30 mL biomass suspension from the enrichment bioreactor and initially 0.16 ($\pm$0.01) MPa $^{13}$CH$_4$, 15 ($\pm$1) mM sulfate and 0.2 ($\pm$0.1) mM sulfide.

Fig. 2 a $^{13}$Carbon, b sulfur, and c reduction equivalent balances over 4-day batch incubations in the absence (control) or in the presence of one candidate-IEC. The 35-mL bottles contained 30 mL biomass suspension from the enrichment bioreactor and initially 0.16 ($\pm$0.01) MPa $^{13}$CH$_4$, 15 ($\pm$1) mM sulfate, and 0.2 ($\pm$0.1) mM sulfide.
In the absence of candidate-IECs (controls), there was a linear accumulation of $^{13}$C-DIC during the 4-day incubation (Fig. 1); this $^{13}$CH$_4$ oxidation was coupled to SR (Fig. 2c), according to the stoichiometry of Eq. 1.

In the presence of acetate, formate, methanol, or hydrogen, there was also a linear accumulation of $^{13}$C-DIC (Fig. 1), the rates (3.9–5.7 µmol bottle$^{-1}$ day$^{-1}$) were comparable with the rates without a candidate-IEC (3.9 and 4.2 µmol bottle$^{-1}$ day$^{-1}$). In the incubations with carbon monoxide, the $^{13}$C-DIC production rate was slightly lower (2.7 and 3.0 µmol bottle$^{-1}$ day$^{-1}$) and methanethiol completely inhibited $^{13}$C-DIC production.

Some acetate (8.8 and 8.3 µmol-C), formate (8.0 and 8.4 µmol-C), and carbon monoxide (9.1 and 10.8 µmol-C) were being removed during the 4-day incubation. Figure 2a shows that the removal of these candidate-IECs was coupled to $^{12}$C-DIC production and not $^{13}$CH$_4$ production, indicating complete oxidation. Methanol (1.0 and 1.0 µmol-C bottle$^{-1}$) and methanethiol (0.4 µmol-C bottle$^{-1}$) were hardly removed. All incubations showed some (up to 2.6 µmol bottle$^{-1}$) background $^{13}$C-DIC production, likely released from the inoculate.

Figure 2c compares oxidation conversions with reduction conversions. In the presence of acetate, formate, carbon monoxide, and hydrogen more sulfate was reduced than $^{13}$CH$_4$ oxidized. This additional SR was coupled to the oxidation of candidate-IECs. Therefore, CH$_4$, acetate, formate, carbon monoxide, and hydrogen were all used as electron donor for SR by the enrichment, although the oxidation of $^{13}$CH$_4$ was, in all incubations, dominant over the oxidation of candidate-IECs. Both SR and $^{13}$CH$_4$ oxidation were inhibited by the presence of methanethiol.

**Thermodynamic calculations**

Table 2 presents the concentrations of candidate-IECs at which their production, under the applied experimental conditions, is no longer thermodynamically possible. To obtain maximum concentrations, the highest measured CH$_4$ partial pressure (0.16 MPa), HS$^-$ concentration (1 mM) and HCO$_3^-$ concentrations (1 mM) were used for the calculations. Only for the calculation of the maximum H$_2$ partial pressure, the lowest HCO$_3^-$ concentration (0.2 mM) was used. The theoretical maximum concentration for the production of each candidate-IEC was always at least 1,000 times lower than the actual concentration measured at the end of the experiment.

**Discussion**

**Exclusion of candidate-IECs**

This research shows that acetate, formate, methanol, carbon monoxide, and hydrogen can be excluded as sole IEC in AOM coupled to SR by an enrichment composed of ANME-2a and bacteria mainly belonging to *Deltaproteobacteria* and *Flavobacteriales*. The $^{13}$CH$_4$ oxidation rates in the presence of these compounds were not or hardly lower than in the controls (Fig. 1). Moreover, during the 4-day incubations, the concentrations of these candidate-IECs were at least 1,000× higher than the candidate-IEC concentrations at which no more Gibbs free energy can be conserved from their production from CH$_4$ at the applied conditions (Table 2). Nauhaus et al. (2002, 2005) already showed that acetate, formate, methanol, carbon monoxide, methylamines, and hydrogen are unlikely IECs in AOM coupled to SR by the ANME-2/bacteria community in Hydrate Ridge sediment because the SR activity on those compounds was lower than on CH$_4$. In Black Sea microbial mats, the SR activity with acetate was also lower than with methane, but with hydrogen and formate this was not the case, which was likely due to the rapid enrichment of SRB not involved in AOM (Nauhaus et al. 2005). Possibly the carbon monoxide concentration (10 kPa CO) used by Nauhaus et al. (2005) was inhibitory for sulfate reduction or the candidate-IECs were consumed by methanogens or homoacetogens. By also assessing the $^{13}$CH$_4$ oxidation rate (Fig. 1), the $^{13}$CH$_4$ production (Fig. 2a), $^{12}$C-DIC production (Fig. 2a), and the candidate-IEC consumption (Fig. 2a and c), those possibilities can be excluded in this study for acetate, formate, methanol, carbon monoxide, and hydrogen.

**Inhibition by carbon monoxide and methanethiol**

$^{13}$CH$_4$ oxidation was slightly hampered by carbon monoxide and completely inhibited by methanethiol (Fig. 1), both

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**Table 2** The concentration of candidate interspecies electron carriers (IECs) at which their production from CH$_4$ is no longer thermodynamically possible ($\Delta G^\prime=0$) at 0.16 MPa CH$_4$, 0.2 mM HCO$_3^-$ (for H$_2$) or 1 mM HCO$_3^-$ (of the other potential-IECs), 1 mM HS$^-$, 30 °C, and a pH of 7.

| IEC              | IEC concentration at which $\Delta G_{IEC \text{ production}}=0$ | IEC concentration on day 4       |
|------------------|-------------------------------------------------|----------------------------------|
| Acetate          | $3.8 \times 10^{-9}$ M                           | $8.5 \times 10^{-4}$, $8.6 \times 10^{-4}$ M |
| Formate          | $1.0 \times 10^{-8}$ M                           | $7.3 \times 10^{-4}$, $7.2 \times 10^{-4}$ M |
| Methanol         | $1.8 \times 10^{-15}$ M                          | $9.3 \times 10^{-4}$, $9.3 \times 10^{-4}$ M |
| Carbon monoxide  | $8.4 \times 10^{-10}$ kPa                        | $4.9$, $4.4$ kPa                 |
| Methanethiol     | $8.4 \times 10^{-12}$ M                          | $9.9 \times 10^{-4}$ M           |
| Hydrogen         | $6.6 \times 10^{-4}$ kPa                         | $3.2$ kPa                        |
compounds have been reported to be toxic. Carbon monoxide hampered SR by sulfate-reducing sludge at a concentration of 5% or higher (van Houten et al. 1995), and sulfate reducers used only methyl sulfides as substrate at low concentrations (<10 µM; Kiene et al. 1986). If these compounds would be produced in situ, the concentrations would remain much lower due to simultaneous consumption, therefore toxic effects would be less profound. Because methanethiol inhibited AOM, it can therefore not be excluded as IEC in AOM coupled to SR. Moran et al. (2007) also reported an inhibition of AOM by methanethiol. If electrons would be transferred via methanethiol, sulfate reducers would be able to utilize these compounds; however that did not occur (Fig. 2b). Possibly, the sulfate reducers were intoxicated by methanethiol.

Local concentration of candidate-IECs

Many of the candidate-IECs tested were consumed (Fig. 2a, c), which can result in a concentration gradient within the biomass flocks. Therefore, the concentration near the responsible organism can be lower than in the bulk liquid. A big difference between the concentration in the bulk liquid at the concentration near the organism mediating AOM is not expected though because the reactor suspension was well mixed (orbital shaker at 100 rpm), the biomass flocks were small (0.1 mm; Meulepas et al. 2009), and the candidate-IEC consumption rates were low (<4 µmol bottle−1 day−1).

Syntrophy between ANME and SRB

The reason that the addition of a candidate-IEC does not affect AOM and SR might be the involvement of more than one intermediate in AOM coupled to SR (Valentine and Reeburgh 2000; Stams and Plugge 2009). Other theories for the shuttling of electrons between ANME and SRB are that reduction equivalents are transferred via extracellular redox shuttles (Widdel and Rabus 2001; Wegener et al. 2008) or via membrane-bound redox shuttles or so-called “nanowires” (Reguera et al. 2005; Stams et al. 2006; Stams and Plugge 2009; Thauer and Shima 2008; Wegener et al. 2008). The extracellular redox shuttle theory requires the shuttle to be transported back to the ANME after donating the electrons to the SRB, giving rise to an additional loss in Gibbs free energy change available for the microorganisms, due to the concentration gradients between the syntrophic partners. The membrane-bound redox shuttles require the ANME and SRB to make direct physical contact, which is not always the case (Michaelis et al. 2002; Knittel et al. 2005; Orphan et al. 2002; Treude et al. 2005; Jagersma et al. 2009). However, Nielsen et al. (2010) showed that electrical currents in marine sediments coupled spatially separated biogeochemical processes, presumably trough nanowires. Such mechanism might also be responsible for interspecies electron transfer in AOM coupled to SR.

At in situ conditions there is only −22.35 kJ mol−1 available for AOM coupled to SR (Harder 1997). Methanogenic archaea and sulfate-reducing bacteria have been shown to require a free energy change under physiological conditions of at least −10 and −19 kJ mol−1, respectively, to support their metabolism in situ (Hoehler et al. 2001; Dale et al. 2006). Therefore, the in situ free energy change of AOM coupled to SR is probably not sufficiently large to fuel the energy metabolism of two microorganisms in tandem (Schink 1997; Thauer and Shima 2008). Further research should also consider the possibility that one microorganism is responsible for AOM coupled to SR.

Alternative electron donors

The enrichment was able to utilize acetate, formate, methanol, carbon monoxide, and hydrogen as electron donor for SR (Fig. 2c), although the enrichment was not fed with any other electron donor and carbon source than CH4 for 512 days (Meulepas et al. 2009). Prior to this, the enrichment was additionally fed with small amounts of acetate (70 µmol L−1 day−1) for a period of 330 days. Possibly, the sulfate reducers involved in AOM coupled to SR are capable of utilizing acetate, formate, methanol, carbon monoxide, and hydrogen as alternative electron donors for the IEC or CH4. If this would be the case, those microorganisms could be enriched on those alternative substrates instead of on CH4. Another explanation is that other SRB, not involved in AOM coupled to SR, survived the enrichment period. This hypothesis would require inactive SRB to become active within the 4-day duration of the experiment, which is a rather short time span.

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