Methanol utilization in defined mixed cultures of thermophilic anaerobes in the presence of sulfate

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Abstract

We studied thermophilic sulfate reduction with methanol as electron donor in continuous cultures. Mixed cultures of selected microorganisms were used, representing different methanol degrading pathways followed by various trophic groups of microorganisms. Our results show that direct competition for methanol between a homoacetogen, *Moorella thermoautotrophica*, and a sulfate reducer, *Desulfotomaculum kuznetsovii*, is in favour of the sulfate reducer due to its affinity for methanol. Methanogenesis as a result of interspecies hydrogen transfer between *D. kuznetsovii* and a hydrogen-consuming methanogenic archaeon, *Methanothermobacter thermoautotrophicus*, occurred only below 5 mM total sulfide. A similar result was obtained when *M. thermoautotrophica* was grown on methanol in the presence of *Mb. thermoautotrophicus*. Interestingly, *D. kuznetsovii* could coexist with a non-methanol-utilizing sulfate reducer (*Thermodesulfovibrio* species). Our data show that it is possible to maintain a dominant sulfate-reducing process with methanol as electron donor at 60 °C in mixed continuous cultures.

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1. Introduction

Methanol is a suitable electron donor for the removal of inorganic sulfur compounds (SO_2 and SO_3) from flue-gases (e.g., of coal-fired electricity-generating plants) in anaerobic bioreactors operated at 60 °C or higher [1]. In reactors with sludge composed of different types of microorganisms, sulfate was converted to sulfide, which can be used for the chemical or biological production of elemental sulfur [2]. However, under certain conditions part of the methanol was converted to methane or acetate. Clearly, a better understanding of the processes in the sludge and of the interactions among the involved microorganisms is needed. Theoretically, in a sulfidogenic process, methanol might be used directly as electron donor by sulfate-reducing bacteria, or indirectly via the combined activities of microorganisms. Moderately thermophilic sulfate-reducing bacteria that can utilize methanol as electron donor have been described [1–4]. Thermophilic homoacetogens can use methanol in the presence of CO_2 for the production of acetate or butyrate. Direct methanogenesis from methanol has not been observed at temperatures of 60 °C or higher. However, methanogens might play a role as hydrogen scavengers in a syntrophic association with methanol utilizers. The conversion of methanol to CO_2 coupled to sulfate reduction is energetically a more favourable process than the production of methane or acetate from methanol. In the absence of an external electron acceptor, the formation of methane is more favourable than acetate formation. The conversion of methanol to H_2/CO_2 is only energetically favourable at low hydrogen concentrations and therefore depends upon hydrogen utilization by methanogens or sulfate reducers (for Gibbs free energy yields per reaction, see Table 1). Little is known about
thermophilic syntrophic methanol degradation. Only two thermophilic sulfidogenic syntrophic associations with methanol have been described and partially characterized [5–8]. Davidova and Stams [5] enriched a stable thermophilic sulfate-reducing culture from granular sludge of a methanogenic bench-scale reactor. Methanol was degraded syntrophically to acetate, H2/CO2, and formate by a homoeotetogen. The isolated syntrophic sulfate reducer was unable to use methanol, but could use hydrogen and formate as growth substrates. This strain was not fully characterized, but will be referred to as “Thermodesulfovibrio species” in this paper. Another syntrophic association with methanol was obtained from a sulfidogenic expanded granular sludge bed reactor (EGSB) operated with methanol at 65 °C [6]. In this reactor, methanol degrading Desulfotomaculum species were present, but part of the methanol was utilized by other species. One methanol degrader was characterized as a Thermotoga species, Thermotoga lettingae [7]. This strain degrades methanol slowly, but degradation rates increased by the addition of thiosulfate or a methanogen. T. lettingae was also able to grow on methanol in coculture with Thermodesulfovibrio yellowstonii. Another methanol degrading strain isolated from this reactor was characterized as Moorella mulderi, a novel species closely related to Moorella thermoacetica [8].

Here, we report on the possible interactions in defined cocultures of the methanol-utilizing thermophilic sulfate reducer Desulfotomaculum kuznetsovii, the methanol-utilizing methanogen Moorella thermoacetica, the hydrogenotrophic methanogen Methanothermobacter thermoautotrophicus and a hydrogenotrophic sulfate-reducing Thermodesulfovibrio species.

2. Materials and methods

2.1. Bacterial strains

Desulfotomaculum kuznetsovii (DSM 6115) and Moorella thermoacetica (DSM 1974) were obtained from the Deutsche Sammlung für Mikroorganismen (DSMZ, Braunschweig, Germany). Methanothermobacter thermoautotrophicus (formerly Methanobacterium thermoautotrophicum ΔH) (DSM 1053) was kindly provided by J.T. Keltjens (University of Nijmegen, The Netherlands). The Thermodesulfovibrio species originated from an enrichment culture of Davidova and Stams [5]. Electron micrograph pictures of this strain showed a gram-negative cell wall, a vibrio shape, and a polar flagellum. A partial 16S rRNA gene sequence (750 bp) showed a 99% sequence similarity with Thermodesulfovibrio TGE-P1 (seq. Accession No.: AB021302, Sekiguchi and Kamagata, 1999), a strain that is not described further.

2.2. Media and cultivation

We used a bicarbonate-buffered basal medium containing (g/l): NaCl (7), NaHCO3 (4), Na2SO4 (2.8), MgCl2·6H2O (1.2), KCl (0.5), NH4Cl (0.3), KH2PO4 (0.2), CaCl2 (0.15), Na2S·7·H2O (0.3). Additions were made from anoxic stock solutions and per liter of medium, we added: 0.5 ml vitamin solution according to Stams et al. [9], 1 g yeast extract (if required), 1 ml trace-element solution SL 6 according to Pfennig and Lippert [10], and 2 mM acetate as carbon source (if required). Filter-sterilized methanol (20 mM, final concentration, or 30 mM in sulfate-limiting experiments) was added from anoxic stock solution. The pH was adjusted to 7.0 with NaOH (if necessary).

In batch incubations, 120-ml bottles were used, with 50 ml medium. Prior to inoculation the gas phase in the headspace was replaced by N2/CO2 (80%/20%). Bottles were closed with butylrubber stoppers and aluminum caps. In all experiments anaerobic cultivation techniques were used.

2.3. Continuous culture experiment

Continuous culture experiments were carried out in a 2.5 l (total volume) Bioflow III fermentor (New Brunswick Sc., Edison, NJ) with a 1:1 working volume. The reactor, pH probes, and all associated tubing and drip tubes were autoclaved for 30 min at 121 °C prior to use. All tubing connections were made with glass connectors (male–female), and all parts that needed to be connected after autoclaving were wrapped in aluminum foil to maintain sterility. Tubings in the vessel, the headplate and the vessel bottom were made of high-grade stainless steel (RMS). All tubings outside the vessel were made of butylrubber or marprene. During the fermentation the working volume was maintained with a tube at a fixed height, providing overflow of the culture medium in a sterilized 20 l effluent vessel, thereby maintaining constant level. The culture was stirred at 100 rpm during operation and a New Brunswick Sc. (Edison, NJ) motor.
and speed controller were used to maintain the speed of the stainless steel impeller. Temperature was maintained at 60 °C by constantly circulating water through the round-bottomed-water-jacketed vessel. The pH was maintained at 7.5 by addition of 0.1 M sodium hydroxide to the fermentation by a single speed peristaltic pump (Watson-Marlow Bredel, Falmouth, UK). Autoclavable pH probes (Ingold, Mettler Toledo AG, Urdorf, Switzerland) suitable for applications with high sulfide concentrations were connected through the headplate. Culture medium was pumped through a drip tube connected to the headplate to prevent back-growth in the culture medium. Watson-Marlow pumps (Watson Marlow Bredel, Falmouth, UK) were used to control the substrate flow rates. The feed carboy was maintained under slight overpressure with N2/CO2 that had passed through a sterilized membrane filter. Samples for analyses were taken with nitrogen-flushed syringes through a butylrubber septum in the headspace. Cultures were supposed to be in a steady state after five volume changes. After every experiment with \textit{D. kuznetsovii} all butylrubber tubings, filters and Teflon parts were replaced.

2.4. Analytical procedures

Culture optical density was determined in a Starcoll colorimeter (R&D Mechatronics) at a wavelength of 660 nm. All optical density measurements were made immediately after sampling and samples were diluted in medium if necessary. Cells in culture samples were counted using a Bürker–Türk counting chamber. Protein concentration was determined according to Bradford [11]. Methanol, methane, and fatty acids were analyzed with GC as described previously [12]. Sulfide was determined colorimetrically using the methylene blue method [13].

3. Results

3.1. Direct competition for methanol by the sulfate reducer \textit{D. kuznetsovii} and the homoacetogen \textit{M. thermoautotrophica} (reactions 1 and 3, Table 1)

We have chosen \textit{M. thermoautotrophica}, one of the best-characterized thermophilic methanol-degrading homoacetogens, for mixed culture experiments with \textit{D. kuznetsovii}. Its $\mu_{\text{max}}$ on methanol is $0.77 \, \text{h}^{-1}$ [14], while the $\mu_{\text{max}}$ of \textit{D. kuznetsovii} on methanol is $0.33 \, \text{h}^{-1}$.

Cells of \textit{D. kuznetsovii}, obtained from batch cultures grown on methanol/sulfate/yeast extract, concentrated 10-fold by centrifugation, washed in medium, and pre-incubated at 60 °C did not convert methanol, despite the use of strict anaerobic techniques throughout the manipulations. Temperature changes during the procedure were inevitable and might have inactivated the cells. Therefore, methanol conversion kinetics of \textit{D. kuznetsovii} was determined in a methanol-limited continuous culture. At steady state ($D = 0.022 \, \text{h}^{-1}$) the methanol concentration was below the detection limit (100 μM). The medium flow was stopped when steady state was reached and methanol (20 mM) was added. Product formation and substrate consumption were monitored with time (Fig. 1). Methanol consumption occurred at a rate of approximately 170 nmol min$^{-1}$ mg protein$^{-1}$. The apparent $K_m$ value for \textit{D. kuznetsovii} was approximately 1 mM as calculated from the substrate depletion curve.

Due to contamination of our fermentors with extremely heat-resistant \textit{D. kuznetsovii} spores, we were not able to grow \textit{M. thermoautotrophica} as a pure culture in the chemostat. When \textit{D. kuznetsovii} and \textit{M. thermoautotrophica} were grown together in a methanol-limited continuous culture, the competition for methanol resulted in a dominancy of the sulfate-reducing process (Fig. 2). A fermentor was inoculated with 4% of fresh cultures from both microorganisms, pre-grown on methanol. After a batch phase of 88 hours, medium supply was turned on. A few millimoles (mM) acetate were produced, but at steady state no acetate was detectable anymore. Low concentrations of methanol (0.6 mM) were still found, maybe due to fluctuations in the sulfide concentration. Microscopic examination of the culture revealed that \textit{D. kuznetsovii} was the dominant microorganism in the fermentor. In batch cultures, growth on acetate by \textit{D. kuznetsovii} is poor, yielding cultures with lower optical densities compared to the mixed culture (results not shown). These observations strongly suggest that sulfate reduction occurred directly from methanol and not indirectly via acetate. Sulfide toxicity experiments in batch culture revealed that sulfide did not inhibit growth of both organisms at concentrations below 10 mM. Because the sulfide concentration in the fermentor was kept below 10 mM, and because the culture was methanol-limited, the

![Fig. 1. Methanol consumption in a steady state culture of \textit{D. kuznetsovii}. Symbols: \textbullet, methanol; \textblacksquare, sulfide.](image-url)
affinity for methanol was considered to be the crucial parameter in the competition.

3.2. Interaction between D. kuznetsovii and Mb. thermoautotrophicus (reactions 1, 5, and 9, Table 1)

In a sulfate-limited coculture with sulfide concentrations below 5 mM, as well as in a methanol-limited coculture of D. kuznetsovii and Mb. thermoautotrophicus methane formation was observed, likely due to interspecies hydrogen transfer. A sulfate-limited culture of D. kuznetsovii was inoculated at \(t = 0\) with 10% of a culture of Mb. thermoautotrophicus pregrown on H\(_2\)/CO\(_2\) (Fig. 3(a)). When the sulfide concentration was kept high (12 mM) no methane was produced. However, when the sulfide concentration decreased due to sparging with N\(_2\)/CO\(_2\) (from \(t = 7\)), methane was produced as soon as the sulfide concentration was lowered to 5 mM. In a methanol-limited culture (Fig. 3(b)) the methanogen still pushed the sulfate reducer to hydrogen transfer, even if methanol concentrations were below 0.5 mM. Mb. thermoautotrophicus is unable to use methanol and can produce methane only with H\(_2\)/CO\(_2\). However, when high sulfide concentrations (above 10 mM) were applied, no methane was produced anymore. The toxicity of sulfide seems to be an important parameter to suppress methane production. Optimal sulfide concentration for this strain of Mb. thermoautotrophicus was reported to be 1–2 mM [15]; high concentrations are known to be inhibitory although detailed quantitative data are scarce. To examine whether methane production could be suppressed irreversibly, sulfide pulses were applied to a sulfate-limited steady state coculture of D. kuznetsovii and Mb. thermoautotrophicus (Fig. 3(c)). We observed that after an extra supply of sulfide to a concentration of 10 mM, methane production did not return, whereas after a sulfide pulse resulting in a sulfide concentration of 7 mM, methane production returned to the steady state level within 5 h.

3.3. Interaction between D. kuznetsovii and the Thermo-desulfovibrio species (reactions 1, 5, and 6, Table 1)

A stable, mixed culture of two sulfate reducers was achieved under methanol limitation with sulfide concentrations up to 12 mM. Although D. kuznetsovii was
the dominant organism in this culture during a steady state, the Thermodesulfovibrio species cells remained at a constant low level (results not shown).

3.4. Interaction between M. thermoautotrophica and the Thermodesulfovibrio species (reactions 3, 5, and 6, Table 1)

Because we were not able to grow M. thermoautotrophica without D. kuznetsovii in a chemostat, we performed batch culture experiments to gain more insight into the possibility of methanol being used for sulfidogenesis via interspecies hydrogen transfer. We added 20 mM sulfate to an exponentially growing aceticogenic culture (20 mM methanol) of M. thermoautotrophica. After incubation with the Thermodesulfovibrio species, 7 mM sulfide was produced. In a control experiment with M. thermoautotrophica growing on methanol and sulfate, sulfide was not produced and acetogenesis continued until the substrate was converted completely. Sulfide production in the coculture must thus be attributed to activity of the Thermodesulfovibrio species growing via interspecies hydrogen transfer with M. thermoautotrophica.

3.5. Competition for hydrogen – D. kuznetsovii, Mb. thermoautotrophicus, and the Thermodesulfovibrio species (reactions 3, 5, 6, and 9, Table 1)

Coculture of D. kuznetsovii and Mb. thermoautotrophicus was grown under sulfate limitation and with a constant sulfide concentration of 5 mM. Mb. thermoautotrophicus produced 3 mM methane due to interspecies hydrogen transfer. After the steady state has been achieved, the coculture was inoculated with the Thermodesulfovibrio species at t = 2. Methane production ceased and Thermodesulfovibrio cells were detectable in the culture at constant low level after 12 hours of growth (Fig. 4). Both at low and high sulfide concentrations (12 mM), a stable sulfidogenic culture of two sulfate reducers was obtained. These observations were confirmed in batch culture experiments. A methanogenic culture of Mb. thermoautotrophicus growing on H2/CO2 inoculated with a Thermodesulfovibrio species culture growing on H2/CO2 produced 8 mM sulfide and no more methane.

4. Discussion

The direct competition for methanol during methanol limitation between D. kuznetsovii and M. thermoautotrophica resulted in a dominance of the sulfate reducer. If we assume the ratio \( \mu_{\text{max}}/K_s \) to be a useful parameter for comparing growth properties of microorganisms on a common substrate, this ratio should be higher for D. kuznetsovii than for M. thermoautotrophica. The \( \mu_{\text{max}} \) on methanol of M. thermoautotrophica is almost twice as high as the \( \mu_{\text{max}} \) of D. kuznetsovii. Thus, the half-saturation constant for methanol of M. thermoautotrophica may be assumed to be higher than 2 mM. At high methanol concentrations, the outcome of the competition might be reverse, due to the higher specific growth rate on methanol of M. thermoautotrophica.

Mixed growth of D. kuznetsovii and Mb. thermoautotrophicus suggests a central role for hydrogen in the transfer of reducing equivalents, as the methanogen uses only hydrogen as energy substrate. Hydrogen transfer to the Thermodesulfovibrio species was more pronounced than to the methanogen Mb. thermoautotrophicus, probably due to differences in affinity and half-saturation constants \( (K_s) \) for hydrogen, and sulfide toxicity. This has also been observed in disintegrated granules from moderately thermophilic upflow anaerobic sludge blanket reactors (UASB) [16]. The addition of hydrogen-utilizing sulfate-reducing bacteria (Desulfovomaculum species) resulted in a higher degradation rate of volatile fatty acids in the granules than the addition of comparable numbers of hydrogen utilizing methanogens (Mb. thermoautotrophicus) [17].

Sulfide concentrations as low as 3.3 mM are already inhibitory for some Desulfovomaculum species [18]. However, for sulfate-reducing bacteria sulfide inhibition may be reversible [19]: complete inhibition for a sulfate-reducing bacterium occurred at a sulfide concentration as high as 16.1 mM [20]. In our experiments with D. kuznetsovii concentrations up to 15 mM sulfide had little effect on the specific growth rate (results not shown). A decrease of 20% of the specific growth rate was observed at a sulfide concentration of 20 mM. On the contrary, our experiments showed that sulfide toxicity for methanogenic archaea could be irreversible depending on the concentration. Uncoupling of growth and activity was not observed for Mb. thermoautotrophicus. Visser [16] showed that a 50% decrease in activity was observed for hydrogenotrophic methanogenesis in methanogenic sludge granules at a concentration of 10 mM sulfide. The high sulfide concentrations (>30 mM) applied in

Fig. 4. Sulfate-limited culture of D. kuznetsovii, Mb. thermoautotrophicus, and the Thermodesulfovibrio species at low sulfide concentration. Symbols: \( \bullet \), methanol; \( \square \), sulfide; \( \Delta \), methane; \( * \), D. kuznetsovii; \( \bigcirc \), Thermodesulfovibrio species.

The coexistence of two sulfate reducers in a stable methanol-limited sulfidogenic culture can be explained by differences in substrate affinity. *D. kuznetsovii* and the *Thermodesulfovibrio* species can both grow on H2/CO2 and/or formate. No yeast extract was present in the growth medium, thus growth of the *Thermodesulfovibrio* species was due to an interaction with *D. kuznetsovii*. Competition for H2/CO2 and/or formate must be in favour of the *Thermodesulfovibrio* species apparently due to higher affinity of the *Thermodesulfovibrio* species for H2/CO2 and/or formate compared to *D. kuznetsovii*.

Methylothrophic growth of *D. kuznetsovii* in mixed cultures with *Mh. thermoautotrophicus* and the *Thermodesulfovibrio* species is in favour of the hydrogenotrophic *Thermodesulfovibrio* species. Experiments were performed at sulfide concentrations that are not inhibitory to the methanogen, indicating that the affinity for hydrogen but not the toxicity of sulfide is the dominant factor in the competition for hydrogen. Threshold values for hydrogen in sediments are assumed to be lower for sulfate reducers than for methanogens [6].

From our experiments it is clear that sulfate reduction with methanol at 60 °C in defined mixed cultures of different trophic organisms can be easily maintained. Moreover, competition for substrates between different sulfate reducers and interspecies hydrogen transfer between different sulfate reducers may be more important in anaerobic environments than has been recognized hitherto.

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**References**


