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Authors

Valentine, DL
Blanton, DC
Reeburgh, WS

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David L. Valentine · Douglas C. Blanton
William S. Reeceburg

Hydrogen production by methanogens under low-hydrogen conditions

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Abstract Hydrogen production was studied in four species of methanogens (*Methanothermobacter marburgensis*, *Methanosaeta thermophila*, *Methanosarcina barkeri*, and *Methanosaeta concilii*) under conditions of low (sub-nanomolar) ambient hydrogen concentration using a specially designed culture apparatus. Transient hydrogen production was observed and quantified for each species studied. Methane was excluded as the electron source, as was all organic material added during growth of the cultures (acetate, yeast extract, peptone). Hydrogen production showed a strong temperature dependence, and production ceased at temperatures below the growth range of the organisms. Addition of polysulfides to the cultures greatly decreased hydrogen production. The addition of bromoethanesulfonic acid had little influence on hydrogen production. These experiments demonstrate that some methanogens produce excess reducing equivalents during growth and convert them to hydrogen when the ambient hydrogen concentration becomes low. The lack of sustained hydrogen production by the cultures in the presence of methane provides evidence against “reverse methanogenesis” as the mechanism for anaerobic methane oxidation.

Keywords Methanogens · Hydrogen production · Storage compounds · Anaerobic methane oxidation

Introduction

Hydrogen (H_2) is an important intermediate in the microbially-dominated degradation of organic material in anoxic environments (Schink 1997; Wolin 1982). The ambient H_2 concentration is dynamically controlled and is generally indicative of the dominant terminal electron-accepting process. In general, organisms that respire NO_3^- , Fe(III), or Mn(IV) are able to outcompete sulfate-reducing bacteria for H_2 , which in turn outcompete methanogens (Conrad and Wetter 1990; Cord-Ruwisch et al. 1988; Hoehler et al. 1998; Lovley and Phillips 1987; Lovley and Goodwin 1988). Hydrogen concentration generally varies by orders of magnitude between such different redox environments, with the highest H_2 levels present in methanogenic environments.

Anaerobes commonly experience changes in their redox environment, with corresponding changes in the ambient H_2 level. In nature, different terminal electron-accepting processes occur in close proximity to each other, both spatially and temporally; both microbial transport and changes in environmental conditions can lead to changes in the redox condition for a given organism. While some anaerobes employ locomotion to deal with such changes, other anaerobes are known to have developed long-term metabolic strategies. Some homoacetogens, for example, are able to reverse their metabolism and convert acetate to CO_2 and H_2 when H_2 levels become too low for homoacetogenic growth (Lee and Zinder 1988). Some sulfate-reducing bacteria are capable of switching to H_2 production and can act as syntrophs under conditions of low sulfate and low H_2 (Bryant et al. 1977). Several methanogens can use non-competitive substrates such as methylamines that may sustain growth even in environments where sulfate reducers scavenge H_2 and acetate (Oremland and Polcin 1982). It has also been proposed that methanogens are capable of reversing their metabolism under low H_2 (i.e., sulfate-reducing conditions) to convert CH_4 to CO_2 and H_2 syntrophically, a process referred to as reverse methanogenesis (Hoehler et al. 1994).

D. L. Valentine (✉) · D. C. Blanton · W. S. Reeceburg
Department of Earth System Science, University of California,
Irvine, 220 Rowland Hall, Irvine, CA 92679-3100, USA
e-mail: davalentine@ucsd.edu,
Tel.: +1-858-8224765, Fax: +1-858-5347313

Present address:

D. L. Valentine
Scripps Institution of Oceanography,
Marine Biology Research Division, Hubbs Hall Room 4305,
8750 Biological Grade, La Jolla, CA 92037, USA

This study was conducted to test the reverse methanogenesis hypothesis for anaerobic methane oxidation by subjecting known methanogens to low- H_2 conditions. This work focuses on observations of H_2 production during these experiments.

Several studies have considered H_2 production and consumption by methanogens during active methanogenesis (Boone and Mah 1987; Boone et al. 1987; Lovley 1985; Zinder and Anguish 1992), during fermentation of pyruvate (Bock and Schönheit 1995), and during the equilibration of formate and H_2 (Wu et al. 1993). However, despite the environmental relevance of low- H_2 settings, few studies have quantified metabolic changes in methanogens transferred from a growth-supporting environment to an H_2 -depleted environment. The present study considers H_2 production from several methanogens grown in batch culture (precultures) with abundant substrate, and subsequently transferred to a low- H_2 environment. Particular emphasis is placed on determining whether methanogenesis can be reversed by providing cultures with CH_4 while maintaining low levels of H_2 .

Materials and methods

Organisms and growth conditions

Four different species of methanogens were utilized in these studies: *Methanothermobacter marburgensis* (OCM 82, formerly referred to as *Methanobacterium thermautotrophicum* strain Marburg, see Wasserfallen et al. 2000), supplied by D. Boone; *Methanosarcina barkeri* strain 227 (OCM 35), also supplied by D. Boone; *Methanosaeta thermophila* strain CALS-1 (DSMZ 3870), supplied by S. Zinder; and *Methanosaeta concilii* strain GP6 (DSMZ 3671), supplied by A. Chidthaisong. All precultures were grown in 1-l crimp-top culture bottles using the technique of Hungate (1969). Precultures of *Methanothermobacter marburgensis* were grown at 65 °C and pH 7.5 in either mineral salts (MS) medium or MS Min medium (Boone et al. 1989). *Methanosarcina barkeri*, a metabolic generalist that can use a variety of substrates, was grown at 37 °C and pH 6.7 in MS Min medium. Both *Methanothermobacter marburgensis* and *Methanosarcina barkeri* were grown with H_2 (7.5×10^4 Pa) and CO_2 (2.5×10^4 Pa) as the sole substrates for growth. Precultures of the obligately acetate-utilizing *Methanosaeta thermophila* strain CALS-1 were grown at 61 °C and pH 6.5 in defined MS medium containing the following (l^{-1}): 2.5 g sodium acetate, 0.4 g KH_2PO_4 , 0.5 g NH_4Cl , 0.1 g $MgCl_2 \cdot 6H_2O$, 0.05 g $CaCl_2 \cdot 2H_2O$, 1 mg resazurin, 1.0 g $NaHCO_3$, 0.36 g $Na_2S \cdot 9H_2O$, 0.15 g mercaptoethanesulfonate, 0.04 mg biotin, 5.0 mg sodium EDTA dihydrate, 1.5 mg $CoCl_2 \cdot 6H_2O$, 1.0 mg $MnCl_2 \cdot 4H_2O$, 1.0 mg $FeSO_4 \cdot 7H_2O$, 1.0 mg $ZnCl_2$, 0.4 mg $AlCl_3 \cdot 6H_2O$, 0.3 mg $Na_2WO_4 \cdot 2H_2O$, 0.2 mg $CuCl_2 \cdot 2H_2O$, 0.2 mg $NiSO_4 \cdot 6H_2O$, 0.1 mg Na_2SeO_3 , 0.1 mg H_3BO_3 , and 0.1 mg $Na_2MoO_4 \cdot 2H_2O$. *Methanosaeta concilii* is obligately acetate-utilizing, and was grown using the same media formulation but at 35 °C and pH 7. The initial gas phase composition of precultures of both *Methanosaeta thermophila* and *Methanosaeta concilii* was 5×10^4 Pa CO_2 , 5×10^4 Pa N_2 . Growth was followed in all precultures by analysis of headspace methane. Biomass was estimated from methane production in the precultures assuming the following growth yields (g of dry cell mass per mol of CH_4 formed): *Methanothermobacter marburgensis* 2 g mol^{-1} (Fuchs et al. 1979; Schönheit et al. 1980), *Methanosarcina barkeri* 6.4 g mol^{-1} (Weimer and Zeikus 1978), and *Methanosaeta thermophila* 1.1 g mol^{-1} (Zinder et al. 1987).

Culture apparatus for hydrogen control

The culture apparatus used in these studies has been described previously (Valentine et al. 2000). Briefly, H_2 -free gas (N_2 , CO_2 , CH_4) is constantly bubbled through the culture, rapidly stripping H_2 from the system. Gas analysis is performed downstream from the culture apparatus so that sampling does not interfere with the culture. The apparatus is capable of maintaining H_2 levels in the culture below 10^{-3} Pa. The maximum liquid working volume of the apparatus is 350 ml, with an additional headspace of 100 ml.

The empty culture apparatus was generally purged for 1–2 days before inoculation, and H_2 levels were always near our detection limit (10^{-3} Pa) before beginning an experiment. Reducing agents, generally 2 ml of 0.1 M sodium sulfide, were added to the culture apparatus 1 h before inoculation. Prior to transfer to the experimental apparatus, H_2 was removed from the preculture by flowing an N_2/CO_2 mixture through the headspace of the bottle for several minutes at room temperature. Immediately before transfer, the bottle containing the preculture was pressurized to 2×10^5 Pa with N_2 . Transfer was achieved aseptically by inserting one end of a sharpened canula through the sampling port of the culture apparatus, while the other end was pierced through the stopper of the inverted bottle containing the preculture. The overpressure in the bottle forced the preculture through the canula and into the culture apparatus. Control experiments demonstrated that small quantities of dissolved H_2 were transferred into the culture apparatus with the preculture, but such H_2 was rapidly sparged from the apparatus and did not interfere with the experiments (data not shown).

Analytical methods

Hydrogen was quantified using a gas chromatograph equipped with a reducing gas analyzer (Trace Analytical, Menlo Park, Calif.). The lower detection limit of H_2 using this technique is about 10^{-3} Pa. Methane was quantified using a gas chromatograph equipped with a flame ionization detector (GC Mini 2, Shimadzu, Kyoto, Japan). Analysis of pH and acetate was performed by removing discrete liquid samples through the sampling port of the culture apparatus. Acetate was analyzed by HPLC (LC-600, Shimadzu) using an organic acids column (Alltech, IOA-1000) and a UV/VIS detector (SPD-6AV, Shimadzu) set at 210 nm. The mobile phase (0.5 mM H_2SO_4) was set at 0.6 ml min^{-1} and a 0.2-ml sample loop was used. The pH was measured using a Cole-Parmer (ChemCadet) pH electrode.

Chemical reagents

All chemical reagents were acquired from commercial suppliers, and stock solutions were sterilized and stored under anoxic conditions. Polysulfides (HS_n^-) were synthesized by boiling an aqueous mixture of sodium sulfide and elemental sulfur (Widdel and Pfennig 1992). Polysulfides are chains of sulfur atoms with acidic hydrogen atoms at both ends and have an oxidation state intermediate between elemental sulfur and sulfide. The average sulfur chain length for the Widdel and Pfennig (1992) synthesis procedure is 3.25. This was not confirmed experimentally.

Results

Hydrogen production

Hydrogen production within the culture apparatus was never observed in the absence of inoculum. Typical results for incubation of pure cultures in the flow-through apparatus, with a gas flow containing methane, are shown in Fig. 1 and Fig. 2. Figure 1 demonstrates patterns of H_2 production by *Methanothermobacter marburgensis* grown

Fig. 1 Hydrogen production by three cultures (200 ml) of *Methanothermobacter marburgensis*: (●) pregrown in mineral media, (○) pregrown in complex media, and (▽) pregrown in complex media and heat-sterilized (30 min at 121 °C). Each experiment was performed by transferring 200 ml of stationary-phase, batch-fed preculture into the culture apparatus, and measuring H₂ in the exhaust gas. The apparatus was maintained at 65 °C and pH 8 with a flow rate of 30 ml min⁻¹ (at 0 °C) and a gas composition of 3.4×10³ Pa CO₂, 9.9×10⁴ Pa N₂, 7.2×10² Pa CH₄ for each experiment. The integrated H₂ production relative to biomass was 0.16 μmol H₂ (mg cell dry mass)⁻¹ for the experiment involving mineral media and 0.25 μmol H₂ (mg cell dry mass)⁻¹ for the experiment involving complex media.

Fig. 2 Hydrogen production by *Methanosaeta thermophila* (Δ), *Methanosaeta concilii* (▽), and *Methanosarcina barkeri* (□). The following conditions were utilized: *Methanosaeta thermophila* (61 °C, pH 7.2, inoculum 250 ml, gas flow 30 ml min⁻¹ (at 0 °C), gas composition 5.8×10⁴ Pa CO₂, 3.4×10⁴ Pa N₂, 10⁴ Pa CH₄), *Methanosaeta concilii* (35 °C, pH 7.2, inoculum 200 ml, gas flow 20 ml min⁻¹ (at 0 °C), gas composition 5.2×10⁴ Pa CO₂, 5.2×10⁴ Pa CH₄), *Methanosarcina barkeri* (45 °C, pH 7, inoculum 190 ml, gas flow 20 ml min⁻¹ (at 0 °C), gas composition 5.2×10⁴ Pa CO₂, 5.2×10⁴ Pa N₂, 13 Pa CH₄). The integrated H₂ production relative to biomass was 0.23 μmol H₂ (mg cell dry mass)⁻¹ for the experiment involving *Methanosaeta thermophila* and 0.21 μmol H₂ (mg cell dry mass)⁻¹ for the experiment involving *Methanosarcina barkeri*.

in both basal and complex media, compared to another culture that had been heat-sterilized. Hydrogen production generally began within 2 h (sometimes within minutes) of inoculation and increased rapidly until a maximum was achieved. The production rate then began to slow and decreased gradually for about 1 day. After 1–2 days, the H₂

production decreased to a point where it became indistinguishable from background levels. Hydrogen production was only observed with *Methanothermobacter marburgensis* when the temperature was within the growth range of the organism; cooling the culture down to room temperature during H₂ production caused H₂ production to cease, while returning the culture to temperature caused H₂ production to resume. The level of H₂ production after the temperature was raised was the same as the initial condition.

Additional experiments were performed using three other methanogens (*Methanosaeta thermophila*, *Methanosarcina barkeri*, and *Methanosaeta concilii*, Fig. 2) to determine if H₂ production is a general feature of methanogens in low-H₂ environments. *Methanosaeta thermophila* demonstrated H₂ production, though the rate was lower than in *Methanothermobacter marburgensis*. Due to the kinetic ability of *Methanosaeta thermophila* to consume acetate to low micromolar levels, it was difficult to ensure that the observed H₂ was not generated during methanogenesis from acetate (Min and Zinder 1989). The preculture was incubated for 2 months to ensure complete acetate consumption, and acetate was below our analytical detection limit (low micromolar) upon transfer; due to the high methane levels (10⁴ Pa) flowing into the culture apparatus, it was not feasible to quantify low-level methane production directly. However, additions of acetate to the culture during H₂ production had no effect on H₂ production. An experiment performed with *Methanosaeta concilii* (Fig. 2) demonstrated no H₂ production even after the culture was incubated for over 1 week. *Methanosaeta concilii* is not known to metabolize H₂, and the lack of H₂ production serves as a possible indication of hydrogenase involvement in the observed H₂ production. Experiments performed with *Methanosarcina barkeri* (Fig. 2) demonstrated H₂ production patterns similar to *Methanothermobacter marburgensis*.

Methane consumption

Further H₂ production experiments were performed to determine whether CH₄ was the source of H₂. Figure 3 shows the concurrent analysis of CH₄ and H₂ for cultures of *Methanothermobacter marburgensis*, *Methanosaeta thermophila*, and *Methanosarcina barkeri* under low CH₄ and low H₂. Although for reverse methanogenesis the expected reaction stoichiometry is 4H₂: 1CH₄ (Hoehler and Alperin 1996), the results demonstrate that CH₄ is not the source of H₂ for *Methanothermobacter marburgensis* or for *Methanosarcina barkeri*. The methane level is too large to detect a small change in the *Methanosaeta thermophila* experiment, but the transient nature of H₂ production strongly indicates that the process behind H₂ production is not sustained. Other experiments with these three organisms further demonstrated that H₂ production occurred independently of the CH₄ partial pressure within the range of 1–10⁵ Pa (data not shown). These results directly demonstrate that CH₄ is not the source of H₂ ob-

Fig. 3 Concurrent analysis of H_2 (○) and CH_4 (●) in cultures of **A** *Methanothermobacter marburgensis*, **B** *Methanosarcina barkeri*, and **C** *Methanosaeta thermophila*. **A** A late-exponential-phase preculture of *Methanothermobacter marburgensis* was used as inoculum with the following conditions: 70 °C, pH 8, inoculum 150 ml, gas flow 30 ml min⁻¹ (at 0 °C), gas composition 4.1×10³ Pa CO_2 , 9.9×10⁴ Pa N_2 , 0.15 Pa CH_4 . **B** A preculture of *Methanosarcina barkeri* from the stationary phase was used for the experiment with the following conditions: 45 °C, pH 7, inoculum 200 ml, gas flow 20 ml min⁻¹ (at 0 °C), gas composition 5.2×10⁴ Pa CO_2 , 5.2×10⁴ Pa N_2 , 0.4 Pa CH_4 . **C** A late-exponential-phase preculture of *Methanosaeta thermophila* was used for the experiment with the following conditions: 61 °C, pH 6.7, inoculum 240 ml, gas flow 30 ml min⁻¹ (at 0 °C), gas composition 5.8×10⁴ Pa CO_2 , 4.5×10⁴ Pa N_2 , 10 Pa CH_4 . Dashed lines indicate the CH_4 level entering the apparatus during each experiment. The time axis refers to time after transfer of culture into the apparatus

served from either *Methanothermobacter marburgensis* or *Methanosarcina barkeri*.

From a kinetic perspective, the low levels of CH_4 shown in Fig. 3 are unlikely to support reverse methanogenesis. Methane is a very stable molecule, and the threshold concentration for CH_4 oxidation is expected to be higher than the levels shown in Fig. 3. From a bioenergetic perspective, the low levels of CH_4 , as shown in Fig. 3, are unlikely to support some of the observed H_2 partial pressures. The free-energy changes ($\Delta G'$) calculated for reverse methanogenesis (Hoehler et al. 1994) for each experiment shown in Fig. 3 (at the maximum observed H_2) are: -5 kJ (mol CH_4)⁻¹, +15 kJ (mol CH_4)⁻¹, and -24 kJ (mol CH_4)⁻¹ for *Methanothermobacter marburgensis*, *Methanosarcina barkeri*, and *Methanosaeta thermophila*, respectively. These free-energy changes are not as exergonic as those under high CH_4 conditions, though yields for both *Methanothermobacter marburgensis* and *Methanosaeta thermophila* become sufficiently exergonic during this experiment (when H_2 levels fall). The independence of H_2 production from both the expected reaction kinetics and the theoretical free-energy yield serves as a further indication that H_2 production is independent of methane oxidation.

Additional tests

Further experiments were performed to constrain the possible sources of H_2 in *Methanothermobacter marburgensis*

Fig. 4 The influence of temperature on H_2 production by *Methanothermobacter marburgensis* (○) and *Methanosarcina barkeri* (□). The experiment involving *Methanothermobacter marburgensis* was performed by inoculating the culture apparatus with 200 ml of stationary phase preculture. Initial conditions were as follows: 50 °C, pH 8, gas flow 30 ml min⁻¹ (at 0 °C), gas composition 3.4×10³ Pa CO_2 , 9.9×10⁴ Pa N_2 , 7.2×10² Pa CH_4 . Temperature was raised incrementally and H_2 production rapidly stabilized at each temperature. Only the stabilized H_2 values are presented here. The experiment involving *Methanosarcina barkeri* was performed in a similar fashion except that the first temperature change took place 85 h after inoculation, and the temperature changes were not performed in an increasing order. Initial conditions were as follows: 37 °C, pH 7, inoculum 215 ml, gas flow 20 ml min⁻¹ (at 0 °C), gas composition 5.2×10⁴ Pa CO_2 , 5.2×10⁴ Pa CH_4

and *Methanosarcina barkeri*. Hydrogen production demonstrated a temperature dependence in both species (Fig. 4). In *Methanothermobacter marburgensis*, this trend is superimposed on a trend of the production rate gradually decreasing with time; the production decreased as the temperature increased. In *Methanosarcina barkeri*, the temperature dependence was determined after several days of H_2 production, and the rate at which H_2 production decreased was small compared to the experimental time, thus no superimposed trend is apparent. The rate of H_2 production in *Methanosarcina barkeri* is low because the temperature dependence was determined late in the incubation. However, the temperature response (i.e., the slope of the line in Fig. 4) is not expected to change.

Additional experiments were performed to determine whether the addition of bioactive chemicals would influence H_2 production by *Methanothermobacter marburgensis* and *Methanosarcina barkeri*. Several chemicals were added, including acetate, bromoethanesulfonic acid, polysulfides, ammonium, cysteine, sulfate, sulfite, and thiosulfate. Of these chemicals, only polysulfides influenced H_2 production (Fig. 5). A rapid drop in H_2 production was observed upon the addition of polysulfides, indicating a link between sulfur metabolism and H_2 metabolism. Similar sulfur-containing compounds are known to interact with H_2 metabolism in methanogens (Stetter and Gaag 1983). Uninoculated control experiments with constant H_2 levels showed no H_2 oxidation in the presence of polysulfides.

Fig. 5 The influence of polysulfide addition on H_2 production by *Methanothermobacter marburgensis* (○) and *Methanosarcina barkeri* (□). The experiment involving *Methanothermobacter marburgensis* was performed using an exponential-phase preculture with the following conditions: 65 °C, pH 8, inoculum 200 ml, gas flow 30 ml min⁻¹ (at 0 °C), gas composition 1.4×10⁴ Pa CO₂, 9.0×10⁴ Pa N₂, 18.5 Pa CH₄. Polysulfides (0.25 ml, ~1 M) were added as indicated. The experiment involving *Methanosarcina barkeri* was performed at the end of a longer experiment ($t=0$ corresponds to 161 h in the culture apparatus) using a preculture from the stationary phase of growth. Initial conditions were as follows: 37 °C, pH 7, inoculum 205 ml, gas flow 20 ml min⁻¹ (at 0 °C), gas composition 5.2×10⁴ Pa CO₂, 5.2×10⁴ Pa CH₄. Polysulfides (0.5 ml, ~1 M) were added as indicated.

Discussion

Possible hydrogen sources

Hydrogen production in *Methanothermobacter marburgensis* and *Methanosarcina barkeri* show similar patterns, and the production mechanisms are likely to be similar. Both organisms also share very similar catabolic pathways for methanogenesis from H_2 and CO₂, though *Methanosarcina barkeri* has a much broader substrate range. Both species of *Methanosaeta* utilize pathways that differ significantly from the H_2 /CO₂ pathway. The differences in H_2 production between the species may be related to such catabolic differences.

Results from control experiments and experiments with different media formulations, coupled with the strong temperature dependence and the influence of polysulfides, indicate that H_2 production in *Methanothermobacter marburgensis* and *Methanosarcina barkeri* is biologically mediated and that H_2 originates from metabolites produced during growth of the preculture. However, these experiments do not differentiate between endogenous metabolites and metabolites excreted during growth. Partially reduced sulfur compounds are bioactive in methanogens and are readily reduced to sulfide in the presence of H_2 (Stetter and Gaag 1983). The action of polysulfides to decrease H_2 production in *Methanothermobacter marburgensis* and *Methanosarcina barkeri* may occur at the same step by drawing reducing equivalents away from the hydrogenase system or from another component involved in electron transport. The lack of inhibition of H_2 production upon addition of bromoethanesulfonic acid in *Methanosarcina barkeri* indicates that the target site of bromoethanesulfonic acid is not involved in H_2 production.

Hydrogen production in *Methanosarcina barkeri* and *Methanothermobacter marburgensis* is likely mediated by the hydrogenase system. The source of reducing equivalents is likely either a direct substrate of hydrogenase or is linked to the hydrogenase system. The integrated H_2 production (Fig. 1, Fig. 2) can be used to constrain possible sources for the observed H_2 . The estimated H_2 production for the three H_2 -producing species ranged from 0.16 to 0.25 $\mu\text{mol } H_2$ (mg cell dry mass)⁻¹. These values are 50- to 100-fold higher than for key metabolic intermediates, including derivatives of methanopterin and sarcinapterin, as well as for coenzyme F₄₂₀ (Gorris and Van Der Drift 1986; Van Beelen et al. 1983). These results indicate that unidentified storage compound(s) are produced during growth and are converted back to H_2 under low- H_2 conditions.

Reverse methanogenesis and anaerobic methane oxidation

Substantial environmental evidence now indicates that anaerobic methane oxidation is performed by a consor-

tium of archaea and sulfate-reducing bacteria (Boetius et al. 2000; Elvert et al. 1999; Hinrichs et al. 1999; Hinrichs et al. 2000; Hoehler and Alperin 1996; Hoehler et al. 1994; Pancost et al. 2000; Thiel et al. 1999; Valentine and Reeburgh 2000). Hoehler et al. (1994) outlined a consortium hypothesis to explain anaerobic methane oxidation, in which they postulated that methanogens operate in reverse to consume methane and produce H_2 when ambient H_2 is held low by sulfate-reducing bacteria. The levels of H_2 and methane achieved in much of this study were sufficient to allow reverse methanogenesis to proceed with energy conservation, though methane oxidation was never observed. Hydrogen production was never sustained and was independent of the methane level. Given the transient nature of H_2 production, the lack of CH_4 oxidation during H_2 production, and the lack of sensitivity of H_2 production to the CH_4 level, it appears that none of the organisms studied are able to oxidize methane under these experimental conditions. While this does not disprove the hypothesis presented by Hoehler et al. (1994), it does indicate that reverse methanogenesis is not a general feature of methanogens that is brought about by low H_2 levels.

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