Hydrogen turnover by psychrotrophic homoacetogenic and mesophilic methanogenic bacteria in anoxic paddy soil and lake sediment


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1. SUMMARY

The effect of temperature on CH$_4$ production, turnover of dissolved H$_2$, and enrichment of H$_2$-utilizing anaerobic bacteria was studied in anoxic paddy soil and sediment of Lake Constance. When anoxic paddy soil was incubated under an atmosphere of H$_2$/CO$_2$, rates of CH$_4$ production increased with time at temperatures higher than 25°C, but decreased at temperatures lower than 20°C. Chloroform completely inhibited methanogenesis in anoxic paddy soil and lake sediment, but did not or only partially inhibit the turnover of dissolved H$_2$, especially at low incubation temperatures. Cultures with H$_2$ as energy source resulted in the enrichment of chemolithotrophic homoacetogenic bacteria whenever incubation temperatures were lower than 20°C. Hydrogenotrophic methanogens could only be enriched at 30°C from anoxic paddy soil. A homoacetogen (strain HP4) and a methanogen (strain Bab1) were isolated from enrichment cultures with lake sediment at 4°C and with anoxic paddy soil at 30°C, respectively. The two strains greatly differed in cardinal temperatures of growth. Whereas the methanogen was mesophilic, the homoacetogen was psychrotrophic. This adaptation possibly allows homoacetogenic bacteria to contribute to the turnover of dissolved H$_2$ at low in situ temperatures of anoxic soils and lake sediments.

2. INTRODUCTION

Various anaerobic chemolithotrophic bacteria are able to utilize hydrogen as an energy source. The list of H$_2$ oxidizers comprises methanogens, homoacetogens, sulfate reducers, and other bacteria which for example use ferric iron as electron acceptor [1–3]. In anoxic freshwater environments, bicarbonate usually is the dominant electron acceptor and thus, H$_2$ is mainly oxidized by methanogenic or homoacetogenic bacteria. Compared to methanogens, cell numbers of homo-
acetogens were found to be low in anoxic habitats [4], e.g. in lake sediments, and their contribution to the sedimentary H₂ turnover seems to be insignificant [5]. Only in slightly acidic sediments or in the hindgut of termites homoacetogenic bacteria apparently play a more important or even the dominant role [6,7]. In general, homoacetogenesis is believed to be of minor importance for the H₂ turnover in most anoxic ecosystems. Consequently, this process has so far not been considered in models of organic matter diagenesis [8].

In the present work, we investigated the influence of temperature on the H₂ turnover and on the development and growth of enrichment cultures and of pure cultures of homoacetogenic and methanogenic bacteria isolated from anoxic paddy soil and lake sediment. The results indicate that homoacetogenic bacteria may contribute significantly to the turnover of dissolved H₂, especially at the low in situ temperatures.

3. MATERIALS AND METHODS

3.1. Soil and sediment samples

Paddy soil was obtained from fields of the Rice Research Institute in Vercelli, Italy, and were handled as previously described [9,10]. The soil slurries had a pH of 7.3. Sediment samples from Lake Constance were collected at a location in the northern arm of the lake close to the Mainau Island at 26 m depth. The in situ temperature was 4°C. The sediment was sampled with a sediment corer. The top 2 cm of the core were discarded and the sediment from 2–12 cm depth was pooled and used for experiments. The pH was 8.1. All handling was conducted under strictly anoxic conditions and at in situ temperatures.

3.2. Measurement of microbial activities

Methane production rates were determined in serum bottles (120 ml) filled with 10–15 ml (about 10 g dry weight) of anoxic paddy soil or sediment under an atmosphere of N₂/CO₂ or H₂/CO₂ (8:2) as described previously [9]. H₂ turnover rate constants (kH₂) were determined from the consumption of dissolved H₂ during incubation of the samples without a gaseous headspace [9]. To inhibit methanogenesis or sulfate reduction, chloroform or sodium molybdate were added to a final concentration of 100 μM or 2 mM, respectively.

3.3. Analytical techniques

Methane and hydrogen were analyzed by gas chromatography using a flame ionization detector and a HgO-Hg conversion detector, respectively [9]. Nitrate and sulfate concentrations were analyzed by ion chromatography (Sykam, Gauting, F.R.G.) and acetate by high pressure liquid chromatography (Sykam) using an Animex HPX-87 H organic acid analysis column (Biorad) and refractory index detection.

3.4. Medium, cultivation, and isolation of bacteria

Enrichment cultures and pure cultures were grown under strictly anaerobic conditions in the defined bicarbonate-buffered and sulfide-reduced mineral medium described for freshwater species of sulfate-reducing bacteria by Widdel and Pfennig [11]. The medium was supplemented with trace element solution SL10 [12], sodium selenite and sodium tungstate (20 nM final concentration), and a mixture of seven vitamins [13]. Organic substrates were added from sterile concentrated stock solutions directly before inoculation.

The cultures were grown in serum bottles (120 ml) or Hungate tubes (17 ml) under an atmosphere of N₂/CO₂ or H₂/CO₂ (8:2). To prevent thermic shock or heat damage of psychrotrophic bacteria enriched at low temperatures (< 20°C), the medium was chilled to 5°C before inoculation. Pure cultures were obtained by repeated application of deep agar dilution series [11]. The purity of cultures was checked by microscopic control and growth tests in complex AC medium (Difco, Detroit, U.S.A.). Stock cultures were kept under N₂/CO₂ or H₂/CO₂ at 4°C. Transfers into fresh medium were made every 8 weeks.

3.5. Growth experiments

Growth of cells was followed by measuring the turbidity at 500 nm in a Spectronic 70 photometer (Bausch and Lomb). All tests were done at least in duplicate. Activation energies of growth were determined from the slope of a semilogarithmic plot of the growth rates (μ) versus the reciprocal tem-
perature according to the logarithmic form of the Arrhenius equation

\[ \ln \mu = \ln A - \left( \frac{E_a}{R} \right) \left( \frac{1}{T} \right) \]

where \( A \) = Arrhenius constant, \( T \) = temperature (K), \( R \) = gas constant, and \( E_a \) = apparent activation energy (kJ mol\(^{-1}\)) of bacterial growth.

4. RESULTS

4.1. \( \text{H}_2 \) turnover in anoxic paddy soil and lake sediment

When anoxic paddy soil was incubated under a \( \text{H}_2/\text{CO}_2 \) (8:2) atmosphere, rates of \( \text{CH}_4 \) production increased with time for the first two days of incubation. Afterwards, increase of \( \text{CH}_4 \) production could only be observed if the incubation temperature was higher than 25 °C. At temperatures lower than 20 °C, however, \( \text{CH}_4 \) production rates decreased (Fig. 1).

\( \text{H}_2 \) turnover rate constants were measured in anoxic paddy soil which had been incubated for up to one month at 17, 25, and 30 °C under an atmosphere of \( \text{N}_2/\text{CO}_2 \) (Table 1). At 17 °C, \( \text{H}_2 \) turnover rate constants increased from 19 to 53 h\(^{-1}\) during the incubation period. At higher temperatures \( \text{H}_2 \) turnover rate constants were in a range of 64–78 h\(^{-1}\) and did not change significantly within one month.

In parallel experiments, the \( \text{H}_2 \) turnover was also measured in presence of chloroform (Table 1). Chloroform was added two hours before the measurements were started. Within one hour, \( \text{CH}_4 \) production had completely stopped while the \( \text{H}_2 \) turnover was only partially inhibited. The residual \( \text{H}_2 \) turnover rates reached values of 60–75% of the rates measured in chloroform-free controls (Table 1). These values decreased with time and increasing temperature and were after one month of incubation in the range of 20–50%.

In sediment samples from Lake Constance the \( \text{H}_2 \) turnover rate constants decreased from 55 to 30 h\(^{-1}\) during one week incubation at 4 °C (Table 2). \( \text{H}_2 \) turnover was only slightly inhibited by chloroform. Both anoxic lake sediment and paddy soil did not contain significant amounts of nitrate (< 10 \( \mu \text{M} \)) or sulfate (< 20 \( \mu \text{M} \)). Neither in lake sediment nor in anoxic paddy soil, addition of

![Fig. 1. Effect of temperature on the temporal change of CH4 production rates in anoxic paddy soil incubated under a H2/CO2 atmosphere.](image-url)
Table 2

<table>
<thead>
<tr>
<th>Preincubation</th>
<th>( k_{\text{H}_2} ) (h(^{-1}))</th>
<th>Contribution of homoacetogens (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>at 4°C (days)</td>
<td>control 100 ( \mu \text{M} ) CHCl(_3)</td>
<td>homologens (%)</td>
</tr>
<tr>
<td>1</td>
<td>54.6</td>
<td>48.9</td>
</tr>
<tr>
<td>3</td>
<td>34.9 ± 8.1</td>
<td></td>
</tr>
<tr>
<td>6–8</td>
<td>29.2 ± 5.9</td>
<td>28.8 ± 4.1</td>
</tr>
</tbody>
</table>

\( a \) Mean values ± SD of triplicate measurements.

molybdate had any inhibitory effect on \( \text{H}_2 \) turnover indicating that sulfate-reducing bacteria were not involved in the oxidation of dissolved \( \text{H}_2 \).

These results suggest that especially at low temperatures a significant part of the anaerobic turnover of dissolved \( \text{H}_2 \) was due to anaerobic bacteria other than methanogens or sulfate reducers, e.g. to homoacetogenic bacteria. This conclusion was further confirmed by enrichment of \( \text{H}_2 \)-utilizing anaerobes.

### 4.2. Enrichment cultures from anoxic paddy soil

Enrichments incubated at 30°C under \( \text{H}_2/\text{CO}_2 \) showed strong and persistent \( \text{CH}_4 \) production and development of rod-shaped bacteria which mainly were methanogens identified by their fluorescence. By contrast, identical enrichments incubated at 17°C showed only slight \( \text{CH}_4 \) production which ceased after 10–25 days. Nevertheless, \( \text{H}_2 \) was further consumed and simultaneously the pH decreased and acetate accumulated in the medium. In these cultures short, non-fluorescent rods with pointed ends resembling the homoacetogenic \text{Acetobacterium} were numerically dominant. Hence, the enrichment of \( \text{H}_2 \)-utilizing methanogens or homoacetogens was dependent on high or low incubation temperatures, respectively. Addition of small amounts (1 mM) of acetate as carbon source to the medium did not influence the result of the enrichment experiments. Enrichments with acetate (20 mM) as sole energy source, on the other hand, generally resulted in \( \text{CH}_4 \) production and development of fluorescent bacteria resembling \text{Methanosarcina}.

From one of the enrichment cultures (30°C under \( \text{H}_2/\text{CO}_2 \)) a \( \text{H}_2 \)-utilizing methanogen, strain Bab1, was isolated. Strain Bab1 is a slender, non-motile rod with blunt-rounded ends (Fig. 2a). It only grew and produced \( \text{CH}_4 \) on \( \text{H}_2/\text{CO}_2 \) as substrate. Formate or acetate were not used as energy substrates. Acetate added in low concentrations (1–2 mM) as carbon source stimulated growth on \( \text{H}_2/\text{CO}_2 \). According to these properties, strain Bab1 resembled \text{Methanobacterium bryantii} [14].

### 4.3. Enrichment cultures from Lake Constance sediment

Enrichment cultures incubated under \( \text{H}_2/\text{CO}_2 \) showed intense acetate production (5–10 mM final concentration) at incubation temperatures of 4, 15 as well as 30°C. No production of \( \text{CH}_4 \) was

![Fig. 2](https://academic.oup.com/femsec/article-abstract/5/5/285/639730/283280)
observed with H₂/CO₂ as substrate. After several transfers, non-fluorescent short rods resembling *Acetobacterium* were observed in all enrichment cultures. Identical results were obtained when formate (12 mM) was used as substrate. Enrichments with methanol (10 mM) at 30°C resulted in CH₄ production and supported the development of *Methanosarcina*-like species; at 4 or 15°C, however, acetate-producing short rods developed on methanol. Enrichments with acetate (15 mM) generally resulted in CH₄ production and development of *Methanosarcina*-like bacteria.

From one of the enrichment cultures (4°C under H₂/CO₂) a short rod-shaped bacterium, strain HP4, was isolated. Strain HP4 formed non-motile cells with pointed ends (Fig. 2b). The isolate was able to grow on H₂/CO₂, methanol, formate, ethylene glycol with acetate being the only product. Good growth was also observed on trimethoxybenzoate which was catabolized to acetate and gallic acid. Sulfate, thiosulfate, or nitrate were not used as electron acceptors. Based on these properties strain HP4 most likely belongs to *Acetobacterium carbinolicum* [15].

### 4.4. Temperature characteristics of strains HP4 and Babl

Both isolates, the homoacetogenic bacterium strain HP4 and the methanogenic bacterium strain Babl, were tested for cardinal temperatures and apparent activation energies (Eₐ) of growth. Whereas methanogenic strain Babl showed a temperature range typical for a mesophilic bacterium, the homoacetogenic strain HP4 had a considerably lower minimum, optimum, and maximum temperature for growth (Table 3). Strain HP4 could even grow at 0°C, but died rapidly at temperatures higher than 25°C. The optimum growth temperatures of both strains were higher than the incubation temperatures used for enrichment and isolation. The apparent activation energies (Eₐ) of growth taken from the Arrhenius plots shown in Fig. 3 were 78 kJ mol⁻¹ for strain HP4 and 43 kJ mol⁻¹ for strain Babl.

### 5. DISCUSSION

Our studies have shown that the turnover of dissolved H₂ in anoxic and nitrate-free paddy soil and sediment of mesotrophic Lake Constance was not or not completely inhibited by the addition of chloroform and molybdate. These observations indicate that a significant part of the anaerobic turnover of H₂ in these slightly alkaline (pH 7.3–8.1) anoxic environments was due to other microbial processes than methanogenesis or sulfate reduction. Nitrate concentrations were too low for nitrate reduction. Assuming that concentrations of oxidized iron or manganese compounds also were too low in these anoxic samples to support a significant iron or manganese reduction, bicarbonate seems to be the main electron acceptor available for H₂ oxidation. Hence, homoacetogen-
esis apparently was the dominant process responsible for the H₂ turnover observed in the presence of chloroform. Similar observations were made by Lovley and Klug [16] in sediment samples of the oligotrophic Lake Lawrence. Jones and Simon [17] observed that H₂-consuming homoacetogens occasionally contribute up to 25% of the acetate produced in sediments of Blelham Tarn. All these observations indicate that homoacetogenic bacteria can play a significant role in the anaerobic turnover of dissolved H₂, at least in some aquatic methanogenic ecosystems.

The dominance of H₂-utilizing homoacetogens versus methanogens is not easily explained. Another biotope where H₂-utilizing homoacetogens play an important role is the hindgut of lower termites [7]. There, the dominant homoacetogen is a *Sporomusa termitida*. Its H₂ consumption kinetics show a $V_{\text{max}}$ of 380 nmol H₂ min⁻¹ mg protein⁻¹ and a $K_{\text{m}}$ of 6 μM H₂ which would not allow a successful competition with methanogens having either a higher $V_{\text{max}}$ or a lower $K_{\text{m}}$ for H₂ [18]. The threshold for H₂ consumption also is significantly higher for *S. termitida* than for methanogenic bacteria [19] and thus, also cannot explain the dominance of homoacetogenesis versus methanogenesis in termite hindguts.

The in situ partial pressures of H₂ in paddy soil usually are in a range of 1–4 Pa [20]. These H₂ concentrations are just within the range of the H₂ thresholds measured in pure cultures of H₂-utilizing methanogens (2–10 Pa) but are significantly lower than those of homoacetogens (43–95 Pa) [19]. Hence, homoacetogens should not be able to utilize the in situ H₂. One can argue that the observed chloroform-insensitive H₂ turnover rates are only potential H₂ turnover rates as they have been measured after the experimental increase of dissolved H₂. However, H₂ concentrations still were in the range below the $K_{\text{m}}$ of methanogenic and homoacetogenic bacteria resulting in a logarithmic consumption of the dissolved H₂ within 5–20 min. It is unlikely that such a small and brief increase of H₂ might have triggered a resident chemoorganotrophic homoacetogenic population to shift to chemolithotrophic metabolism. It is possible, however, that under in situ versus defined culture conditions homoacetogens can consume H₂ to much lower concentrations because of the availability of additional organic energy sources. Compared to methanogens, homoacetogenic bacteria can use a wide range of substrates, possibly by mixotrophic metabolism, and this versatility may be of ecological importance [21]. Mixotrophic H₂ utilization has been reported for *A. woodii* [22] but the effect of a second substrate on the efficiency of H₂ utilization is unknown. Similarly, suitable organic electron acceptors (e.g. caffeate) could allow a more efficient H₂ utilization [19] but it is unknown whether they play a role under in situ conditions.

It also must be emphasized that measurements of H₂ turnover rate constants can only detect the turnover of the H₂ which is dissolved in pore water, but not that of the H₂ which is directly transferred between juxtaposed H₂-producing and H₂-consuming bacteria. In fact, turnover of dissolved H₂ apparently makes up only a small percentage of the H₂ transfer in juxtaposed bacterial associations [10, 23, 24]. Therefore, the homoacetogenic consumption of dissolved H₂, though significant in itself, may be small compared to the total flow of electrons towards methanogenesis and also may contribute little to total acetate turnover. On the other hand, H₂ may also be directly transferred between juxtaposed H₂-producing and homoacetogenic bacteria, thus rendering the homoacetogens important H₂ scavengers. Further research is needed to elucidate the quantitative importance of chemolithotrophic homoacetogens for H₂ turnover and acetate production.

Another strong support for the assumption that homoacetogenic bacteria are of biogeochemical importance in the soil and sediment samples studied is the outcome of the enrichment cultures. From sediments of Lake Constance, for example, no methanogenic bacteria could be enriched and isolated with H₂ as energy substrate. Instead, H₂-utilizing homoacetogens developed in general. Similar results were obtained, when enrichment cultures for H₂-utilizing sulfate reducers were initiated (Bak, PhD thesis, Konstanz, 1988). The prevalence of homoacetogens cannot be explained by selectivity of the culture medium, since
this medium is successfully used since many years in our laboratory for enrichment and cultivation of all kinds of sulfate-reducing and methanogenic bacteria. Hence, homoacetogens seem to be relatively significant \(H_2\)-utilizing bacteria in Lake Constance sediments, that are able to outgrow the \(H_2\)-utilizing methanogens, at least at the high \(H_2\) partial pressures used for enrichment cultures.

It is mostly unknown which environmental factors render homoacetogenic bacteria the successful \(H_2\) utilizers. In situ temperature apparently is one important factor determining whether the resident population of homoacetogenic bacteria can successfully compete for \(H_2\) with methanogens or not. Temperature control is undoubtedly very important in those environments which show large temperature changes, e.g. paddy fields, littoral sediments, peat. The enrichment studies with anoxic paddy soil have shown that \(H_2\)-utilizing homoacetogenic bacteria were able to overgrow \(H_2\)-utilizing methanogenic bacteria at \(17^\circ C\) and vice versa at \(30^\circ C\). The critical temperature which determined the outcome of the competition was around \(20\) to \(25^\circ C\) (Fig. 1). This is exactly the temperature range which is observed during most of the year in Italian paddy fields [25]. Hence, the dissolved \(H_2\) in an anoxic ecosystem may be consumed by homoacetogenic and methanogenic bacteria to varying degrees during the season or even during the day, depending on the actual in situ temperature. The contribution of \(H_2\) to methanogenesis in an Italian rice paddy varied between 28 and 51% during the season [26].

That homoacetogens possibly are better adapted to growth at lower temperatures than methanogens is reflected by the different temperature optima of the isolated strains. Whereas *Acetobacterium* strain HP4 had a temperature optimum at \(18^\circ C\) and a range of 0 to \(25^\circ C\), *Methanobacterium* strain Bab1 showed its optimum growth at \(35^\circ C\) and a range of 18 to \(45^\circ C\). \(CH_4\) production rates showed a similar range and optimum of temperature as growth (unpublished results). Recently, Rajagopal et al. [27] also isolated a *Methanobacterium* as the dominant \(H_2\)-utilizing methanogen from Louisiana paddy soils. This isolate has similar characteristics as our isolate and shows a similar temperature range.

Although strain HP4 could tentatively be determined as an *Acetobacterium carbinolicum*, the cardinal temperatures of growth greatly differed from those of the type strain [15] or those of other species of homoacetogens [28]. These are either typical mesophiles having a temperature minimum of about \(15^\circ C\) and an optimum of more than \(25^\circ C\) or are thermophiles. In fact, strain HP4 is the first psychrotrophic homoacetogen isolated in pure culture (psychrotrophic as defined by Morita [29]). It cannot be excluded that other isolates might have slightly different temperature ranges and that the temperature ranges of the in situ sediment activity might be slightly different from those of pure cultures. In general, however, the enriched homoacetogenic bacteria seemed to be of psychrophilic-psychrotrophic and the methanogenic bacteria of mesophilic nature.

The observation that \(H_2\)-utilizing methanogenic bacteria from paddy fields are better adapted to higher temperatures is consistent with the observation that contribution of \(H_2\)-dependent \(CH_4\) production increases with increasing temperature [9]. Conrad et al. [9] observed that at low temperatures methanogens were mainly limited by the supply of \(H_2\), since \(H_2\)-producing bacteria seemed to be more sensitive to low temperature than methanogens. Now, it seems also possible that psychrotrophic homoacetogenic bacteria successfully compete with mesophilic \(H_2\)-producing fermenting bacteria for common organic substrates and thus decrease \(H_2\) production at low temperature. Limitation of \(H_2\) production by temperature-sensitive fermenting bacteria affects juxtaposed methanogenic bacteria as well as free-living ones. The free-living methanogens could additionally be limited by the \(H_2\) competition of psychrotrophic homoacetogens. The thus produced acetate would then only serve acetotrophic *Methanosarcina*-like methanogens as substrate.

Svensson [30] observed two different methanogenic activities in peat. One bacterial community used \(H_2\) and had a temperature optimum at \(28^\circ C\), the other used acetate and had a temperature optimum at \(20^\circ C\). It may be that the adaptation of the acetate-dependent \(CH_4\) production to low temperature is due to a response to the temperature adaptation of a psychrotrophic homoaceto-
methanogenic bacterial metabolism in methanogenic environments.

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