H₂ oxidation in the presence of thiosulfate, by a *Thermoanaerobacter* strain isolated from an oil-producing well

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Abstract: A thermophilic rod (strain SEBR 5268), isolated from an oil-producing well, was identified as a *Thermoanaerobacter* strain that was phenotypically related to *T. fumii*. Both SEBR 5268 and *T. fumii* oxidized H₂ by reducing thiosulfate to sulfide using yeast extract as growth substrate. H₂ oxidation in the presence of thiosulfate was significant at the end of the exponential growth of SEBR 5268 and was maintained during the lysis phase. In the absence of thiosulfate, H₂ was inhibitory for both strains. The role of H₂ consumption by these bacteria is discussed with regard to their metabolism on organic compounds.

Keywords: Thermophily; Anaerobiosis; *Thermoanaerobacter*; H₂-oxidation; Thiosulfate; Oil well

Introduction

In anaerobic ecosystems, H₂ is known to be oxidized when a mineral or an organic electron acceptor is available. Fumarate is used as organic electron acceptor and is reduced to succinate by some sulfate-reducing bacteria [1,2]. Among the mineral electron acceptors, CO₂ and the oxidized forms of sulfur ranging from SO₄ to elemental sulfur (S⁰), are reduced with the electrons resulting from H₂ oxidation. CO₂ is reduced to methane or to acetate by methanogenic [3] or homoacetogenic bacteria [4] respectively, while several sulfate reducers may produce sulfide from H₂ metabolism by reducing sulfate, thiosulfate, sulfite or elemental sulfur [1].

Extreme thermophiles, belonging to the Archae or to the Bacteria domains, show a sulfur-dependent metabolism when grown on H₂ [5]. In this case, S⁰ is the only sulfur compound that can be reduced concomitantly with H₂ oxidation [5].

Although methanogenesis, homoacetogenesis, and sulfate-reduction from H₂ are energy-yielding reactions, S⁰ reduction does not necessarily involve ATP synthesis. Whereas *Thermoproteus* generate energy when using S⁰ as electron acceptor [5,6], *Pyrococcus* [7] and *Thermotoga* [8] do
not produce energy while oxidizing H₂, but this oxidation does alleviate an inhibitory effect on their growth [5]. *Thermoanaerobacter* (*T.*) species are thiosulfate-reducing bacteria [9] producing sulfide. In the closely related genus *Thermoanaerobium* [10,11], S° reduction was observed and the presence of a cytoplasmic hydrogenase catalysing both H₂ evolution and uptake was evident [11].

Although attention has been paid to the sulfur metabolism of these two genera, little emphasis has been placed on their H₂ metabolism in relation with thiosulfate reduction, particularly within the genus *Thermoanaerobacter*.

Strain SEBR 5268 was recently isolated from an oil-producing well [12] and can be affiliated to the genus *Thermoanaerobacter*. This isolate was related phenotypically to *T. finnii* [13]. We intend to show that both microorganisms oxidize H₂, when yeast extract is supplied in the culture medium.

**Materials and Methods**

*Origin of strains*

Strain SEBR 5268 was isolated from an oil-producing well in France [12]. It was deposited at the Elf-Aquitaine Collection of Microorganisms. *Thermoanaerobacter finnii* was obtained from DSM (No. 3389).

*Culture medium*

Media were prepared by the techniques of Hungate [14]. The organisms were cultivated under strict anaerobic conditions at 60°C. The basal medium used for metabolic studies contained the following constituents in distilled water (g per liter): NH₄Cl, 1; K₂HPO₄, 0.3; KH₂PO₄, 0.3; MgCl₂·6H₂O, 1.3; CaCl₂·2H₂O, 0.1; KCl, 0.2; CH₃COONa·3H₂O, 0.5; NaCl, 2.0; resazurin, 0.001; cysteine · HCl, 0.5; Balch minerals [15] solution, 10 ml; pH was adjusted to 7.0 with 10 M KOH. The medium was boiled under a stream of O₂-free N₂, cooled at room temperature, and distributed into Hungate tubes (5 ml) or bottles (50 ml), which were outgassed with N₂-CO₂ (80/20%). After autoclaving (110°C for 40 min), 0.2 ml of 2% Na₂S·9H₂O and 1·ml of 10% NaHCO₃ (sterile, anaerobic conditions), were added for 20 ml of culture medium before inoculation. The basal medium was supplemented with yeast extract or thiosulfate at appropriate concentrations from sterile stock solutions.

*Isolation*

Pure cultures were obtained by repeated use of the agar shake dilution method in anaerobic Hungate tubes as previously described [2]. Purity was checked by microscopic examinations.

*Analytical techniques*

Duplicate vessels were used throughout this work. Optical density was determined at 580 nm with a UV-160A spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Sulfide was determined by the method of Cord-Ruwisch [16]. H₂ was measured by gas chromatography at 150°C, on the catherometer detector of a Girdel 30 gas chromatograph (Delsi, Argenteuil, France), with N₂ as the carrier gas, equipped with a carbosphere SS 60/80 mesh column. Fermentation products were quantified by HPLC on a ORH 801 column (Interaction Chemicals, Inc., Mountain View, CA).

*Results*

*Enrichment and isolation*

Oil-field water, collected at the wellhead, was inoculated into the basal medium containing glucose as energy source and was incubated at 60°C for 48 h. Microscopic examinations showed the presence of motile rods. Direct isolation of the organism from enrichment cultures was performed in roll tubes containing glucose enriched medium with 2% agar. Colonies developed within 48 h and were diluted by repeated application of the agar shake dilution method, in anaerobic Hungate tubes. All colonies observed were smooth, non-pigmented and flat. Strain SEBR 5268 was chosen for further studies.

A range of experiments was performed to classify the isolate. Because this work focuses on H₂ utilization in the presence of thiosulfate as electron acceptor, we present here only data suffi-
cient to establish the taxonomical position of the microorganism.

Characteristics of SEBR 5268

The isolate was a motile rod by peritrichous flagella. Protuberances were observed after the end of exponential growth. Positive Gram reaction was confirmed by electron microscopic examination. The optimum temperature for growth was around 60°C. Growth was observed at 75°C but stopped at 80°C. Strain SEBR 5268 used different carbohydrates only in the presence of yeast extract. Products of glucose metabolism were lactate, ethanol, acetate, H₂ and presumably CO₂. It reduced thiosulfate, but not sulfate, to sulfide when oxidizing carbohydrates.

Hydrogen metabolism

Strain SEBR 5268 and *Thermoanaerobacter finnii* were grown in the basal medium enriched with yeast extract which served as energy sources for both strains. Two concentrations (2 and 4 g/l) were used to study the influence of cell density on the reduction of thiosulfate by H₂.

In the absence of H₂, growth was better at 4 g yeast extract/l than 2 g/l for either strains. At both concentrations, the thiosulfate addition did not modify the growth of SEBR 5268 but slightly decreased the growth of *T. finnii* (Table 1).

In the absence of thiosulfate, the growth of both strains was significantly reduced when H₂ (2 bars) was added in the medium (Table 1). The addition of thiosulfate in the presence of H₂ led to a high sulfide production concurrent with H₂ oxidation. No H₂ oxidation occurred without yeast extract or in the absence of thiosulfate (Fig. 2) via homoacetogenesis.

The dynamic study was performed with SEBR 5268 at 2 g yeast extract/l, since no further information could be produced by increasing yeast extract concentration.

Results showed an inhibitory effect of H₂ on strain SEBR 5268 during the exponential growth (Fig. 1). Differences in absorbance were also observed during lysis (Fig. 1). Furthermore, oxidation of H₂ appeared only in the presence of thiosulfate (Fig. 2). This reaction resulted in the production of sulfide (Figs. 3 and 4). H₂ oxidation was clearly evidenced at the end of the

Table 1

<table>
<thead>
<tr>
<th>Yeast Extract (g/l)</th>
<th>Strain SEBR 5268</th>
<th>T. finnii</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ΔOD (580 nm)</td>
<td>H₂S (mM)</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>0.345</td>
</tr>
<tr>
<td></td>
<td>M+H₂</td>
<td>0.025</td>
</tr>
<tr>
<td></td>
<td>M+TS</td>
<td>0.326</td>
</tr>
<tr>
<td></td>
<td>M+TS+H₂</td>
<td>0.192</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>0.352</td>
</tr>
<tr>
<td></td>
<td>M+H₂</td>
<td>0.047</td>
</tr>
<tr>
<td></td>
<td>M+TS</td>
<td>0.404</td>
</tr>
<tr>
<td></td>
<td>M+TS+H₂</td>
<td>0.304</td>
</tr>
</tbody>
</table>

1 Results after 3 days of incubation at 60°C; 2 Results after 5 days of incubation at 60°C; M: Basal medium without yeast extract; H₂: Hydrogen (initial pressure was 2 bars); TS: Thiosulfate (initial concentration was 20 mM); Inoculation was realized with bacteria grown on basal medium containing yeast extract (2 g/l) and thiosulfate (20 mM).
Strain SEBR 5268, isolated from an oil-producing well, presented all the phenotypical characteristics which justify its taxonomical affiliation to the genus *Thermoanaerobacter* [9,17]. It grew between 42°C and 80°C, with an optimum around 60°C. It was a Gram-positive peritrichous rod which converted sugars to acetate, lactate, ethanol, H₂, and presumably CO₂. Furthermore, it reduced thiosulfate to sulfide. This biochemical property was used by Lee et al. [9] to differentiate the genus *Thermoanaerobacter* from *Thermoaerobacterium* which reduce thiosulfate to elemental sulfur [9]. Phenotypically, SEBR 5268 was closely related to *Thermoanaerobacter finnii* [13] as it differed only by the use of one sugar (melibiose) and the optimum temperature for growth (60°C instead of 65°C).

To our knowledge this is the first report of H₂ oxidation by *Thermoanaerobacter* strains (SEBR 5268 and *T. finnii*). This occurred only when thiosulfate and yeast extract (or possibly another energy source) were present in the medium. Our results showed also that H₂ was inhibitory for growth under the culture conditions as already observed with *T. ethanolicus* [17].
H₂ oxidation in the presence of thiosulfate was probably a non energy-yielding reaction, since no increase in growth was obtained after H₂ addition. However, since H₂ was significantly used after the exponential growth, we can also hypothesize that the energy which could be obtained from H₂ metabolism was not available, due to unfavourable physico-chemical conditions after exponential growth (limiting growth factors, pH, etc.). Nevertheless, the reduction of thiosulfate to sulfide is known to be coupled to ATP synthesis in Desulfovibrio vulgaris [18]. Uncoupled growth with both strains could indicate that their H₂ metabolism could be a detoxifying process as suggested for the genus Pyrococcus or Thermotoga [5]. This would result in an optimal growth when cultivated on carbohydrates or possibly other organic compounds. The use of H₂ might also allow unfavourable thermodynamic reactions to occur. In situ, the hyperthermophile genera Pyrococcus and Thermotoga did not produce energy when reducing elemental sulfur to sulfide with H₂ as electron source. From the energetic point of view and with regard to the sulfur metabolism, these genera are closely related to the the two strains of Thermoanaerobacter studied.

When considering the evolution of the earth’s atmosphere, it can be hypothesized that the appearance of oxygen resulted in the sulfide oxidation producing different intermediary forms, including thiosulfate and sulfate, which became the major sulfur components of the seas [19]. These Thermoanaerobacter strains (SEBR 5268 and T. finnii) might have appeared between the strict elemental sulfur reducers and the sulfate-reducing bacteria.

The environmental origin of the strain isolated (oil-producing well) and its ability to oxidize H₂ indicates that it may compete for H₂ with methanogens or sulfate-reducing bacteria when thiosulfate is present in the ecosystem. Biochemical studies are needed to determine the affinity of its hydrogenase(s) for H₂. Finally, sulfide production by thiosulfate-reducing strains other than sulfate-reducing bacteria may have a significant impact on bacterial corrosion of oil-producing facilities. It has been recently hypothesized that the presence of thiosulfate could increase bacterial corrosion risks [20].

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References

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moireobacter thermohydrosulfuricus comb. nov., respectively; and transfer of Clostridium thermohydrosulfuricum 39E to Thermoanaerobacter ethanolicus. Int. J. Syst. Bacteriol. 43, 41–51.


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