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H_2 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. finnii*. Both SEBR 5268 and *T. finnii* oxidized H_2 by reducing thiosulfate to sulfide using yeast extract as growth substrate. H_2 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, H_2 was inhibitory for both strains. The role of H_2 consumption by these bacteria is discussed with regard to their metabolism on organic compounds.

Key words: Thermophily; Anaerobiosis; *Thermoanaerobacter*; H_2-oxidation; Thiosulfate; Oil well

Introduction

In anaerobic ecosystems, H_2 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, CO_2 and the oxidized forms of sulfur ranging from SO_4 to elemental sulfur (S°), are reduced with the electrons resulting from H_2 oxidation. CO_2 is reduced to methane or to acetate by methanogenic [3] or homoacetogenic bacteria [4] respectively, while several sulfate reducers may produce sulfide from H_2 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 H_2 [5]. In this case, S° is the only sulfur compound that can be reduced concomitantly with H_2 oxidation [5].

Although methanogenesis, homoacetogenesis, and sulfate-reduction from H_2 are energy-yielding reactions, S° reduction does not necessarily involve ATP synthesis. Whereas *Thermoproteus* generate energy when using S° as electron acceptor [5,6], *Pyrococcus* [7] and *Thermotoga* [8] do

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not produce energy while oxidizing H_2, 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 H_2 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 H_2 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 H_2, 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): NH_4Cl, 1; K_2HPO_4, 0.3; KH_2PO_4, 0.3; $MgCl_2 \cdot 6H_2O$, 1.3; $CaCl_2 \cdot 2H_2O$, 0.1; KCl, 0.2; $CH_3COONa \cdot 3H_2O$, 0.5; NaCl, 2.0; resazurin, 0.001; cystein · 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 O_2-free N_2, cooled at room temperature, and distributed into Hungate tubes (5 ml) or bottles (50 ml), which were outgassed with N_2-CO_2 (80/20%). After autoclaving (110°C for 40 min), 0.2 ml of 2% $Na_2S \cdot 9H_2O$ and 1 ml of 10% $NaHCO_3$ (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]. H_2 was measured by gas chromatography at 150°C, on the catharometer detector of a Girdel 30 gas chromatograph (Delsi, Argenteuil, France), with N_2 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 H_2 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, H_2 and presumably CO_2. 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 H_2.

In the absence of H_2, 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).

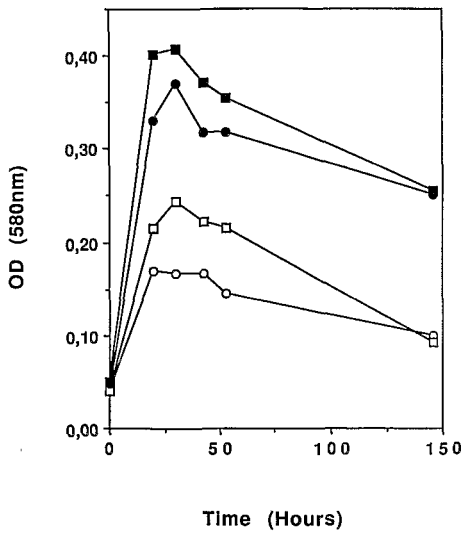

Fig. 1. Effect of H_2 addition on the growth of *Thermoanaerobacter* SEBR 5268, in the presence and in the absence of thiosulfate. (●) Yeast extract (2 g/l); (■) Yeast extract (2 g/l); Thiosulfate (20 mM); (○) Yeast extract (2 g/l); Hydrogen (2 bars); (□) Yeast extract (2 g/l); Thiosulfate (20 mM); Hydrogen (2 bars).

In the absence of thiosulfate, the growth of both strains was significantly reduced when H_2 (2 bars) was added in the medium (Table 1). The addition of thiosulfate in the presence of H_2 leaded to a high sulfide production concurrent with H_2 oxidation. No H_2 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 H_2 on strain SEBR 5268 during the exponential growth (Fig. 1). Differences in absorbance were also observed during lysis (Fig. 1). Furthermore, oxidation of H_2 appeared only in the presence of thiosulfate (Fig. 2). This reaction resulted in the production of sulfide (Figs. 3 and 4). H_2 oxidation was clearly evidenced at the end of the

Table 1

Effect of hydrogen addition on growth of strain SEBR 5268 and *T. finnii* in the presence and the absence of thiosulfate

Yeast Extract (g/l)	Treatment	Strain SEBR 5268 [1]		*T. finnii* [2]	
		ΔOD (580 nm)	H_2S (mM) Final	ΔOD (580 nm)	H_2S (mM) Final
2	M	0.345	1.32	0.223	3.32
	M+H_2	0.025	0.54	0.000	3.52
	M+TS	0.326	1.64	0.100	2.06
	M+TS+H_2	0.192	31.22	0.210	35.31
4	M	0.352	1.04	0.248	3.37
	M+H_2	0.047	1.01	0.010	4.13
	M+TS	0.404	5.21	0.179	7.41
	M+TS+H_2	0.304	28.90	0.150	36.40

[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; H_2: 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).

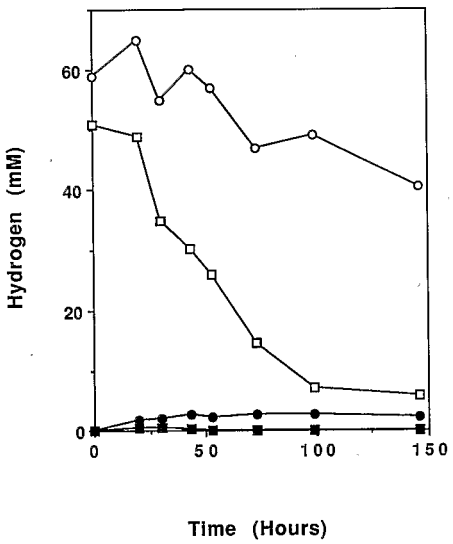

Fig. 2. H_2 metabolism by *Thermoanaerobacter* SEBR 5268, in the presence and in the absence of thiosulfate. (●) Yeast extract (2 g/l); (■) Yeast extract (2 g/l); Thiosulfate (20 mM); (○) Yeast extract (2 g/l); Hydrogen (2 bars); (□) Yeast extract (2 g/l); Thiosulfate (20 mM); Hydrogen (2 bars).

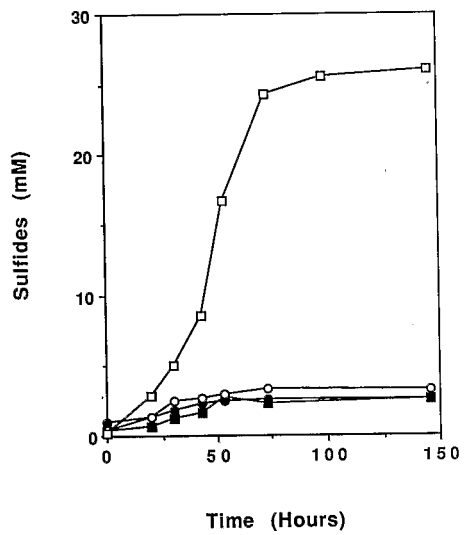

Fig. 3. Effect of H_2 addition on sulfide production by *Thermoanaerobacter* SEBR 5268, in the presence and in the absence of thiosulfate. (●) Yeast extract (2 g/l); (■) Yeast extract (2 g/l); Thiosulfate (20 mM); (○) Yeast extract (2 g/l); Hydrogen (2 bars); (□) Yeast extract (2 g/l); Thiosulfate (20 mM); Hydrogen (2 bars).

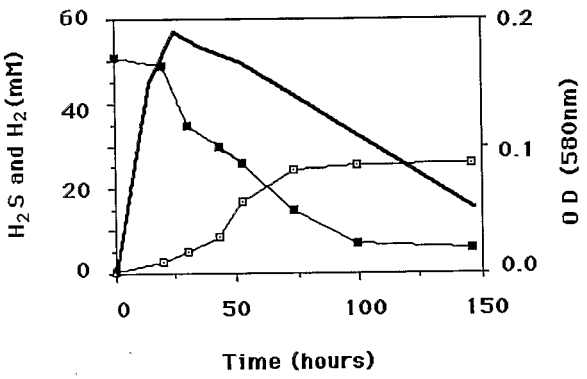

Fig. 4. H_2 oxidation by *Thermoanaerobacter* SEBR 5268, in the presence of thiosulfate. (□) Sulfides; (■) Hydrogen; (___) Optical Density (OD 580 nm).

exponential growth and was not affected during the lysis phase (Fig. 4).

Discussion

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, H_2 and presumably CO_2. Furthermore, it reduced thiosulfate to sulfide. This biochemical property was used by Lee et al. [9] to differentiate the genus *Thermoanaerobacter* from *Thermoanaerobacterium* 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 H_2 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 H_2 was inhibitory for growth under the culture conditions as already observed with *T. ethanolicus* [17].

H_2 oxidation in the presence of thiosulfate was probably a non energy-yielding reaction, since no increase in growth was obtained after H_2 addition. However, since H_2 was significantly used after the exponential growth, we can also hypothesize that the energy which could be obtained from H_2 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 synthetisis in *Desulfovibrio vulgaris* [18]. Uncoupled growth with both strains could indicate that their H_2 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 H_2 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 H_2 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 H_2 indicates that it may compete for H_2 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 H_2. 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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