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Pure culture and cytological properties of '*Syntrophobacter wolinii*'

Christina Wallrabenstein [1], Elisabeth Hauschild and Bernhard Schink *

Fakultät für Biologie der Universität Konstanz, Postfach 5560, D-78434 Konstanz, FRG

Abstract: The syntrophically propionate-oxidizing bacterium *Syntrophobacter wolinii* was grown in binary methanogenic coculture with *Methanospirillum hungatei* as sole partner, free of contaminating *Desulfovibrio vulgaris*. Substrate tests revealed that *S. wolinii* could grow also without any syntrophic partner, either with pyruvate as sole substrate or with propionate plus sulfate. Pyruvate was fermented to acetate, propionate and presumably CO_2; propionate in the presence of sulfate was oxidized incompletely to acetate and CO_2, with stoichiometric sulfide formation. The pure culture contained cytochromes *b* and *c* and menaquinone-7. Desulfoviridin or desulforubidin could not be detected.

Key words: Syntrophic fermentation; Methanogenesis; Propionate degradation; Pyruvate fermentation; Sulfate reduction; Syntrophobacter wolinii

Introduction

In the absence of external electron acceptors such as oxygen, nitrate, iron(III), or sulfate, anaerobic oxidation of propionate occurs only in syntrophic association with hydrogen-scavenging methanogenic bacteria [1–3]. Oxidation of propionate to acetate and hydrogen is a highly endergonic process (calculations of free energy changes after [4]):

$$CH_3CH_2COO^- + 2\ H_2O \longrightarrow CH_3COO^- + CO_2 + 3\ H_2$$

$\Delta G^{0\prime} = +76.0$ kJ per mol propionate

The hydrogen partial pressure has to be kept low by an, e.g., methanogenic partner organism to make the overall reaction energetically feasible:

$$4\ CH_3CH_2COO^- + 2\ H_2O \longrightarrow 4\ CH_3COO^- + CO_2 + 3\ CH_4$$

$\Delta G^{0\prime} = -26.5$ kJ per mol propionate

However, the amount of free energy liberated during syntrophic propionate oxidation is still very low and just in the range of the minimum energy quantum needed for ATP formation by each partner bacterium [3]. Until now, only one defined syntrophic propionate degrading culture, *Syntrophobacter wolinii*, has been described [2] which contains both a methanogenic and a sulfate-reducing partner bacterium. Because of the unusual difficulties with growth of such 'obligately syntrophic' bacteria, studies on the kinetics and biochemistry of fermentative propionate

* Corresponding author. Tel: +49 (7531) 882–140; Fax: +49 (7531) 882-966; email: bernhardschink@uni-Konstanz.de.

[1] Present address: Gesellschaft zur Förderung der naturwissenschaftlich-technischen Forschung e.V., Schiepziger Str. 59, 06120 Halle/Salle, FRG.

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degradation have mostly been carried out with enrichment cultures [5–7] or sediment and sludge samples [8].

Here we report on cultivation of *Syntrophobacter wolinii* in defined binary coculture with a methanogenic bacterium and in pure culture, which will allow detailed biochemical studies on this bacterium in the future.

Materials and Methods

Sources of organisms

Syntrophobacter wolinii (DSM 2805), a binary coculture with *Desulfovibrio vulgaris* G11, was obtained from Deutsche Sammlung von Mikroorganismen (DSM) in Braunschweig, FRG. *Methanospirillum hungatei* SK was kindly provided by Prof. Dr. F. Widdel, Bremen, FRG.

Cultivation and isolation

All procedures for cultivation and isolation were essentially as described in earlier papers [9]. The mineral medium for cultivation and isolation contained 30 mM sodium bicarbonate as buffer, 1 mM sodium sulfide as reducing agent, the trace element solution SL 10 [10], a selenite-tungstate-solution [11] and a 7-vitamins solution [9]. The medium contained 0.5 g NaCl and 0.4 g $MgCl_2 \times 6$ H_2O per liter. The pH was 7.2–7.4. Addition of small amounts of sodium dithionite [9], either as dry powder or from a sterile stock solution, to a final concentration of about 50–100 μM, made growth much more reproducible and shortened lag times considerably. For isolation of pure cultures, the agar shake culture method [12] or analogous dilution series in liquid medium in the presence of 5 mM bromoethanesulfonate were applied. Incubation temperature was 28°C.

M. hungatei was grown in half-filled serum bottles in the above medium with 5 mM sodium acetate under H_2/CO_2 (80/20).

Characterization

Cytochromes were determined in cell-free extracts and cell subfractions obtained by ultracentrifugation (1 h at 120,000 $\times g$).

Fractions were subjected to difference spectroscopy (dithionite-reduced minus air-oxidized) in an Uvikon 860 spectrophotometer (Kontron, Zürich, Switzerland), and quantified after [13]. Isoprenoid quinones were extracted from dry cells according to [14] using petroleum ether : methanol (2 : 1 v/v) as solvent. They were separated and isolated by analytical and preparative thin layer chromatography. Quinones were identified by HPLC analysis after [15].

For determination of desulforubidin, cells grown on propionate plus sulfate (6 g wet weight) were suspended in 6 ml 10 mM Tris/HCl, pH 7.6, and broken in a French pressure cell. The supernatant obtained after centrifugation at 5000 $\times g$ for 15 min and ultracentrifugation (1 h at 120,000 $\times g$) was applied to an anion exchange column HiTrap™Q (Pharmacia BioSysterms, Freiburg, FRG) equilibrated with the same buffer. Proteins were eluted with a linear gradient (10–500 mM Tris/HCl in 2 h, HiLoad™System, Pharmacia). Desulforubidin was identified by its typical absorption spectrum at 300–700 nm [16]. Desulfoviridin was determined according to [9].

Chemical determinations

Propionate and acetate were assayed by gas chromatography as described [17], methane by gas chromatography according to [18]. Sulfide was determined after [19].

Chemicals

All chemicals used were of analytical grade quality and obtained from Fluka, Neu-Ulm, Merck, Darmstadt, Sigma, Deisenhofen and Serva, Heidelberg, FRG.

Results

Growth of Syntrophobacter wolinii

The binary mixed culture *Syntrophobacter wolinii / Desulfovibrio vulgaris* could be grown in a defined mineral medium without complex additions. From the constantly growing sulfidogenic coculture transfers were made into sulfate-free medium by adding 10% volume of a well-grown *Methanospirillum hungatei* culture as hydrogen scavenger. Growth of *S. wolinii* in this methano-

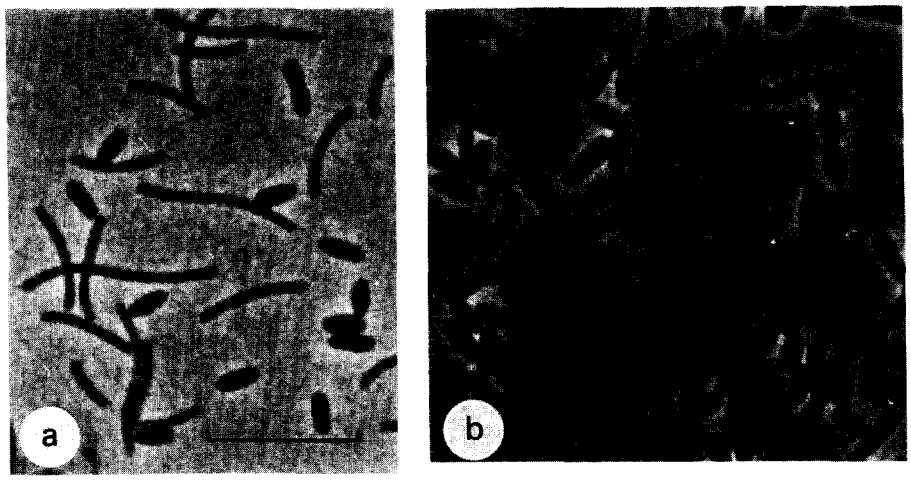

Fig. 1. Phase contrast photomicrograph of *Syntrophobacter wolinii* (a) in binary mixed culture with *Methanospirillum hungatei*, grown with 20 mM propionate, (b) in pure culture, grown with 10 mM pyruvate. Bar equals 10 μm for both panels.

genic coculture was distinctly slower than in association with *D. vulgaris*, but a reproducibly growing culture could be obtained if cultures were reduced with sodium dithionite. The number of highly motile *Desulfovibrio* cells decreased significantly with every transfer. After 10–12 transfers in medium without sulfate, sulfate-reducing bacteria could no longer be detected by microscopic control. Since *S. wolinii* did not grow in agar shake cultures, the enrichment culture thus obtained was diluted in 1 : 10 steps in liquid medium in the presence of a background lawn of *M. hungatei* cells. Transfers were made into a second dilution series from the last positive tube grown up after 12 weeks of incubation (Fig. 1a).

After inoculation into a test medium containing 10 mM propionate, 10 mM sulfate, 5 mM bromoethansulfonate as inhibitor of methanogenesis, and 0.05% yeast extract, turbidity developed after 4 weeks of incubation. To our surprise, the culture mainly contained *S. wolinii*-cells together with few non-motile *M. hungatei*-cells; cells of *Desulfovibrio* could not be detected. Acetate and sulfide were determined as products of substrate conversion (Table 1).

Further substrate tests with other medium additions revealed that *S. wolinii* could also use pyruvate as energy and carbon source, in the absence of a syntrophic partner. Several transfers into medium with pyruvate and 5 mM bro-

Table 1

Stoichiometry of propionate, propionate plus sulfate, and pyruvate metabolism by mixed and pure cultures of *Syntrophobacter wolinii*

Substrate	Substrate added [μmol]	Products formed [μmol]				Electron recovery [a] [%]
		Acetate	Propionate	Methane	Sulfide	
Propionate [b]	1920	1790	0	1390	n.d. [c]	94.8
Propionate [d]	850	800	0	n.d.	640	97.2
+ Sulfate	1000					
Pyruvate [d]	1000	930	150	n.d.	n.d.	85.0

Experiments were carried out in 120 ml infusion bottles in duplicates. [a] Cell dry matter formed was not included; [b] in syntrophic methanogenic coculture with *Methanospirillum hungatei*; [c] not determined; [d] in pure culture.

methanesulfonate increased the number of *Syntrophobacter* cells over those of *Methanospirillum* in a way that separation of the former from the latter was possible again in liquid dilution series as described above. The pure culture fermented pyruvate to acetate, propionate and CO_2; no formate formation could be detected (Fig. 1b; Table 1).

The binary methanogenic mixed culture grew at a very low growth rate of $\mu = 0.022\ d^{-1}$ ($t_d = 31$ d, Fig. 2) as compared to growth with pyruvate as substrate or with propionate plus sulfate ($\mu = 0.062\ d^{-1}$, $t_d = 11$ d; Fig. 3). Fermentation balances were measured with all substrates (Table 1).

Cytochromes and quinones

Syntrophically grown cells of *S. wolinii* contained cytochromes of the *b*- and *c*-type. Cytochrome *c* (maxima at 419, 523, and 552 nm) could be detected in the soluble fraction of crude cell extract, whereas a cytochrome *b* (maxima at 428, 530 and 560 nm) was membrane-associated.

From cells grown in methanogenic coculture, menaquinone-7 could be isolated as sole isoprenoid quinone. In cells of a pure culture of *M. hungatei*, no lipophilic substance with similar chromatographic properties could be detected.

Neither desulfoviridin nor desulforubidin could be detected in cell-free extracts and enriched fractions of the pure culture grown with propionate and sulfate.

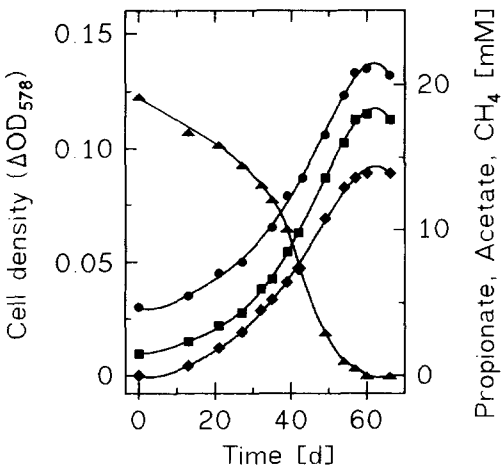

Fig. 2. Growth of *Syntrophobacter wolinii* in binary methanogenic coculture with propionate as substrate. Symbols: (●) cell density; (▲) propionate; (■) acetate; (◆) sulfide.

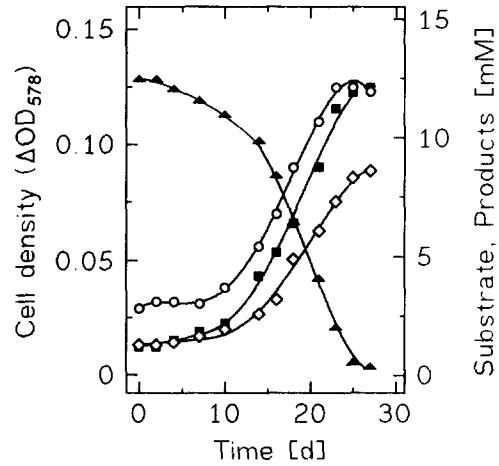

Fig. 3. Growth of *Syntrophobacter wolinii* with propionate plus sulfate. Symbols: (○) cell density; (▲) propionate; (■) acetate; (◇) methane.

Discussion

Our studies show that *S. wolinii* can couple propionate oxidation to reduction of sulfate instead of proton reduction. Reduction of external electron acceptors by a syntrophic propionate oxidizer had been demonstrated recently also for an enrichment culture which couples propionate oxidation with fumarate reduction to succinate [20]. Our results confirm that *S. wolinii* is actually a sulfate-reducing bacterium which can couple propionate oxidation as well with proton reduction. 16 S-rRNA analysis has revealed recently that this bacterium indeed is phylogenetically closely related to *Desulfomonile, Desulfobulbus*, and other sulfate-reducing bacteria [21]. It is unclear why this bacterium prefers electron release to hydrogenotrophic partners even in the presence of sulfate as the sulfidogenic coculture with *D. vulgaris* demonstrates. Like other sulfate reducers, *S. wolinii* can also ferment pyruvate in the absence of sulfate; in this case, the propionate oxidation pathway is obviously used in the opposite direction.

Neither desulfoviridin nor desulforubidin could be detected in the sulfate-grown pure culture; the type of sulfite reductase present in this strain is still unknown.

The question remains now what really differentiates *S. wolinii* from other incompletely propionate-oxidizing sulfate reducers such as *D. propionicus* [22] or *D. elongatus* [23]. *D. propionicus* could not couple propionate oxidation with proton reduction in syntrophic coculture (Widdel, pers. commun.). Obviously, this bacterium lacks suitable electron carrier systems for reduction of protons ($E'_0 = -414$ mV) with electrons released, e.g., in the succinate dehydrogenase reaction ($E'_0 = +30$ mV). Involvement of a reversed electron transport has been discussed earlier for syntrophic butyrate oxidation [24] and was also postulated for syntrophic propionate oxidation [3]. Whether the cytochromes and quinones detected in *S. wolinii* in this study really act as electron carriers in such a reversed electron transport system will have to be revealed in the future.

The pure culture of *S. wolinii* has been deposited with the Deutsche Sammlung von Mikroorganismen (DSM) GmbH, Braunschweig, under the number DSM 2805.

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