

Fructose Degradation by *Desulfovibrio* sp. in Pure Culture and in Coculture with *Methanospirillum hungatei*

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Abstract. In a mineral medium containing sulfate, the sulfate-reducing bacterium *Desulfovibrio* sp. strain JJ degraded 1 mol of fructose stoichiometrically to 1 mol of H₂S, 2 mol of acetate, and presumably 2 mol of CO₂. The doubling time was 10 h, and the yield was 41.6 g dry weight/mol fructose degraded. In the absence of sulfate, the hydrogenophilic methanogen *Methanospirillum hungatei* replaced sulfate as hydrogen sink. In such cocultures, 1 mol of fructose was converted to acetate, methane, succinate, and presumably CO₂ in varying concentrations. The growth yield of the H₂-transferring association was 33 g dry weight/mol fructose. In the absence of sulfate, *Desulfovibrio* strain JJ slowly fermented 1 mol of fructose to 1 mol of succinate, 0.5 mol of acetate, and 0.5 mol of ethanol. The results are compared with those of other anaerobic hexose-degrading bacteria.

Sulfate-reducing bacteria (SRB) are known as typical organisms which end-oxidize organic matter to CO₂ in strict anaerobic sulfate-containing natural environments [11, 13, 21, 26]. Their metabolism is specialized in degrading the products excreted by fermenting bacteria: acetate, propionate, butyrate, and hydrogen are their main substrates in anaerobic sediments [23]. They also degrade other fermentation products like alcohols, long-chain fatty acids, and lactate [20, 27]. The carbohydrate degradation in natural environments has never been shown to occur directly via SRB.

Only *Desulfotomaculum nigrificans* has been documented to degrade fructose [12] or autoclaved glucose [1], but no other carbohydrates. (Filter-sterilized glucose was not degraded by this species [12]; this indicates isomerization of glucose by heat, partially to fructose [12, 17].) However, growth of *D. nigrificans* on fructose is very poor and slow (R. Klemps, personal communication). Furthermore, *Desulfotomaculum* species, which are all sporulating rods, are more closely related to the fermenting and homoacetogenic bacteria of the genus *Clostridium* than the other SRB, as evidenced by 16S-rRNA homology studies [12], by their gram-positive cell wall structure [22], by their capability to reduce CO₂ to acetate [12], and by the fact that they are sporulated.

Members of the genus *Desulfovibrio* have never been shown to degrade carbohydrates in pure culture [20].

Materials and Methods

Organisms. *Desulfovibrio* strain JJ was obtained by repurification of a culture of a *Desulfovibrio* strain isolated by J.W. Jones (University of Illinois) [10]. *Methanospirillum hungatei* (DSM 864) was isolated from the defined syntrophic association with *Syntrophus bushwellii* (DSM 2612 TB).

Medium and growth conditions. The anaerobic Hungate technique [8] as modified for the use of syringes [14] was used throughout this study. The anaerobic bicarbonate-buffered, sulfide-reduced medium contained fructose and vitamins as sole organic substances. This medium was composed as described for SRB [27] and prepared as previously described [5]. In the coculture and fermentation experiments, sulfate was omitted. Stock solutions of fructose (0.5 mol/liter) were autoclaved separately.

The cells were cultivated in 300 ml medium with and without gas phase for coculture and monoculture experiments respectively. Cell dry weight of the centrifuged and washed (phosphate buffer, 50 mmol/liter) cells was determined after drying at 60°C. All chemicals used were of reagent quality.

Analytical methods. All determinations were repeated twice. Sulfide was determined spectrophotometrically as colloidal CuS [4]. Methane and alcohols were determined by gas chromatography (Varian Aerograph 2700; injection: 250°C; column: 3 m × ¼ in. stainless steel, Porapak Q 80-100 mesh, 215°C; carrier gas: N₂, flow rate: 30 ml/min; detection: flame ionization 245°C).

Fructose and organic acids were measured by HPLC

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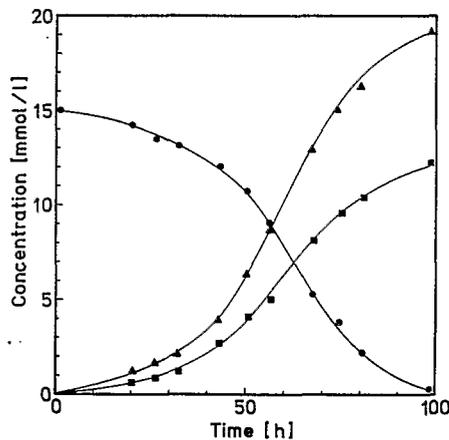


Fig. 1. Time course of fructose degradation and end-product formation by *Desulfovibrio* sp. strain JJ in the presence of sulfate: ●—●, fructose; ▲—▲, acetate; and ■—■, sulfide.

(pump: Analprep 93, Touzart et Matignon, Vitry sur Seine, France; flow rate: 0.6 ml/min; injection loop: 100 μ l; column: Amines Ion-Exclusion HPX-87H, 300 \times 7.8 mm, Bio-Rad, Richmond, California; column temperature: 40°C; detection: differential refractometer, Knauer, Berlin; recorder integrator: Chromatopak C-R3A, Shimadzu, Kyoto).

Purity tests. The purity of the isolate was tested in the same medium as described above, with addition of 1% glucose + 1% yeast extract + 1% biotrypcase. Furthermore, all isolated colonies were examined microscopically.

Purification technique. The strain was repurified by repeated application of the agar shake dilution technique [27], modified by using Hungate tubes and sterile syringes after each dilution step. Agar was washed five times with distilled water. The strain was also repurified on H_2 , according to the anaerobic roll tube technique [9]. In this case, "Noble-Agar" (Difco), acetate (1 mM), and vitamins were the only organic compounds.

Results

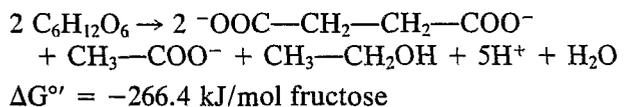
The *Desulfovibrio* sp. isolated by J.W. Jones [10] and called *D. vulgaris* strain JJ was previously used to serve as H_2 consumer in mixed defined cultures with the homoacetogenic *Sporomusa acidovorans* [17] on fructose (unpublished results). Sulfate was reduced to sulfide by this coculture as well as by the control essay, containing fructose and inoculated only with *Desulfovibrio* strain JJ. After two days of incubation, strain JJ was grown to a higher optical density (OD = 0.9 at 580 nm) than the pure culture of *S. acidovorans* (OD = 0.7).

The *Desulfovibrio* strain also grew well on lactate and hydrogen (+1 mM acetate) and poorly on ethanol and methanol. In order to confirm the purity

of the culture, strain JJ was cultivated parallel on lactate, ethanol, methanol, hydrogen (+1 mM acetate), and fructose; and after three transfers of each essay, the strain was repurified on the relative substrate as described in *Materials and Methods*. All isolates were morphologically uniform (motile vibrios). No isolate grew in the purity test medium. After transfer into fructose medium, all isolates degraded fructose without lag phase. The isolate finally used was obtained from a single colony of the last (7th) dilution step of a rolled agar tube [17] with H_2 as the energy source. The colony was suspended aseptically in liquid mineral medium and was diluted by successive transfers into a series of sterile tubes containing H_2 as the sole energy source. The last positive tube (11th) of this third dilution series served as the inoculum for the following experiments.

Fructose was degraded to acetate, sulfide, and presumably to CO_2 (Fig. 1). Traces of succinate were found after the growth in fructose-rich (20 mM) medium (Table 1). The high cell yield (about 41.6 g dry weight/mol fructose) accounted for about 28% of the whole reducing equivalents from the substrate incorporated into cell mass. The doubling time of strain JJ as calculated from fructose disappearance was 10 h.

In the absence of sulfate, strain JJ fermented fructose to acetate, succinate, and ethanol (Table 1) according to the following equation:



Under these conditions the growth of *Desulfovibrio* sp. strain JJ was extremely slow (t_d 450 h). However, even after two months of incubation most of the cells were motile. The addition of sulfate to such a two-month-old culture of strain JJ led to the degradation of the ethanol produced but not to succinate degradation.

In the absence of sulfate, the hydrogen-consuming methanogenic bacterium *Methanospirillum hungatei* served as an alternative acceptor of reducing equivalents liberated by strain JJ from fructose degradation to acetate. At the beginning of the incubation, this coculture produced only acetate, CH_4 , and presumably CO_2 as end-products (Fig. 2). When about 2 mmol of fructose per liter were degraded, succinate appeared as a further end-product. However, the ratio of the end-products was not

Table 1. Results of experiments of anaerobic fructose degradation by *Desulfovibrio* strain JJ in the presence of sulfate (lines 1–4) in the absence of sulfate (lines 9–10), and in the absence of sulfate in the presence of *Methanospirillum hungatei* (lines 5–8)

Line	Fructose degraded	OD (580 nm)	Dry weight (g/liter)	Y (g/mol)	Fructose assim.	Acetate	H ₂ S (S) or CH ₄ (M)	Succinate	Ethanol	e ⁻ Recovery
1	5.0	0.45	0.207	41.4	1.42	6.9	3.9 (S)	0.0		100
2	10.0	0.80	0.417	41.7	2.87	14.7	8.3 (S)	0.0		105
3	15.0	0.97	0.577	38.5	3.97	18.8	12.8 (S)	0.0		96
4	20.0	1.10	0.593	29.7	4.08	27.3	16.2 (S)	0.2		93
5	5.0	0.42	0.177	35.4	1.22	5.1	3.5 (M)	1.0		93
6	10.0	0.70	0.313	31.3	2.15	9.2	6.5 (M)	3.4		93
7	15.0	0.90	0.365	24.3	2.51	12.4	8.5 (M)	6.3		87
8	17.8 ^a	0.98	0.438	24.6	3.01	15.0	9.0 (M)	11.4		99
9	5.0	0.15	n.d. ^b	13.2 ^c	0.53	2.0		4.2	1.3	91
10	6.9 ^a	0.22	n.d. ^b	14.0 ^c	0.66	2.6		5.8	2.1	85

Fructose was the sole carbon and energy source. Highest growth yield was obtained on 2.5 mM fructose (45 g/mol calculated from optical density [OD]).

^a Fructose given (20 mM and 10 mM) was not completely degraded.

^b Not determined.

^c As calculated from optical density.

constant: The relative portion of succinate increased with the amount of fructose degraded (Fig. 2 and Table 1). Besides acetate, methane, and succinate, no other organic compound was produced. The doubling time of this coculture was 21 h. The maximum cell yield was 35.4 g dry weight/mol fructose degraded.

Discussion

In natural anoxic environments, carbohydrates are generally degraded by fermentative bacteria, resulting in the formation of fermentation products such as fatty acids, H₂, lactate, or alcohols.

Our results show that the direct use of carbohydrates by a strain of the genus *Desulfovibrio* is possible: *Desulfovibrio* strain JJ oxidized fructose stoichiometrically to acetate and presumably CO₂ during sulfate reduction to sulfide. The growth rate obtained (10 h) corresponded to that of fructose-degrading homoacetogenic bacteria [24, 28].

Because of the high yield of 41.6 g dry weight/mol fructose, the assimilated substrate had to be considered before the equation of the reaction was established. The empirical formula for bacterial dry mass is C₄H₈O₂N [7]. A simpler formula with the same carbon-oxidation state and nearly the same molecular weight (102:103) can be obtained by replacing the bound NH₃ by H₂O: C₄H₇O₃. This formula is used in assimilation equations [24, 25]. The assimilation equation for fructose is:

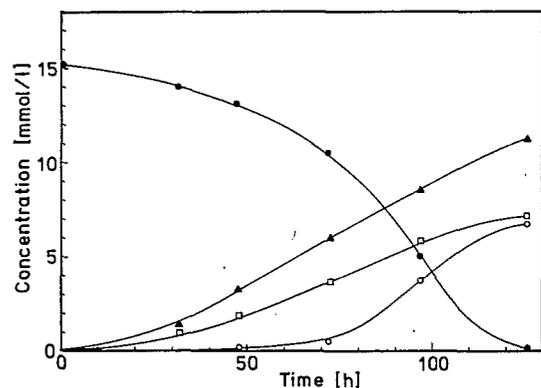
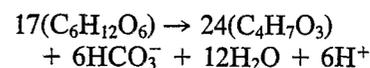
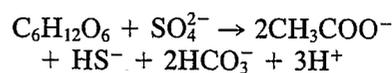


Fig. 2. Time course of fructose degradation and end-product formation by the coculture of *Desulfovibrio* sp. strain JJ and *Methanospirillum hungatei* in the absence of sulfate: ●—●, fructose; ▲—▲, acetate; □—□, methane; and ○—○, succinate.



Corresponding to this equation, 1 g of cell dry weight equals 6.88 mmol of fructose assimilated. When 41.6 g dry weight were produced/mol fructose degraded, this corresponded to 28.6% of fructose assimilated.

If one considers 28.6% of fructose assimilated, the pure dissimilatory fructose degradation corresponded approximately to the equation:



Therefore, 1 mol of fructose seems formally to be fermented to 4 mol of H₂ and 2 mol of acetate, and the hydrogen then oxidized with 1 mol of SO₄²⁻ as the electron acceptor. This assumption corresponds to the theory of hydrogen cycling involved in *Desulfovibrio* species [16, 19], and was verified by the coculture experiment with the H₂-oxidizing *M. hungatei*, which served as an alternative hydrogen sink. The way of fructose degradation via acetate, hydrogen, and CO₂ appears also in homoacetogenic bacteria [6, 18, 28].

The growth yield obtained was comparable to that of homoacetogenic bacteria grown on fructose [24]. This indicates similar ATP gains for both sulfate reduction to sulfide (1 to 1.3 ATP/sulfate [2, 15]) and CO₂ reduction to acetate, provided glycolysis and pyruvate degradation to acetate yield generally the same amounts of ATP. The rate of fructose degradation by *Desulfovibrio* strain JJ was similar to that of homoacetogenic bacteria (Table 1).

Hydrogenophilic methanogens are able to remove the intermediary H₂ formed from homoacetogenic bacteria-degrading organic products [6, 28]. In the absence of sulfate and in the presence of hydrogen-consuming methanogens, sulfate-reducing bacteria also transfer their reducing equivalents from lactate or ethanol degradation to the methanogen [3]. All these interspecific H₂-transferring cocultures produce only acetate, CO₂, and CH₄.

When grown on fructose in the absence of sulfate, *Desulfovibrio* strain JJ was able to use the H₂-consuming *M. hungatei* as alternative H₂ sink. However, this coupling was not perfect: besides CO₂, CH₄, and acetate, succinate was also produced. Therefore, *Desulfovibrio* sp. strain JJ used a part of the reducing equivalents to reduce oxaloacetate to malate and fumarate to succinate rather than protons to H₂. This is thermodynamically more favorable than ethanol or lactate production and should gain a further ATP via electron transport phosphorylation. (In the presence of both sulfate and fumarate as external electron acceptors, strain JJ reduced fumarate to succinate instead of sulfate to sulfide [data not shown].) This incomplete interspecific hydrogen-transfer indicates a limitation of the process by H₂-consumption rather than by glycolysis.

In the absence of a suitable external electron sink, the growth of *Desulfovibrio* strain JJ on fructose was extremely slow (t_d = 250 h). The fermentation of fructose to succinate, acetate, and ethanol by this strain could not play a significant ecological role. However, in sulfate-free anaerobic environ-

ments, the hydrogen partial pressure is lowered by methanogenic bacteria; this would allow a more rapid degradation of the sugar by means of an interspecific hydrogen transfer.

Desulfovibrio strain JJ has been deposited in the *Deutsche Sammlung für Mikroorganismen* (DSM), Göttingen, FRG, under the number DSM 3604. Further taxonomical studies of this strain are in progress.

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