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Desulfovibrio alcoholovorans sp. nov., a sulfate-reducing bacterium able to grow on glycerol, 1,2- and 1,3-propanediol

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Abstract. *Desulfovibrio* strain SPSN was isolated from an anaerobic industrial fermenter fed with waste water from the alcohol industry. The isolate was a gram-negative, non-spore-forming, curved organism, the motility of which is provided by a single polar flagellum. The oxidation of substrates was incomplete and included glycerol and 1,3-propanediol. Sulfate, sulfite, thiosulfate, and sulfur were utilized as electron acceptors. Pyruvate, fumarate and malate could be fermented. The DNA base composition was $64.5 \pm 0.3\%$ G + C. Cytochrome c_3 and desulfoviridin were present. On the basis of these characteristics and because strain SPSN could not be ascribed to any of the existing species, the isolate is established as a new species of the genus *Desulfovibrio*, and the name *Desulfovibrio alcoholovorans* is proposed.

Key words: Sulfate reduction – Fermentation – Glycerol – 1,2-Propanediol – 1,3-Propanediol – *Desulfovibrio*

Among the sulfate-reducing bacteria (SRB), the "classical" *Desulfovibrio* species are known to have a limited range of oxidizable substrates including hydrogen, ethanol, lactate, formate, malate, fumarate and succinate (Postgate 1984). With the description of new isolates, the number of energy substrates known to be utilized by the various sulfate-reducing bacteria has increased by now to over 75 compounds (Hansen 1988; Widdel 1988). Recently, *Desulfovibrio* strains have been isolated that utilize amino acids (Stams et al. 1985), methanol and other alcohols (Braun and Stolp 1985; Nanninga and Gottschal 1986, 1987; Kremer and Hansen 1987), fructose (Ollivier et al. 1988) and saccharose (Joubert and Britz 1987). Sulfate-dependent glycerol oxidation has quite recently been proved unequivocally (Stams et al. 1985; Nanninga and Gottschal 1986, 1987; Kremer and Hansen 1987; Esnault et al. 1988; Ollivier et al. 1988; Qatibi and Garcia 1989; Qatibi et al. 1989), but few reports have been published to date on the diol utilization by *Desulfovibrio* (Qatibi 1990).

The following recently isolated species resemble the "classical" *Desulfovibrio* strains in all but some of their properties: *Desulfovibrio carbinolicus* (Nanninga and Gottschal 1986, 1987) is non-motile and is the only species with *Desulfovibrio fructosovorans* (Ollivier et al. 1988) able to utilize methanol as an energy source. Like *D. fructosovorans*, it disproportionates glycerol in the absence of sulfate into 3-hydroxypropionate and 1,3-propanediol. In the presence of sulfate, *D. carbinolicus* oxidizes glycerol to 3-hydroxypropionate, whereas *D. fructosovorans* oxidizes glycerol to acetate in the presence of sulfate. Both strains oxidize 1,3-propanediol to 3-hydroxypropionate (Nanninga and Gottschal 1987; this study). In the course of our study on the role of sulfate-reducing bacteria in anaerobic glycerol and 1,3-propanediol degradation in waste water from bioethanol production plants, several strains of *Desulfovibrio* able to grow on alcohols were isolated (Qatibi 1990). In the present study we report the isolation and characterization of *Desulfovibrio* strain SPSN which is able to oxidize 1,3-propanediol to acetate.

Materials and methods

Source of organisms

Desulfovibrio strain SPSN was isolated from a pilot fermenter containing alcohol distillery waste water. Apart from lactate, glycerol is the chief by-product present in the waste water of bioethanol production plants (INRA, Narbonne, France); *Desulfovibrio carbinolicus* (DSM 3852) and *Desulfovibrio fructovorans* (DSM 3604) were purchased from the German Collection of Microorganisms in Braunschweig, FRG.

Media and growth conditions

The anaerobic Hungate technique (Hungate 1960) as modified for use with syringes (Macy et al. 1972) was used throughout this study.

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Strain SPSN was cultured on medium containing per liter: Na_2SO_4, 3.0 g; KH_2PO_4, 0.2 g; NH_4Cl, 0.3 g; KCl, 0.3 g; $CaCl_2 \cdot 2H_2O$, 0.15 g; $MgCl \cdot 6H_2O$, 0.4 g; NaCl, 1.2 g; 1 ml trace element solution SL10 (Imhoff-Stuckle and Pfennig 1983); 1 mg resazurin. After autoclaving at 110°C for 40 min, the medium was cooled under a stream of O_2-free N_2-CO_2 (80–20%) and then reduced with 0.1 ml of $Na_2S_2O_4$ solution (0.2 mM). $NaHCO_3$ (30 mM) and $Na_2S \cdot 9H_2O$ (1.5 mM) from separately sterilized anoxic solutions, and 1 ml/l of filter-sterilized vitamin solution (Pfennig 1978) were added. The medium was finally adjusted to pH 7.2–7.3 and distributed into Hungate tubes as described previously (Widdel and Pfennig 1981). Growth was monitored in Hungate tubes at 580 nm. All characterization tests were carried out in at least duplicate cultures.

Isolation

Pure cultures were obtained by repeatedly applying the agar shake culture method (Pfennig et al. 1981) in anaerobic tubes.Tubes were gassed with N_2/CO_2 (80/20 v/v) and closed with butyl rubber stoppers. Purity was routinely checked by examining wet mounts and inoculating the basal sulfate free medium containing 1% glucose, 1% yeast extract and 1% Biotrypcase (Biomérieux, Craponne, France).

Cell fractionation

Cells (3 g wet weight) were suspended in 10 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mg pancreatic deoxyribonuclease I and disrupted by passing them 3 times through a French pressure cell (Aminco) at 1atm. The broken cell suspension was centrifuged at 25000 *g* for 20 min to remove cell debris. The resulting cell-free extract was separated into a supernatant and a particulate fraction by centrifugation at 140000 *g* for 2 h. The supernatant was taken to constitue the soluble fraction. The dark gelatinous pellet was resuspended in the same buffer and taken to constitute the particulate fraction. Cell-free extracts were examined for cytochrome and desulfoviridin using a Carry 219 recording spectrophotometer. Protein was estimated using the method described by Lowry et al. (1951) with bovine serum albumin as the standard.

DNA extraction

Whole-cell DNA was extracted after disruption of the cells and purified as described by Marmur (1961) at the German Collection of Microorganisms (DSM), Braunschweig, FRG; the mol% G + C of the DNA was determined using HPLC technique. HPLC was calibrated with non-methylated LAMBDA virus DNA (= 49.858 mol% G + C).

Transmission electron and phase contrast micrographs

A culture from the exponential growth phase was used. A drop of the centrifuged culture was placed on a Formvar-coated, carbon reinforced grid (200 mesh), and excess fluid was drawn off with filter paper. Cells were stained with 1% (w/v) sodium phosphotungstate solution (pH 7) for 5 s. Preparations were examined using a TEM Hitachi H600. Phase contrast micrographs were taken on agar slides with a Nikon Optiphot.

Analytical techniques

Glycerol, diols and non-volatile fatty acids were analysed by HPLC measurements [column: Aminex HPX-87H (BIORAD, Richmond); detector: differential refractometer (Knauer); external standard]. Volatile fatty acids were analysed as described previously (Qatibi et al. 1990). Sulfide was measured spectrophotometrically in the form of collodial CuS (Cord-Ruwisch 1985). All chemicals used were of reagent quality.

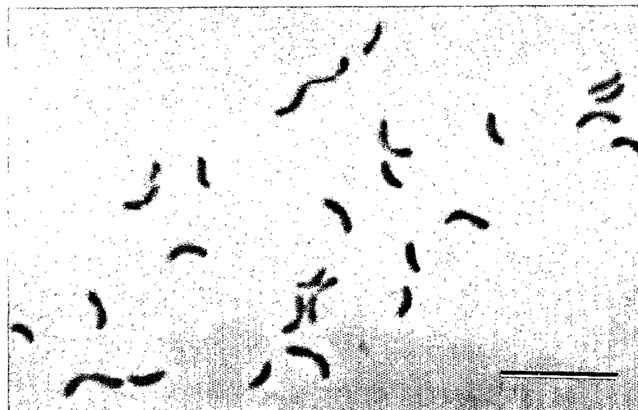

Fig. 1. Phase contrast photomicrograph of strain SPSN grown on 1,3-propanediol (10 mM) with sulfate (15 mM). *Bar,* 10 µm

Results

Purification of a 1,3-propanediol-degrading sulfate reducer

Purification of strain SPSN via agar dilution series was carried out from the last positive tubes used in MPN counting of bacteria containing 1,3-propanediol in the presence of sulfate (Qatibi et al. 1990) using the repeated agar shake serial dilutions in 1,3-propanediol in the presence of sulfate. Under these conditions, lens-shaped, maroon-coloured colonies were observed after 1 week at 35°C; the selected colonies were capable of growing on this substrate; acetate, sulfide and presumably carbon dioxide were produced. The bacterial counts indicated the predominance of morphologically very similar types of SRB (Qatibi 1990). Three strains were isolated and subsequently found to be physiologically similar.

Morphology

When growing on 1,3-propanediol (10 mM) as carbon and energy source in the presence of sulfate (15 mM), the prevailing organisms were motile vibroid rods (Fig. 1) possessing single polar flagella (Fig. 2). They became spirilloid in aging cultures. The cells were 0.7–0.9 µm in diameter and 2.8–3.2 µm in length. They occurred either single or in pairs. With a mixture of glycerol (10 mM) and lactate (10 mM) and in the presence of sulfate (20 mM), their morphology changed and became elongated; in this case the cells were 0.5–0.7 µm in diameter and 12–16.4 µm in length (Fig. 3). The cells stained gram-negative. No spores were ever observed.

Substrates and optimal conditions

Nutritional studies were performed at 35°C. Growth occurred in the absence of vitamins. Elemental sulfur,

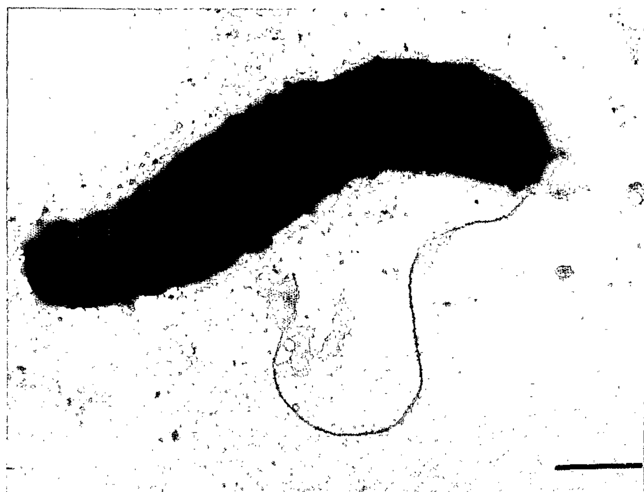

Fig. 2. Transmission electron micrograph of strain SPSN growing on 1,3-propanediol (10 mM) with sulfate (15 mM). *Bar*, 1 μm

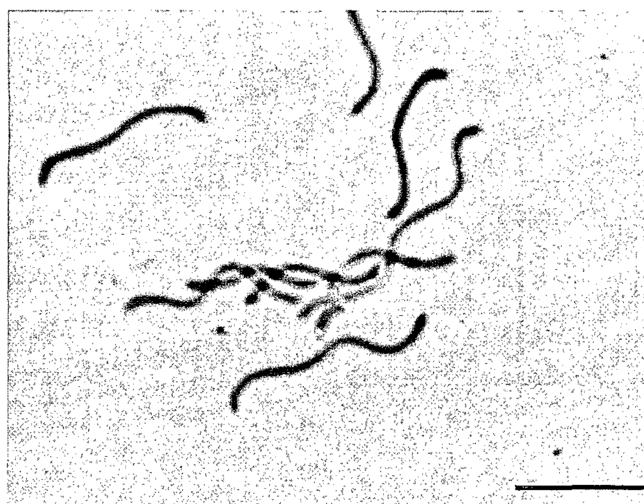

Fig. 3. Phase contrast photomicrograph of strain SPSN growing on a mixture of glycerol (10 mM) and lactate (10 mM) in the presence of sulfate (20 mM). *Bar*, 10 μm

sulfate, thiosulfate and sulfite served as electron acceptors. The isolate did not reduce nitrate. The compounds used as electron donors in sulfate-containing medium are summarized in Table 1. No growth was observed on sugars, amino acids or saturated fatty acids. The utilization of molecular hydrogen, formate and methanol required acetate as carbon source. However with the latter substrate, a slight increase in the turbidity was observed (Table 1). In the presence of sulfate, glycerol, DHA (dihydroxyacetone), 1,3-propanediol, ethanol, pyruvate, fumarate, succinate, malate and lactate were incompletely oxidized into acetate and carbon dioxide. 1,2-propanediol was oxidized to acetate and propionate. Propanol-1, butanol-1 and pentanol-1 were oxidized to propionate, butyrate and valerate, respectively. Formate and methanol were oxidized to carbon

Table 1. Utilization of various substrates as electron donors by strain SPSN in the presence of sulfate

Substrates [mM]	Final OD 580 nm	Products
Glycerol (10)	0.26	acetate
DHA (10)	0.32	acetate
1,3-Propanediol (10)	0.28	acetate
1,2-Propanediol (10)	0.20	acetate + propionate
1,4-Butanediol (10)	0.16	succinate
Pentanol-1 (10)	0.14	valerate
Propanol-1 (10)	0.14	propionate
Butanol-1 (10)	0.17	butyrate
Ethanol (10)	0.17	acetate
Methanol (10)	0.08	carbon dioxide
Ethylene glycol (10)	0.09	acetate
Formate (10)	0.07	carbon dioxide
H2 + CO2 (3 bars)	0.15	nd
Lactate (10)	0.32	acetate
Pyruvate (10)	0.26	acetate
Fumarate (10)	0.35	acetate
Succinate (10)	0.20	acetate
Malate (10)	0.28	acetate
Control	0.03	nd

The growth medium contained 15 mM sulfate; hydrogen, formate, and methanol were tested in the presence of 5 mM. Substrates tested but not utilized by strain SPSN were as follows (concentrations in mM); 2,3-butanediol (10); butanol-2 (10); propanol-2 (10); cellobiose (10); glucose (10); fructose (10); inositol (10); saccharose (10); xylose (10); mannose (10); lysine (10); tyrosine (10); phenylalanine (10), tryptophan (10); arginine (10); ornithine (10); alanine (10); oxamate (10); oxalate (10); choline (10); mannitol (10) and ribose (10). Growth was also measured by sulfide determination after an incubation period of 3 weeks.
nd: not determined

dioxide. The growth of strain SPSN was extremely slow with formate, methanol and ethyleneglycol. Succinate was found to be one product of 1,4-butanediol oxidation. Fumarate, malate and pyruvate could be fermented. Fumarate and malate were fermented into succinate, acetate and carbon dioxide. Pyruvate was fermented mainly to acetate. The optimum growth temperature of the strain SPSN was estimated to be between 35 and 37°C. No growth was observed at temperatures above 42°C or below 20°C. Growth of the strain was optimal at pH 7. The strain developed between pH 5.5 and 8.5. With various NaCl-concentrations, growth was observed between 0 and 20 g/l with an optimum at 5–10 g/l.

Pigments

When reduced with sodium dithionite, the soluble extract of *Desulfovibrio* strain SPSN exhibited absorption bands characteristic of cytochrome c_3 with maxima at 418, 523 and 552 nm. The oxidized extract showed the cytochrome Soret peak of 408 nm. Sodium ascorbate was unabel to modify the spectrum of oxidized extract. This indicated that the cytochrome present had a low midpoint redox potential. In addition, the spectrum showed a characteristic absorption band at 628 nm indicating the presence

Table 2. Physiological and morphological comparisons between strain SPSN, *Desulfovibrio fructosovorans* and *D. carbinolicus*

Properties	*Strain SPSN*		*D. fructosovorans*		*D. carbinolicus*	
	Growth	Products	Growth	Products	Growth	Products
Electron donors						
Glycerol	+	C2	+[a]	C2	+[a]	3-OHC3
1,2-Propanediol	+	C2	–	nd	–	nd
1,3-Propanediol	+	C2	+	3-OHC3	+	3-OHC3
1,4-Butanediol	+	Succinate	+	Succinate	+	Succinate
Ethylene glycol	(+)	C2	+[b]	C2	+[b]	C2
DHA	+	C2	+	C2	+	C2
Succinate	+	C2	–	nd	+	C2
Fructose	–	nd	+	C2	–	nd
Electron acceptors						
Fumarate	–		+		–	
Mobility	+		+		–	
Length [μm]	2.8–3.2		2.0–4.0[c]		1.5–5.0[d]	
Diameter [μm]	0.7–0.9		0.5–0.7[c]		0.6–1.1[d]	
GC [mol%]	64.5±0.3		64.13[c]		65 ±1.0[d]	

C2, acetate; 3-OHC3, 3-hydroxypropionate; nd, not determined; (+), utilization without growth

[a] During additional experiments, glycerol dissimilation by *D. fructosovorans* and *D. carbinolicus* were found to be quite complex: after several transfers on glycerol in presence of sulfate, glycerol was oxidized into acetate and 3-hydroxypropionate mixture (Qatibi and Garcia 1989)

[b] In the absence of sulfate, *D. fructosovorans* and *D. carbinolicus* fermented ethylene glycol without growth (see the text)

[c] From Ollivier et al. 1988

[d] From Nanninga and Gottschal 1987

of desulfoviridin (Postgate 1956; Lee and Peck 1971). This was confirmed by the fluorescence test for desulfoviridin (Postgate 1959).

DNA base composition

The mol% G+C content of the DNA of strain SPSN was 64.5 ± 0.3% (mean value of three determinations).

Discussion

Strain SPSN is a curved, non spore-forming sulfate-reducing rod. The isolate incompletely oxidizes pyruvate and lactate to acetate and carbon dioxide; neither acetate, propionate nor butyrate are oxidized. On the basis of these characteristics, the isolate can be classified as a member of the genus *Desulfovibrio* (Pfennig et al. 1981; Widdel 1988). As in other strains of this genus, the soluble extract of strain SPSN exhibits absorption bands characteristic of cytochrome c_3 and desulfoviridin (Postgate 1956; Widdel and Pfennig 1984). The isolate does not belong to the *sapovorans* group since it does not use saturated fatty acids. The morphological and physiological characteristics of strain SPSN are similar to those of the species *Desulfovibrio desulfuricans* and *D. vulgaris* (Widdel and Pfennig 1984) or those of the newly described *D. fructosovorans* (Ollivier et al. 1988) and *D. carbinolicus* (Nanninga and Gottschal 1986, 1987). It differs from *D. desulfuricans* in being able to oxidize glycerol and unable to use choline. Unlike *D. vulgaris*, strain SPSN is able to ferment fumarate and malate. Table 2 gives some of the characteristics that clearly distinguish strain SPSN from *D. fructosovorans* and *D. carbinolicus*. The main difference between strain SPSN and *D. fructosovorans* is that the latter is the only known species of *Desulfovibrio* able to use a carbohydrate. Furthermore, in the presence of sulfate, the isolate differs from *D. fructosovorans* in being able to oxidize succinate and to convert 1,2-propanediol or 1,3-propanediol to acetate and propionate or acetate, respectively, and in being unable to use fumarate as terminal electron acceptor.

When grown on glycerol in the absence of sulfate, strain SPSN performed an interspecies hydrogen transfer with the hydrogenotrophic *Methanospirillum hungatei* as hydrogen scavenger (Qatibi et al. 1989) like *D. fructosovorans* with the same substrate and in coculture with *M. hungatei* (Qatibi and Garcia 1989). The isolate differs from *D. carbinolicus* by converting glycerol and 1,3-propanediol in the presence of sulfate, to acetate and sulfide, and in using 1,2-propanediol; glycerol is not fermented and the cells are motile. Furthermore, unlike *Desulfovibrio* strain SPSN, *D. carbinolicus* and *D. fructosovorans* fermented ethyleneglycol in the absence of sulfate, to acetate, ethanol and H_2 without growth. Because ethyleneglycol is generally converted via dehydratase into acetaldehyde, these results indicate that no substrate level phosphorylation is involved in the conversion of acetaldehyde to acetate in these strains (Kremer et al. 1988).

A recent study of the cellular fatty acid composition of about 35 strains of non-sporeforming sulfate reducers (mostly type strains) showed that strain SPSN falls into the same group as some other *Desulfovibrio* species such as *D. carbinolicus, D. giganteus*, a sulfate-reducer able to use glycerol (Esnault et al. 1988) and *D. fructosovorans*

(Hippe, personal communication). These differences seem to justify the proposal that this isolate should be classified as a new species for which the name *Desulfovibrio alcoholovorans* is proposed.

Description of Desulfovibrio alcoholovorans species nova

Desulfovibrio alcoholovorans sp. nov.; al. co. holo. vor. ans. E.m. alcohol; L.V. *voro* to devour; M.L. part. adj. *alcoholovorans* devouring alcohol.

Morphology

Cells are curved rods 0.7–0.9 μm wide and 2.8–3.2 μm long, motile by a single polar flagellum; they occur either singly or in pairs and become spirilloid in aging cultures. Cells are gram-negative; spores were not observed.

Physiology

Optimum growth occurs between 35°C and 37°C. The optimum pH for growth is seven.

Nutrition

Strictly anaerobic. Sulfur, sulfate, sulfite and thiosulfate serve as electron acceptors and are reduced to sulfide. Nitrate is not reduced. Molecular hydrogen, formate, lactate, pyruvate, fumarate, malate, succinate, DHA, glycerol, 1,2-propanediol, 1,3-propanediol, 1,4-butanediol, pentanol-1, butanol-1, propanol-1, ethanol serve as electron donors. Growth with hydrogen, formate and methanol requires acetate as carbon source. Ethyleneglycol, formate and methanol can be used but cause only a slight increase in turbidity. Pyruvate, fumarate and malate are fermented. Not used: acetate, propionate, butyrate, fructose, oxalate, oxamate, choline. Vitamins are not required for growth. Optimum growth with 0.75% (w/v) NaCl, inhibition at 2% and above.

Pigments

Desulfoviridin and cytochrome c_3 are present.

DNA base ratio

$64.5 \pm 0.3\%$ mol% G + C.

Type strain

Strain SPSN has been deposited with the German Collection of Microorganisms, Braunschweig, FRG, under the collection number DSM 5433.

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