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Isolation and characterization of an anaerobic benzoate-degrading spore-forming sulfate-reducing bacterium, *Desulfotomaculum sapomandens* sp. nov.

(Complete oxidation; sporulation; gas vacuoles; H₂S toxicity; sulfate reduction)

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1. SUMMARY

Spore-forming sulfate-reducing bacteria (SRB) were enriched selectively from various kinds of aerobic soils with fatty acids as the sole carbon and energy source. A Gram-negative motile rod-shaped bacterium, which produced gas vacuoles during sporulation was isolated. It degraded alcohols, aromatic and n-fatty acids (up to C₁₈) except for propionate, completely to CO₂. Sulfate, sulfite, thiosulfate or elemental sulfur served as electron acceptors. Because of its sensitivity to H₂S, the isolate never produced more than 8 mM dissolved sulfide at pH 7.0. G + C-content of the DNA was 48.0 mol %. The isolated strain Pato is described as a new species *Desulfotomaculum sapomandens*.

been investigated recently. Strains of the genera *Desulfovibrio*, *Desulfococcus*, *Desulfonema* and *Desulfosarcina* have been isolated using these carboxylic acids as sole carbon and energy source [1,2]. Within spore-forming SRB, only *D. acetoxidans* is able to degrade the shorter fatty acids acetate, butyrate and valerate [3,4]. This species does not oxidize longer-chained fatty acids nor aromatic compounds. The other formerly described *Desulfotomaculum* species do not degrade fatty acids nor acetate [5].

Spore-forming SRB have never been observed in enrichments on long-chained fatty acids when anaerobic sediments samples had served as inocula (F. Widdel, personal communication). Anaerobic enrichments on acetate, propionate, butyrate or palmitate in presence of sulfate yielded only spore-forming SRB when non-pasteurized aerobic soils of rice fields were used as inoculum (unpublished results; V. Jacq, personal communication).

2. INTRODUCTION

The anaerobic degradation of aromatic or long-chained saturated fatty acids by SRB has

The present paper reports on the isolation and characterization of such a new type of spore-forming SRB from aerobic soil enriched on palmitate as energy source.

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3. MATERIALS AND METHODS

3.1. Source of organism

Strain Pato was enriched from an aerobic, gasoline-contaminated soil at a gasoline station.

3.2. Medium and growth conditions

The basal medium had the following composition (values in g/l): Na_2SO_4 , 3.0; KH_2PO_4 , 0.2; NH_4Cl , 0.3; KCl , 0.5; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.15; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.4; NaCl , 1.0. 1 ml/l trace element solution SL10 [6] and resazurin 1 mg/l were added. The solution was autoclaved, immediately cooled

under N_2 to avoid dissolving of oxygen and was reduced with a few crystals of solid $\text{Na}_2\text{S}_2\text{O}_4$ until resazurin turned from pink to colourless. Separated autoclaved anoxic solutions of NaHCO_3 (1 mol/l) and Na_2S (0.5 mol/l) were added to final concentrations of 30 mmol/l and 0.2 mmol/l. 4 ml/l sterile filtered vitamin solution of Pfennig [7] was added and pH was adjusted to 7.2.

Anoxic medium was prepared and filled into Hungate tubes as described previously [8]. All tubes were filled to the top to avoid oxidation of the medium by air bubbles and sealed with butyl-rubber septa. Stock solutions of substrates were

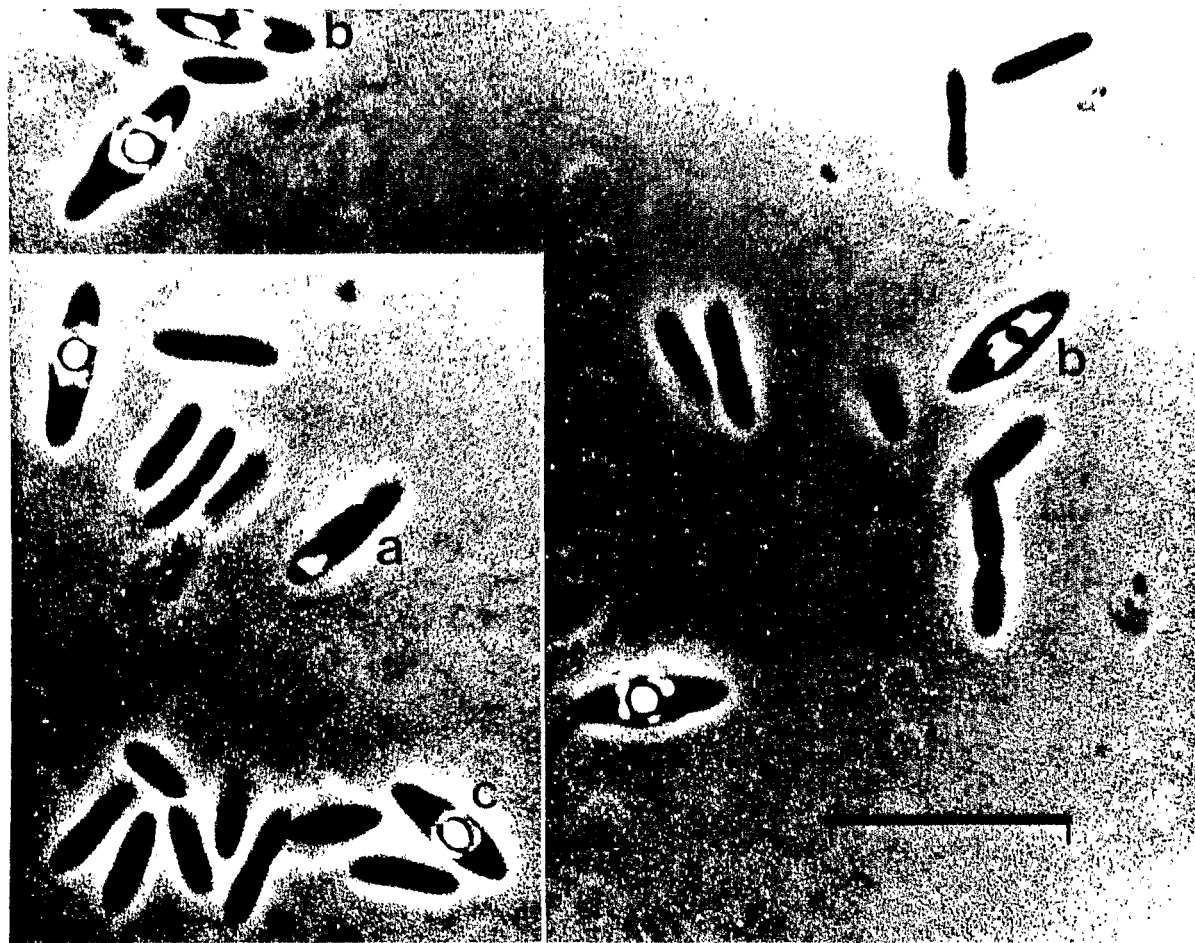


Fig. 1. Phase contrast photomicrograph of *Desulfotomaculum sapomandens*. Some cells contain gas vacuoles and (or) spores. Bar equals 10 μm . (a) Vegetative cell containing a terminal positioned gas vacuole; (b) beginning of sporulation inside a package of gas vesicles; (c) cell with fully developed spore surrounded by gas vesicles.

autoclaved separately in butyl-rubber-sealed serum bottles and immediately cooled under N_2 . The transfer of substrates and inocula was carried out using one-way syringes.

3.3. Isolation

Pure cultures were obtained after repeated application of the agar shake dilution method as described previously [1]. The tubes were outgassed with a mixture of 80% N_2 and 20% CO_2 and sealed with butyl-rubber stoppers. An isolated colony was pasteurized 15 min at $90^\circ C$ before it was transferred into liquid medium.

3.4. Chemical and biochemical determinations

Sulfide was determined photometrically as colloidal CuS [9]. Volatile fatty acids and alcohols were analysed with a Varian Aerograph 2700 gas chromatograph (column Porapak Q 80–100 mesh, $2.0m \times 1/8''$, $190^\circ C$; carrier gas N_2 70 ml/min; detector FID).

The DNA base ratio was determined by Dr. H. Hippe, Göttingen, F.R.G. as buoyant density by centrifugation in a density gradient.

4. RESULTS

4.1. Enrichment and isolation

When culture media containing palmitate as sole energy and carbon source were inoculated with aerobic soils, formation of about 5 mM H_2S and growth of large spore-forming slowly or non-motile rods took place within 2 weeks. The same types of bacteria were observed when 5-year-old dry stored tropical soils (rice soil and BAN soil from Senegal) or gasoline-contaminated aerobic soil were used as the inoculum. Other types of SRB, such as *Desulfovibrio*, *Desulfococcus* or *Desulfosarcina*, were not observed.

In none of the enrichments more than 8 mM sulfide was formed in excess of electron acceptor and donor. Longer incubation times did not lead to further growth but to lysis of the cells. Cultures incubated for more than 3 weeks often failed to grow when transferred into fresh medium. The fastest growing bacteria were those enriched from the gasoline-contaminated soil and were, therefore,

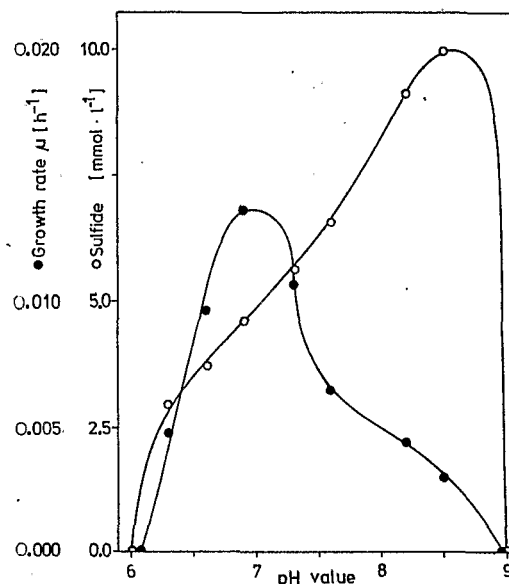


Fig. 2. Influence of the pH value on the assimilation (shown as growth rate) and dissimilation (shown as final sulfide concentration) during growth of *Desulfotomaculum sapomandens* in presence of excess electron donor (butyrate) and electron acceptor (sulfate).

isolated in pure culture (strain Pato). The colonies in the last dilution step were lens-shaped and of brownish colour. Most of the cells of the colony were sporulated.

4.2. Morphology and sporulation

Vegetative cells of strain Pato were straight or slightly curved motile rods (Fig. 1). After more than 2 days of incubation, an increasing number of cells became thicker and spindle-shaped. In most of them, highly refractile inclusions developed at both ends and later grew together in the center of the cell (Fig. 1). The optical appearance of these inclusions and the fact that they disappeared under pressure during 5 min centrifugation at 12000 rev./min indicated that they were gas vacuoles as already observed in *Desulfotomaculum acetoxidans* [3].

The spore formation took place inside the package of gas vesicles in the center of the cell. So the fully developed spore was surrounded by the vacuole even after lysis of the cell. The vegetative cells stained Gram-negative.

Table 1

Physiological characteristics of *Desulfotomaculum sapomandens*

In parentheses: substrate concentrations (in mmol/l).

	Substrates used and completely oxidized to CO ₂ + H ₂ S	Substrates tested but not used
Linear fatty acids	Formate (10) + Acetate (1) Acetate ^a (10) Butyrate (5) Valerate (5) Caproate (2) Heptanoate (2) Octanoate (2) Pelargonate (1) Decanoate (1) Laurate (1) Myristate (1) Palmitate (1) Stearate (1)	Propionate (10) Behenate (1) Arachinate (1)
Branched chained fatty acids	Methyl-propionate (5) 3-Methyl-butyrate (5) 4-Methyl-valerate (5)	2-Methyl-butyrate (5)
Aromatic acids	Benzoate (3) Phenyl-acetate (3) Phenyl-propionate (3) 4-Hydroxy-benzoate (3)	<i>trans</i> -Cinamate (3) Hippurate (3) <i>p</i> -Aminobenzoate (3) Vanillate (3) Syringate (3) Catechol (0.1) Phenol (0.1)
Alcohols	Ethanol (10) Propanol ^a (10) Butanol (10) Pentanol (5) <i>iso</i> -Butanol (5)	<i>iso</i> -Propanol (5) <i>iso</i> -Pentanol (5) Glycerol (5)
Other organic compounds	Pyruvate (10) Malate ^a (10) Succinate ^a (10) Fumarate ^a (10)	Lactate (10) Fructose (10) Glucose (10) Cellobiose (10)

^a Degraded slowly under H₂S production but negligible or no growth occurred.

4.3. Growth conditions

Strain Pato grew in freshwater medium up to 5 g/l NaCl. Growth was observed in mineral medium supplemented with vitamins and energy source. Low concentrations of rumen fluid (0.5%) improved growth rate and final density of the culture. The pH optimum for maximal growth rate was 7.0 but highest final density and highest amount of produced sulfide occurred at pH 8.5 (Fig. 2).

Various carbonic acids and alcohols served as energy source for strain Pato (Table 1). All substrates used were oxidized completely to CO₂, neither volatile fatty acids nor alcohols were formed as end products. In the presence of butyrate, the strain grew with sulfite, thiosulfate or elemental sulfur instead of sulfate as electron

acceptor. In excess of butyrate and electron acceptor, sulfite reducing cultures reached higher absorbances than cultures with sulfate as electron donor. However, the final amounts of sulfide formed were the same in both cases (7.8 mM). N₂ and CO₂ (homoacetogenesis) did not serve as electron acceptors. Pyruvate was not fermented in the absence of inorganic sulfur compounds.

The DNA G + C content was 48.0 mol %.

5. DISCUSSION

5.1. Physiology

The SRB described in this paper grew in defined media supplemented with vitamins. If added as sole substrate, acetate was slowly oxidized. There-

fore strain Pato belongs to the physiological group of complete fatty acid-oxidizing SRB [10]. Unlike the other members of this physiological group (*Desulfovibrio baarsii*, *Desulfococcus multivorans*, *Desulfosarcina variabilis* [1], *Desulfonema* sp. [2] and *Desulfococcus niacini* [5], the isolated strain did not degrade propionate.

During β -oxidation of odd-numbered fatty acids like valerate and heptanoate, the last link not to be degraded is propionate. Therefore, it is expected to find the excretion of 1 mol propionate per mol odd-numbered fatty acid which is degraded by strain Pato. In assays with C₄, C₅, C₆ and C₇ fatty acids, strain Pato produced, in any case stoichiometrically, amounts of H₂S, according to complete oxidation of odd- and even-numbered fatty acids. The excretion of propionate was not observed. The negative growth result with propionate may be due to lack of a transport system. In comparison to this, the complete oxidizer *Desulfotomaculum acetoxidans* excretes propionate during slight growth on valerate [4].

At pH 7.0, strain Pato never produced more than 8 mM H₂S, neither with sulfate, nor with sulfite as electron acceptor. But as the reduction of sulfite is thermodynamically more efficient than the reduction of sulfate [11], growth yield was higher during sulfite reduction. These observations indicate an inhibition of metabolism by dissolved sulfide. This assumption was confirmed by an assay with 10 mmol/l Fe²⁺ added to trap dissolved sulfide as FeS: 16 mmol/l sulfide were produced.

The toxicity of dissolved sulfide decreased with increasing pH values (Fig. 2). Therefore H₂S is considered to be the toxic agent in the sulfide equilibrium rather than HS⁻ and S²⁻.

5.2. Ecology

Among the strictly anaerobic SRB which are all highly oxygen-sensitive, only sporulating species can survive during oxygenation and dryness of the environment. Those sporulating SRB are well adapted to environments of changing redox conditions like gley soils or rice fields. But also anaerobic micro-niches in well aerated highly organic soils, can serve as habitat for the described type of bacterium. Therefore, anaerobic enrichments on

fatty acids with sulfate as electron acceptor from aerobic soils, were selective for spore-forming strains of the type Pato. Their lack of competence in anaerobic sediments and digesters may be due to their slow growth and their high sensitivity to H₂S.

5.3. Taxonomy

The new isolate described in the present paper is a strictly anaerobic spore-forming SRB which, by the current nomenclature, has to be classified as *Desulfotomaculum* sp. [12]. It differs from all other *Desulfotomaculum* species described so far by its morphology and its range of substrates utilized (Table 1), especially by the ability to degrade long-chained fatty acids and aromatic acids. Therefore a new species *D. sapomandens* is proposed.

5.4. Description of *D. sapomandens* sp. nov.

D. sapomandens sp. nov.: *sapo*. *mandens* n. *sapo* soap; *L.V. mando* to eat, to consume; *M.L.* part. adj. *sapomandens* eating soap.

5.4.1. *Morphology*. Straight motile rods, 1.2–2.0 μ m by 5–7 μ m, with pointed ends. Spores round, 1.6 μ m in diameter, central, causing swelling of the cells. Spore-forming cells typically spindle-shaped. Gas vacuoles present in sporulated cells. Gram-negative.

5.4.2. *Culture conditions*. Strict anaerobe; T_{min} , 20°C; T_{opt} , 38°C; T_{max} , 43°C; pH range for growth 6.3–8.5, pH optimum at 7.0.

5.4.3. *Nutritional characteristics*. Chemoorganotroph which grows in mineral media with sulfide or dithionite as reductants. Vitamins are required. Sulfate, sulfite, thiosulfite and elemental sulfur are reduced to H₂S. Not reduced: nitrate and carbonate. Electron donors used: ethanol and higher alcohols, pyruvate, formate, acetate, butyrate, and higher fatty acids up to 18 carbon atoms, methyl and phenyl-substituted organic acids. Oxidation is complete. Growth on acetate is extremely slow. No fermentation of organic compounds.

5.4.4. *Type strain*. Strain "Pato" is deposited in the Deutsche Sammlung von Mikroorganismen (DSMZ), Göttingen, F.R.G., under the number DSM3223.

ACKNOWLEDGEMENTS

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REFERENCES

- [1] Widdel, F. and Pfennig, N. (1984) in *Bergey's Manual of Systematic Bacteriology* (Krieg, N.R. and Holt, J.G., Eds.), Vol. 1, pp. 663-679, Williams and Wilkins, Baltimore.
- [2] Widdel, F. and Pfennig, N. (1983) *Arch. Microbiol.* 134, 286-294.
- [3] Widdel, F. and Pfennig, N. (1977) *Arch. Microbiol.* 112, 119-122.
- [4] Widdel, F. and Pfennig, N. (1981) *Arch. Microbiol.* 129, 401-402.
- [5] Campbell, L.L. and Postgate, J.R. (1965) *Bacteriol. Rev.* 29, 359-363.
- [6] Imhoff-Struckle, D. and Pfennig, N. (1983) *Arch. Microbiol.* 136, 194-198.
- [7] Pfennig, N. (1978) *Int. J. Syst. Bacteriol.* 28, 283-288.
- [8] Widdel, F. and Pfennig, N. (1981) *Arch. Microbiol.* 129, 393-400.
- [9] Cord-Ruwisch, R. (1985) *J. Microbiol. Methods* 3, (in press).
- [10] Widdel, F. (1984) in *Anaerobic Bacteria in Habitats Other than Man* (Barnes, H.G., Ed.), Blackwell, Oxford.
- [11] Thauer, R.K., Jungermann, K. and Decker, R.K. (1977) *Bacteriol. Rev.* 41, 100-180.
- [12] Pfennig, N., Widdel, F. and Trüper, H.G. (1981) in *The Prokaryotes* (Starr, M.P., Stolp, H., Trüper, H.G., Balows, A. and Schlegel, H.G., Eds.) Vol. 1, pp. 926-940. Springer Verlag, Berlin.