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A New Anaerobic, Sporing, Acetate-Oxidizing, Sulfate-Reducing Bacterium, *Desulfotomaculum* (emend.) *acetoxidans*

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Abstract. A new strictly anaerobic, polarly flagellated, sporing, acetate-oxidizing, sulfate-reducing bacterium was isolated from anaerobic fresh or sea water mud samples. The oxidation of acetate to CO_2 is stoichiometrically linked to the formation of H_2S from sulfate. Ethanol, butanol and butyrate are also used. Hydrogen, lactate or pyruvate are not used as electron donors; organic substances are not fermented. A cytochrome of the *b*-type and a supposed sulfite reductase, P582, were detected spectrophotometrically. An emended description of the genus *Desulfotomaculum* is proposed which includes the new bacterium as the species *Desulfotomaculum acetoxidans*.

Key words: Desulfotomaculum acetoxidans – Emendation of *Desulfotomaculum* – Species description – Anaerobic acetate oxidation – Sulfate reduction – Electron donors – *b*-type cytochrome – Sulfite reductase P582.

Dissimilatory reduction of sulfate to sulfide with acetate as the only electron donor was first reported by Rubentschik (1928). Later on, Baars (1930) observed the same process and described a bacterium, *Vibrio rubentschikii*, morphologically resembling *Desulfovibrio desulfuricans* but differing from the latter in its capacity to use acetate or butyrate as carbon and energy source. In an attempt to reisolate *V. rubentschikii*, Selwyn and Postgate (1959) did not detect sulfate-reducing bacteria capable of growth on these substrates. In all authenticated sulfate-reducing bacteria known until now, the anaerobic oxidation of organic substrates is incomplete and leads to the accumulation of acetate as a regular end product.

Recently, in acetate- and sulfate-containing enrichment cultures for phototrophic bacteria, the formation of surprisingly high concentrations of hydrogen sulfide was occasionally observed, although anaerobic conditions had been established solely by evacuation and flushing with nitrogen. Subsequent transfers of such enrichments as well as others freshly inoculated with mud samples of different origin were incubated in the dark; they yielded characteristic populations of fat, motile rod-shaped bacteria. In older cultures many cells became spindle-shaped and formed spores. In view of this striking morphological difference to Baar's *Vibrio rubentschikii*, a pure culture of the bacterium is described here as a new species of the emended genus *Desulfotomaculum*: *D. acetoxidans*.

MATERIALS AND METHODS

Sources of Organisms. Acetate-utilizing sulfate reducers of the new type were obtained in enrichments inoculated with anaerobic samples of the following sources: 1. Mud from a peat bog, 2. Mud from a village ditch, 3. Mud flat of the Jadebusen (Nordsee), and 4. Piggery waste (source of strain 5575).

Media and Conditions of Cultivation. The basal medium had the following composition (values in mmol/l of distilled water): Na_2SO_4, 20.0; KH_2PO_4, 5.0; $MgCl_2$, 2.0; NH_4Cl, 6.0; $CaCl_2$, 0.6; trace element solution of Pfennig (1974), 10 ml/l. To the sterile, cool medium the following components were added from sterile stock solutions: $FeCl_2$, 0.002 (from acidified solution); $NaHCO_3$, 20.0; Na_2S, 1.5; vitamin solution of Pfennig (1965), 5 ml/l; vitamin B_{12}, 20 µg/l. The pH was adjusted to 7.1 with sterile phosphoric acid. For marine isolates, 350 mmol NaCl/l and 12.0 mmol $MgCl_2$/l were added. The sterile medium was placed into sterile screw cap bottles. Substrates were added to the culture bottles from sterile stock solutions to give the concentrations desired. For enrichments, agar shakes and stock cultures 15.0 mmol acetate/l were used as sole carbon and energy source. After inoculation, 0.15 mmol $Na_2S_2O_4$/l were added from a freshly prepared, anaerobically filter-sterilized solution. To test utilization of hydrogen, culture vessels were filled to one fourth with inoculated medium, gased with a mixture of 4 parts by volume hydrogen and 1 part by volume carbon dioxide and sealed with black rubber stoppers. Cultures were incubated at 36° C.

Isolation. Pure cultures were obtained by repeated application of the agar shake culture method in anaerobically sealed test tubes.

Cell Material Determination. Cells were harvested by centrifugation, washed once with phosphate buffer (7.5 mmol/l; pH 6.0), transferred with distilled water to small glass beakers and dried at 80° C for 12 h.

Chemical Determinations. Sulfide was determined with the methylene blue reaction as described by Pachmayr (1960). Acetic acid was determined with a Perkin-Elmer gas chromatograph (column: Porapak QS 100–120 mesh, 2.0 m, 160° C; carrier gas: 70 ml N_2/min; detector: FID).

Characterization of Pigments. Cells were sonicated in phosphate buffer, 50 mmol/l, pH 7.0; cell debris was removed by centrifugation at $4 \cdot 10^5$ m $\cdot$ s^{-2} for 15 min. The cell free extract was recentrifuged at $15 \cdot 10^5$ m $\cdot$ s^{-2} for 90 min; the supernatant and the washed cell membrane pellet were used for measurements with a Zeiss DMR 21 spectrophotometer.

DNA Base Composition. The moles per cent guanine plus cytosine of the DNA were determined by Dr. H. Hippe, Göttingen, by the thermal denaturation method.

RESULTS AND DISCUSSION

Anaerobic enrichments with sulfate and acetate as sole carbon and energy source yielded the same characteristic type of motile, rod shaped bacteria. After several transfers, the medium inoculated with piggery waste was used to obtain a pure culture. After 2 weeks of incubation, colonies of the characteristic cell type in agar shake cultures were large (about 1 mm in diameter), whitish to greyish and of granular surface structure. After 3 weeks, most cells had spores; to get a pure culture, one of the colonies was isolated and pasteurized for 20 min at 80° C in sterile liquid culture medium.

Vegetative cells were straight or slightly curved rods with pointed ends (Fig. 1 a), 1–1.5 µm wide and 3.5–9 µm long; they were motile by a single, polar flagellum (Fig. 2) of unusual diameter (27 nm). Spherical spores of 1.5 µm diameter occurred subterminal to paracentral in spindle shaped cells (Fig. 1 b). Next to the highly refractile spore a conic refractile area was situated, reminiscent of a gas vacuole.

Tests with different carbon and energy sources in the presence of sulfate showed that *Desulfotomaculum acetoxidans* was able to grow with ethanol, butanol or butyrate instead of acetate; no growth occurred with lactate, pyruvate, hydrogen or formate plus yeast extract and succinate, propionate, valerate, caproate, propanol, fumarate, malate, glucose, fructose, maltose and cellobiose. In the presence of acetate, sulfate was replacable by thiosulfate or fumarate but not by elemental sulfur. Vitamins, but no other growth factors were required.

Growth was obtained between pH 6.6 and 7.6 with an optimum at pH 7.1. The temperature range was between 20 and 40° C, the temperature optimum was 36° C. Doubling times from 12 to 24 h were observed.

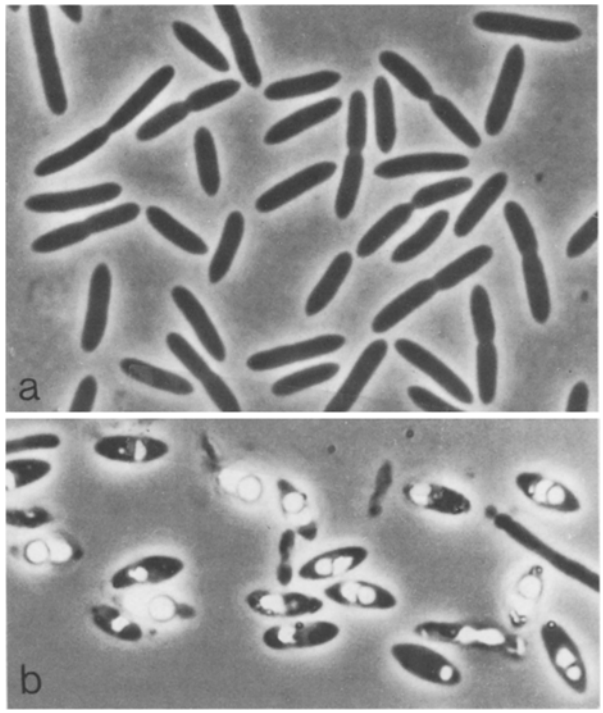

Fig. 1 a and b. Phase contrast photomicrographs (×2000) of *Desulfotomaculum acetoxidans* grown with acetate and sulfate. (a) Vegetative cells from a growing culture in liquid medium. (b) Cells from agar colony with spores and areas reminiscent of gas vacuoles

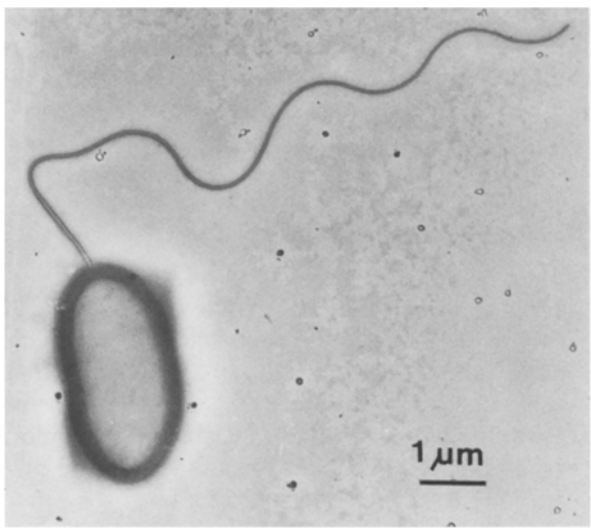

Fig. 2. Negatively stained (phosphotungstic acid) cell of *Desulfotomaculum acetoxidans* grown in liquid medium with acetate and sulfate. Electron micrograph made by R. Lurz, Göttingen

The membrane fraction of cell-free extracts of *D. acetoxidans* exhibited a difference spectrum (dithionite reduced minus oxidized) with absorption

Table 1. Acetate consumed and cell material and hydrogen sulfide formed by *Desulfotomaculum acetoxidans* at different stages of growth with sulfate and acetate as the sole carbon and energy source. The measurements were stopped before the growth-inhibitory H_2S concentration of about 7 mmol/l was reached. The data represent the differences between the amounts measured in grown cultures and inoculated controls maintained at 4° C

Acetate consumed (mmol/l)	Cell material formed (mg/l)	Acetate consumed for cell material[a] (mmol/l)	Acetate consumed for sulfate reduction (mmol/l)	Hydrogen sulfide formed (mmol/l)
1.63	10.8	0.22	1.41	1.43
2.08	11.9	0.25	1.83	1.76
3.05	15.5	0.32	2.73	2.48
3.85	19.0	0.39	3.46	3.44

[a] Acetate consumed for cell material was calculated by the following equation: $17\ CH_3COO^- + 11\ H_2O \rightarrow 8\ (C_4H_7O_3) + 2\ HCO_3^- + 15\ OH^-$, thus, 0.0206 mol acetate are required for 1.0 mg of cell material

maxima at 426, 530 and 559 nm resembling a *b*-type cytochrome. Neither a *c*-type cytochrome, nor desulfoviridin was detected. The carbon monoxide difference spectrum of the reduced soluble cell fraction showed absorption peaks at 412, 550 and 596 nm and troughs at 432, 572 and 621 nm. The same absorption characteristics were observed for the supposed sulfite reductase P 582 of *Desulfotomaculum nigrificans* by Trudinger (1970).

The complete reduction of sulfate with acetate as the electron donor was demonstrated in parallel growth experiments with limited sulfate concentrations (3.0 mmol/l) and excess acetate (15.0 mmol/l). After growth ceased, no turbidity occurred when the acidified supernatant was mixed with $BaCl_2$-solution; when treated in the same way, the inoculated controls, maintained at 4° C, became turbid at once by the precipitation of $BaSO_4$.

To prove the stoichiometry of sulfate reduction with acetate as the electron donor by *D. acetoxidans*, the amounts of acetate consumed and cell material and H_2S formed, were measured in 1 l cultures at different growth stages. Data in Table 1 show that per mol of acetate dissimilated, 1 mol of H_2S was formed, indicating a complete oxidation of acetate to CO_2, as was expected according to the equation:

$$CH_3COO^- + SO_4^{2-} \rightarrow 2\ HCO_3^- + HS^-.$$

Desulfuromonas acetoxidans (Pfennig and Biebl, 1976) and the present new species are the only anaerobic chemoorganotrophs so far found that are able to grow with acetate as sole carbon source and electron donor and with inorganic sulfur compounds as electron acceptors. *Desulfuromonas* grows by electron transport to sulfur while *Desulfotomaculum acetoxidans* utilizes more oxidized sulfur compounds. The free energy change is more favourable for growth on the latter compounds than on sulfur. In agreement with this, the cell yield obtained per mol of acetate consumed (Table 1) is higher than found in *Desulfuromonas*.

The genus *Desulfotomaculum* Campbell and Postgate, 1965 currently includes spore-forming sulfate-reducing bacteria that are peritrichous and do not oxidize acetate; they incompletely oxidize organic compounds with the formation of acetate or homologue and CO_2. The bacterium described in this paper has the following properties in common with *Desulfotomaculum*: straight or curved rod-shaped cells, spore formation, negative Gram reaction, sulfate reduction, cytochrome of the protoheme class, absence of *c*-type cytochrome, sulfite reductase P582, and absence of desulfoviridin. Therefore, we do not advocate the creation of a new genus for the new sporing sulfate reducer, but propose an emended description of the genus *Desulfotomaculum* to include polarly flagellated, acetate-oxidizing species.

Genus Desulfotomaculum

Straight or curved rods, 0.3 – 1.5 by 3 – 9 µm, with rounded or pointed ends, usually single but sometimes in chains. Motile with peritrichous or polar flagella. Spores oval to round, terminal to paracentral, causing slight swelling of the cells. Gram-negative. Produce black colonies in agar containing a suitable carbon source, sulfate and ferrous salts.

Chemoorganotrophs; metabolism respiratory. Sulfates, sulfites and other reducible sulfur compounds serve as electron acceptors and are reduced to H_2S. In species using lactate and pyruvate, oxidation incomplete, leading to formation of acetate or homologue and CO_2; when acetate is used it is completely oxidized to CO_2. Limited range of substrates used, rarely includes carbohydrates. Cells contain a cytochrome of the protoheme class. Specialized media containing a reducible sulfur compound and organic growth factors are required for growth. Strict anaerobes. Temperature optimum 35 – 55° C; maximum 70° C. Some strains grow at 20 – 30° C.

Desulfotomaculum (emend.) *acetoxidans* sp. nov. Acet. oxidans. L. n. *acetum* vinegar; M. L. noun *acidum*

aceticum acetic acid; M. L. v. *oxido* make acid, oxidize; M. L. part. adj. *acetoxidans* oxidizing acetate.

Straight or slightly curved rods, 1.0–1.5 µm by 3.5–9.0 µm, with pointed ends. Motile by means of single, polar flagella; flagellum twice as thick (27 nm) as usual flagella. Spores spherical, 1.5 µm in diameter, subterminal, causing swelling of the cells. Spore-forming cells typically spindle-shaped. Gram negative.

Strict anaerobic chemoorganotroph, which grows in mineral media with sulfide and dithionite as reductants; marine forms require 1–2% NaCl. Vitamins are required.

Acetate, ethanol, butanol or butyrate are used as electron donors and carbon sources in the presence of 20 mmol/l bicarbonate; sulfate or thiosulfate serve as electron acceptors and are reduced to H_2S. Elemental sulfur is not reduced. Fumarate can also serve as electron acceptor; no growth on fumarate alone. Unable to ferment organic substances; not utilized as electron donors: hydrogen, lactate, pyruvate, sugars, higher fatty acids. pH-range: 6.6–7.6. Temperature range of growth: 20–40° C.

Pigments: Cell membrane fractions exhibit absorption spectra characteristic for *b*-type cytochromes (abs. max. at 426, 530 and 559 nm). No soluble *c*-type cytochrome present. The CO-difference spectrum of soluble cell fraction exhibits absorption peaks characteristic for P582 (sulfite reductase; abs. max. at 412, 550 and 596 nm).

DNA base ratios: 37.5 moles % guanine + cytosine (thermal denaturation).

Habitat: Anaerobic, sulfide-containing water and mud of freshwater and marine environments; manure and feces of higher animals.

Type strain: "Göttingen", 5575, DSM 771 deposited in: Deutsche Sammlung von Mikroorganismen, Göttingen.

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