

***Desulfotomaculum geothermicum* sp. nov., a thermophilic, fatty acid-degrading, sulfate-reducing bacterium isolated with H₂ from geothermal ground water**

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Abstract. A strictly anaerobic, thermophilic, fatty acids-degrading, sporulating sulfate-reducing bacterium was isolated from geothermal ground water. The organism stained Gram-negative and formed gas vacuoles during sporulation. Lactate, ethanol, fructose and saturated fatty acids up to C₁₈ served as electron donors and carbon sources with sulfate as external electron acceptor. Benzoate was not used. Stoichiometric measurements revealed a complete oxidation of part of butyrate although growth with acetate as only electron donor was not observed. The rest of butyrate was oxidized to acetate. The strain grew chemolithoautotrophically with hydrogen plus sulfate as energy source and carbon dioxide as carbon source without requirement of additional organic carbon like acetate. The strain contained a *c*-type cytochrome and presumably a sulfite reductase P582. Optimum temperature, pH and NaCl concentration for growth were 54°C, pH 7.3-7.5 and 25 to 35 g NaCl/l. The G + C content of DNA was 50.4 mol %. Strain BSD is proposed as a new species of the spore-forming sulfate-reducing genus *Desulfotomaculum*, *D. geothermicum*.

Introduction

The sulfate-reducing bacteria (SRB) are taxonomically classified into two large subgroups: the non-sporulating genera *Desulfovibrio*, *Desulfomonas*, *Desulfococcus*, *Desulfobacter*, *Desulfobulbus*, *Desulfosarcina*, *Desulfobacterium*, *Desulfonema*, *Thermodesulfobacterium* (Widdel 1987) and the sporulating species of the genus *Desulfotomaculum* (Campbell & Singleton 1986). The non-sporulating group was described as physiologically much more versatile, especially by the capacity of certain species to degrade saturated fatty acids and hydrogen, which are important fermentation products, and aromatic acids.

However, recently new strains of *Desulfotomaculum* were described, which were also able to degrade aromatic and fatty acids (Cord-Ruwisch & Garcia

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1985; Klemps et al. 1985) and hydrogen (Klemps et al. 1985). In environments which do not always provide conditions for the survival of the strict anaerobic SRB, the sporulating species of the genus *Desulfotomaculum* are the dominant species, as evidenced by enrichment cultures from aerobic soils, and dry soils of rice fields (Cord-Ruwisch & Garcia 1985).

Other environments which do not always permit microbial growth may be, due to the extreme temperature changes, the water circuit of heat exchangers or of geothermal heat-plants.

Here we report on the isolation and characterization of a new *Desulfotomaculum* species with hydrogen plus sulfate from a geothermal ground water.

Materials and methods

Origin of strain

Strain BSD was isolated from an anoxic geothermal ground water dating to the Dogger period and used in geothermal heat-plants (GCR4 Creil production well). The sample came from a reservoir situated at a depth of 2,500 m where the in situ temperature was about 58° C and the hydrostatic pressure between 200 and 300 bars. The NaCl concentration was similar to that of sea water (28 g/l) and the pH was about 7.0.

Media and conditions of cultivation

The basal medium was composed by considering the salts concentrations of the water sample and contained in g/l: Na₂SO₄, 3.0; KH₂PO₄, 0.2; NH₄Cl, 0.3; KCl, 0.3; CaCl₂·2H₂O, 0.2; MgCl₂·6H₂O, 3.0; NaCl, 20.0. 1 ml/l trace elements solution SL 10 (Imhoff-Stuckle & Pfennig 1983) and 1 mg/l resazurin were added. This medium was sterilized for 40 min at 110° C. After autoclaving, the medium was immediately cooled under a mixture of N₂-CO₂ (80–20%) and then, reduced with 0.1 ml of Na₂S₂O₄ solution (0.2 mol/l). NaHCO₃ and Na₂S were added from separately sterilized anoxic solutions of 1 mol/l and 0.5 mol/l respectively to final concentrations of 30 mmol/l and 0.5 mmol/l. 1 ml of vitamins solution (Pfennig 1978), sterilized by filtration, was also added. The medium was finally adjusted to pH 7.2–7.3 and distributed into Hungate tubes as described by Widdel & Pfennig (1981b).

Isolation

A pure culture was obtained by repeated application of the agar shake dilution method in anaerobically sealed test tubes as described by Widdel & Pfennig (1984). Purity was tested on a complex sulfate-free medium containing 0.5% glucose, 0.5% trypticase and 0.5% yeast extract.

Chemical analyses

H₂S was determined photometrically as colloidal CuS after reaction with a mixture of HCl [50 mM], CuSO₄ [5 mM] (Cord-Ruwisch 1984). Disappearance and formation of fatty acids were measured by HPLC (Cord-Ruwisch et al. 1986). For determination of cytochromes and desulfovirdin, cells from a culture with lactate (51) were collected by centrifugation and suspended in 5 ml of 50 mM potassium phosphate buffer (pH 7) containing 1 mg pancreatic deoxyribonuclease I and disrupted by passing them twice through a French pressure cell (Aminco) at 15,000 lb/in². Cell debris was removed by centrifugation of the broken cell suspension at 25,000 g for 20 min. The resulting cell-free extract was separated into a cytoplasmic (supernatant) and a membrane fraction (cell pellet) by centrifugation at 140,000 g for 2 h. The membrane pellet was resuspended in phosphate buffer. The cell-free extract and both resulting fractions were used for measurements with a Cary 219 recording spectrophotometer. In addition, the presence of desulfovirdin was studied using the fluorescence test of Postgate (1959).

The mol percent guanine plus cytosine of the DNA was determined by the D.S.M., Göttingen, FRG, by buoyant density centrifugation.

Transmission electron microscopy

A drop of centrifuged culture from the exponential growth phase was put on a copper grid covered by carbon vaporisation solidified Formvar film (150–200 Å). Negative staining was performed by exposing cells to 1% sodium phosphotungstate solution (pH 7) for about 5 s. A TEM Hitachi H600 electron microscope was used.

Results

Enrichment and isolation

The sample was collected from a geothermal ground-water at the discharge pipe from the drilling production of a site dating from the Dogger period (CGR 4 Creil well) in the Paris Basin. The enrichment culture medium containing H_2 and sulfate as the only energy sources and 1 mmol/l acetate as carbon source, was inoculated with geothermal water. After incubation at 55° C for one week, about 8.5 mmol/l H_2S were formed. Microscopically, the prevailing organisms were large motile sporulating rods, similar to members of the genus *Desulfotomaculum*. After several transfers into sterile medium under hydrogen atmosphere, the enrichment culture was directly diluted into agar-tubes containing lactate (20 mM) plus sulfate (20 mM) as energy and carbon source. Only black, lens-shaped colonies developed after two weeks of incubation at 55° C. Microscopic examination revealed sporulating rods of the described morphotype. Colonies were successively picked and rediluted into a second agar dilution series. Finally, the isolate, originating from the last positive dilution step of the second dilution series, was pasteurized for 15 min at 80° C. After heat shock, 1 ml of culture was transferred into liquid medium and after 4 days of cultivation at 55° C, the same spore-forming rods as before were observed. After inoculation of the isolate on the complex sulfate-free medium incubated at 37 and 55° C, no contaminants were observed. The pure culture was designated as strain BSD.

Cell morphology and sporulation

Vegetative cells of the strain were straight rods (2.3–2.5 μm in length and 0.5 μm in width) (Fig. 1). The strain stained Gram-negative. In transmission electron micrographs, cells exhibited at least two flagella (Fig. 2). Cells usually appeared singly or in pairs during the exponential growth. Sporulation was observed at the end of growth on both agar and liquid media. In the first phase of sporulation, the vegetative cells appeared spindle-shaped (2.1 \times 1.2 μm) (Figs. 2–3) due to the presence of spores. These were spherical and located subterminally. The development of the spore was always accompanied by the formation of adjacent highly refractile areas. These structures have been identified as gas vacuoles because they disappeared after centrifugation at 5,000 rpm during 5 min. The simultaneous formation of spores and gas vacuoles has been already observed in *Desulfotomaculum acetoxidans* (Widdel & Pfennig 1977, 1981a) and *Desulfotomaculum sapomandens* (Cord-Ruwisch & Garcia 1985).

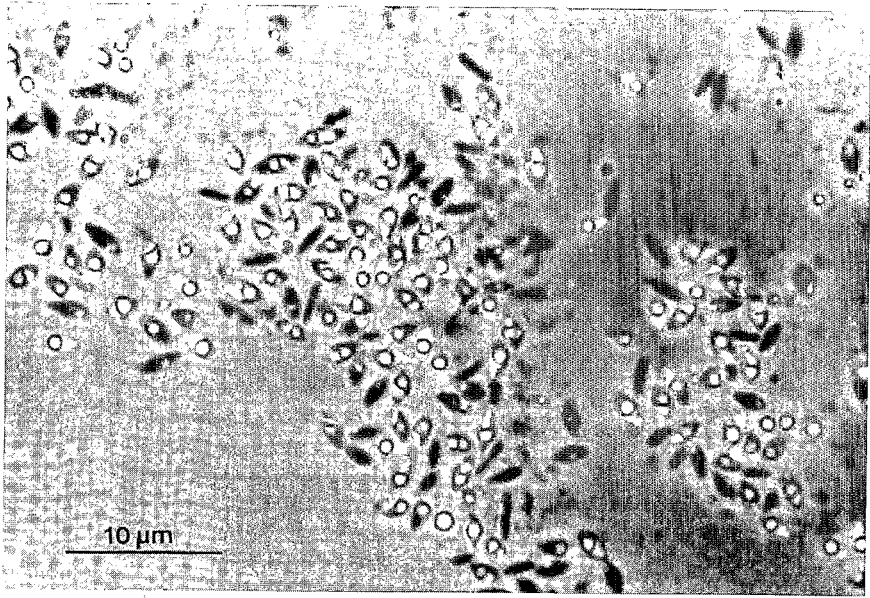


Fig. 1. Spores and gas vacuoles of strain BSD (phase contrast).

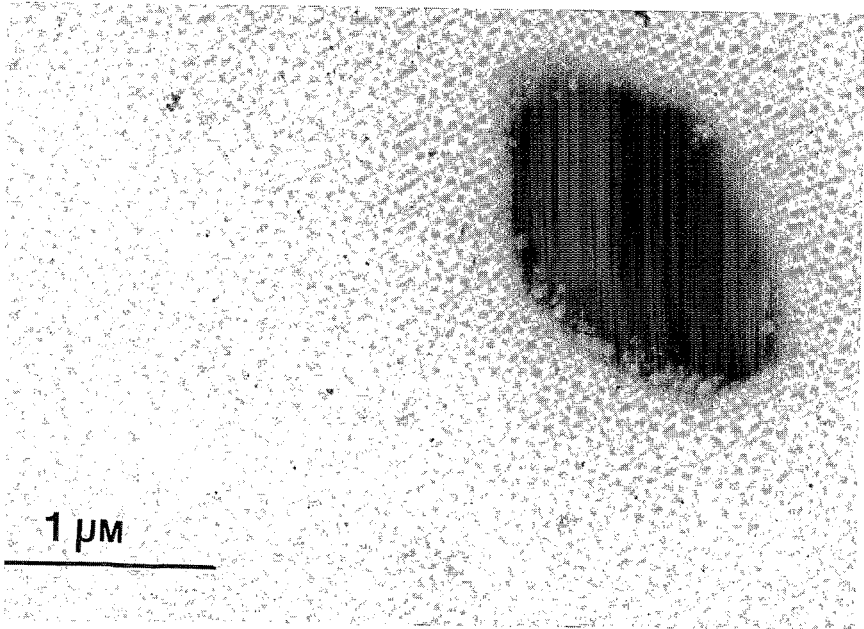


Fig. 2. Gas vacuoles and flagella of strain BSD (electron transmission microscopy).

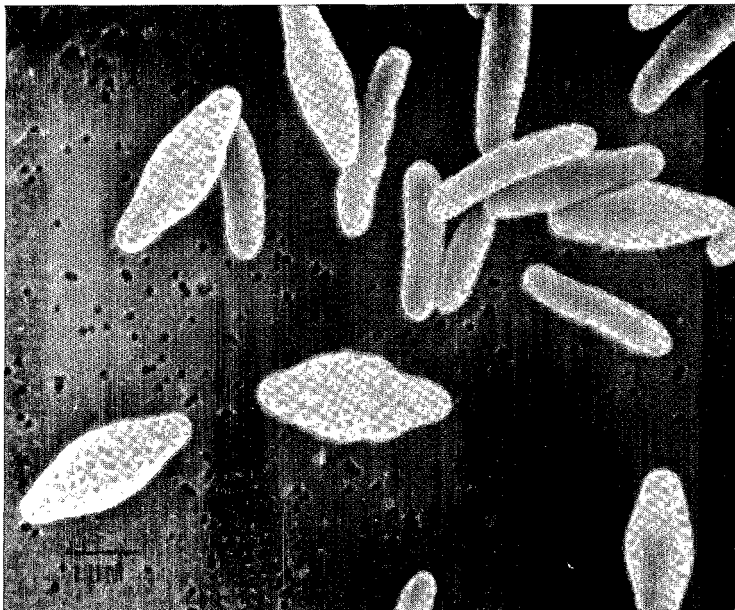


Fig. 3. Non-sporulated and sporulated cells of strain BSD (scanning microscopy).

Pigments and DNA base ratio

Dithionite reduced/air oxidized difference spectra obtained with the cell-free extract of strain BSD showed characteristic absorption bands of *c*-type cytochrome with maxima at 418.5, 523 and 552.5 nm. *b*-type cytochrome was not detected. The redox difference spectrum of the cytoplasmic fraction exhibited a maximum at 582 nm. This absorption band was characteristic for sulfite reductase P582 first described in *D. nigrificans* (Trudinger 1970; Akagi & Adams 1973). Fluorescence tests (Postgate 1959) confirmed that the sulfite reductase, desulfoviridin, was not present. The guanine plus cytosine content of DNA of strain BSD was determined to be 50.4 mol %.

Growth conditions

On the basis of doubling time, the optimum growth temperature of the strain was estimated to be 54°C (Fig. 4). No growth was observed at temperatures higher than 57°C or lower than 37°C. Growth of the strain was optimal at pH 7.2–7.4 (Fig. 5). The strain developed between pH 6 and 8. Growth was observed in media with 2 to 50 g/l NaCl with an optimum at 24–34 g/l (Fig. 6).

In Table 1, the nutritional capacities and physiological characteristics of our

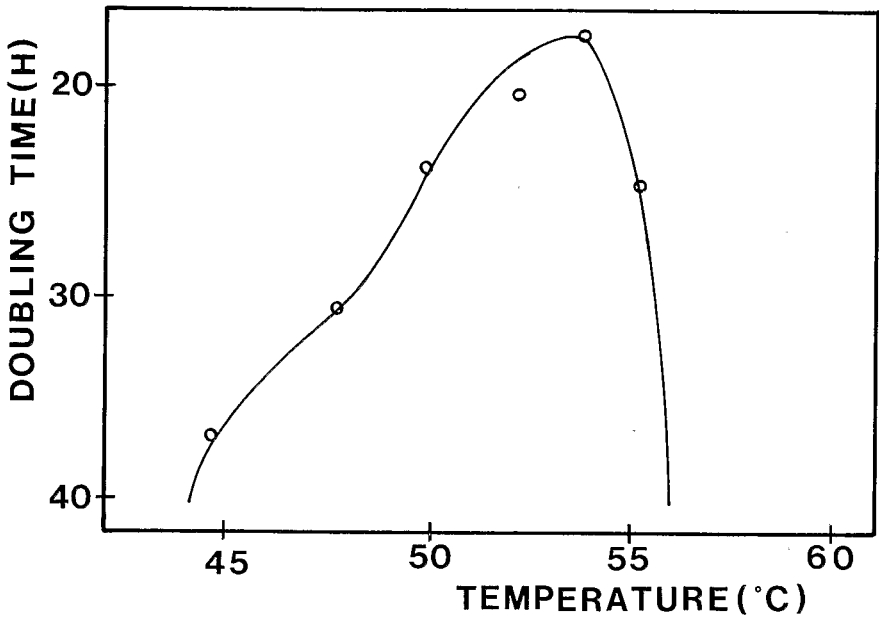


Fig. 4. Influence of temperature on the growth of strain BSD.

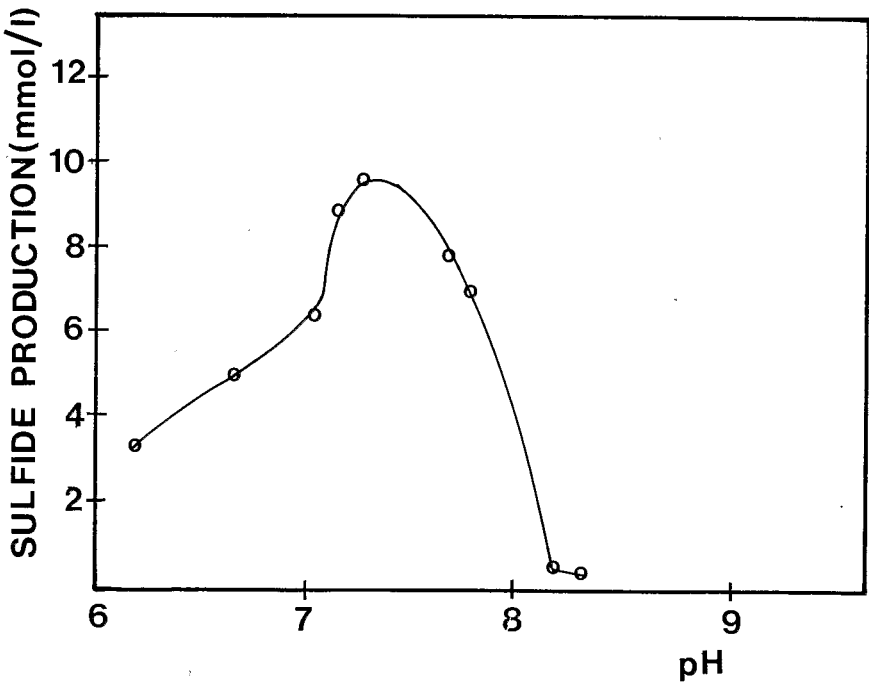


Fig. 5. Influence of pH on the sulfide production by strain BSD.

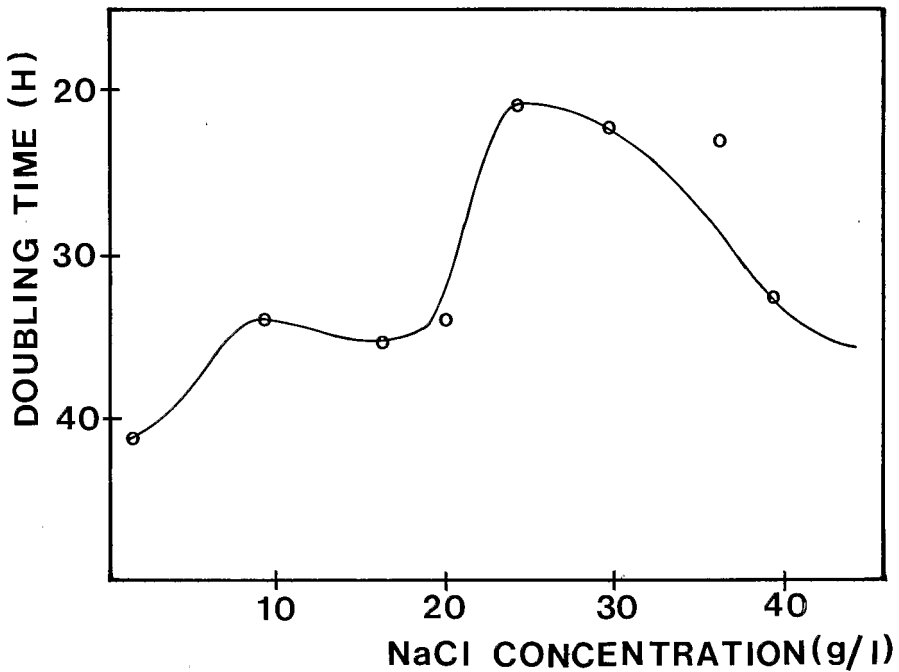


Fig. 6. Influence of NaCl on the growth of strain BSD.

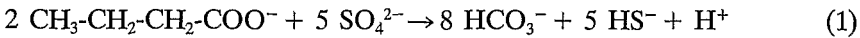
isolate are compared to those of the classical and recently newly isolated spore-forming sulfate-reducers. The studied organism grew reproducibly in subcultures with hydrogen and sulfate as sole energy source, supplemented with 1 mM acetate as carbon source. The strain also developed on hydrogen under strict autotrophic conditions in the absence of acetate and with CO_2 as only carbon source. Strain BSD degraded fructose in the presence or absence of sulfate. Lactate, ethanol and a large number of linear short and long-chain fatty acids, except acetate, were used as electron donors. Benzoate was not utilized.

The excretion of acetate during butyrate degradation (Tab. 2) may indicate that the strain was oxidizing butyrate incompletely to acetate (eq. 2). However, repeated quantitative experiments with butyrate as electron donor showed that less acetate and more sulfide were formed than theoretically possible from incomplete butyrate oxidation (eq. 2).

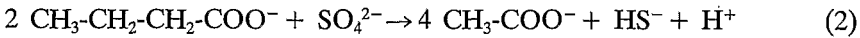
Discussion

Physiology

Among the non-sporulating SRB, the capacity to completely oxidize organic substrates to CO₂ is used to separate the complete oxidizers:



from the incomplete oxidizers (Widdel, 1987; Widdel & Pfennig 1984):



Applying this physiological scheme also to the genus *Desulfotomaculum*, one can separate the classical *Desulfotomaculum* species *D. nigrificans*, *D. orientis* and *D. ruminis* as the incomplete oxidizers (substrate degradation up to acetate, no acetate degradation) from the more recently described *Desulfotomaculum acetoxidans* and *D. sapomandens* as the complete oxidizers.

Stoichiometric measurements revealed that the newly isolated strain BSD produced more H₂S and less acetate than expected for incomplete oxidation. Because of acetate excretion it is difficult to conclude that strain BSD realized a complete oxidation of its substrates. Furthermore, no growth or sulfide formation on acetate as electron donor was observed. This observation suggests an obligatory cooxidation of acetate and butyrate since a part of the acetate produced was completely oxidized during butyrate degradation. Oxidation of acetate by *Desulfonema* species is also promoted by an additional electron donor (Widdel et al. 1983). The formation of acetate from organic substrate degradation by completely oxidizing sulfate reducers was observed with *Desulfococcus niacini* (Imhoff-Stuckle & Pfennig 1983); it was concluded that terminal oxidation of excreted acetate was a slower process than the degradation of the initial substrate to the level of acetate (acetyl-CoA) and that bacteria probably died off before further complete oxidation of acetate took place. The lack of acetate utilization by strain BSD may also suggest that it may not have an effective mechanism of acetate activation to acetyl-CoA. Klemps et al. (1985) also isolated a spore-forming sulfate-reducing bacterium (strain TEP) that performed complete oxidation but which was unable to use acetate.

Anyhow, the capability of strain BSD of partial acetate oxidation during butyrate metabolism clearly assigns it to the physiological group of completely oxidizing sulfate reducers (Tab. 2, eqs. 1, 2).

The described strain has no physiological capacity which is new within the genus *Desulfotomaculum*. However, the combination of such marked features

Lactate (20)	+	+	+	+	+	+	-	-	-
Methanol (20)	-	+	-	-	(+)	-	-	-	-(g)
Ethanol (20)	+	+	+	+	+	+	+	+	+
Fructose (20)	+	-	-	+	-	-	-	-	-
Fructose without SO ₄ ²⁻	+	NT	NT	+	-	-	-	-	-
Electron acceptors (e)									
Elemental sulfur	-	-	-	-	NT	NT	NT	-	+
CO ₂	-	+	-	-	NT	NT	NT	NT	-
Sulfate (20)	+	+	+	+	+	+	+	+	+
Sulfite (20)	+	+	+	+	NT	NT	NT	-	+
Thiosulfate (20)	NT	+	+	+	NT	NT	NT	-	+
Nitrate (10)	-	-	-	-	NT	NT	NT	-	-
Fumarate (40)	-	NT	NT	NT	NT	NT	NT	-	+
Optimum T° (°C)	54	30-37	36-40	55	35-37	35-37	35-37	36	38
Gas vacuoles	+	-	-	-	-	-	-	+	+
GC mol % of DNA	50.4	44.7-45.9 (a)	48.5-49.9 (a)	48.5-49.9 (a)	NT	NT	NT	37.5	48

(a) Campbell & Singleton 1986. (b) Klemms et al. 1985. (c) Cord-Ruwisch & Garcia 1985. (d) Electron donors were tested in the presence of 20 mmol lactate per l. (e) Electron acceptors were tested in the presence of excess sulfate. (f) In the presence of 1 mmol acetate per l. (g) Results not previously published. + Turbidity increase and production of H₂S. (+) Production of H₂S without turbidity increase. NT Not tested.

as thermophily and fatty acid degradation, does not allow the assignment of strain BSD to one of the existing species of the genus *Desulfotomaculum*. On the basis of these nutritional and physiological properties, one can consider that strain BSD differs from all other representatives of the genus. We therefore propose the new species *Desulfotomaculum geothermicum* to accommodate strain BSD.

Taxonomy

According to the current bacterial taxonomy, the described spore-forming sulfate-reducing bacterium has to be classified in the genus *Desulfotomaculum* (Widdel & Pfennig 1984; Campbell & Singleton 1986). Strain BSD contains only *c*-type cytochrome whereas most *Desulfotomaculum* species contain both *b*- and *c*-type cytochrome. However, with *D. guttoideum*, it is the second species which shows this characteristic (Widdel 1987).

After *D. nigrificans*, our isolate represents the second thermophilic species within this genus. However, in contrast to *D. nigrificans* the spectrum of substrates used by the described isolate is broader due to its capability to use fatty acids. Furthermore the formation of gas vacuoles as described in our isolate has not been described in *D. nigrificans*. Yet, the G + C % content of DNA of this strain and strain BSD seems closely related if we refer to determination of Nazina & Rozanova (1978).

Due to the cell shape and the characteristic formation of gas vacuoles, strain BSD resembles morphologically to *D. acetoxidans* or *D. sapomandens*.

Metabolically it appears more related to the recently isolated mesophilic fatty acid degrading *D. sapomandens* (Cord-Ruwisch & Garcia 1985) or to the unnamed *Desulfotomaculum* species described by Klemps et al. (1985, Table 1). However, also apart from the temperature ranges of growth, our isolate differs from *D. sapomandens* by its ability to use hydrogen, lactate, fructose

Table 2. Degradation of butyrate (mmol/l) by strain BSD.

Butyrate degraded	Acetate formed	Acetate/Butyrate	Sulfide formed	Sulfide/Butyrate	Electron balance (%)
4.5	5.5	1.2	4.5	1	89%
6.5	11.5	1.8	4.6	0.71	99.2%
10	15.2	1.5	6.8	0.65	89%

For comparison, theoretical production per mole butyrate during: complete oxidation - 0 acetate, 2.5 sulfide; incomplete oxidation - 2 acetate, 0.5 sulfide.

and propionate and by its inability to oxidize benzoate, and from the other species by its property of vacuole formation.

Species description

Desulfotomaculum geothermicum sp. nov. Geo. thermicum. gr. n. *geo* earth; gr. n. *Therme* heat; M.L. n. adj. *geothermicum* geothermal.

Morphology. Straight motile rods, single or in pairs, 2.3–2.5 μm by 0.5–0.8 μm . Sporulation occurs and gas vacuoles are always present during this process, giving a spindle-shaped form ($2.1 \times 1.2 \mu\text{m}$) of the cells. Vegetative cells stain Gram-negative.

Culture conditions. Strict anaerobe; thermophile; temperature range of growth: T_{min} , 37° C; T_{opt} , 54° C; t_{max} , 56° C; pH range of growth is 6.0 to 8.0 and optimum is between 7.2–7.4.

Nutritional characteristics. Chemoorganotroph, able to grow chemoautotrophically when $\text{H}_2\text{-CO}_2$ and sulfate are the only energy and carbon sources. Does not degrade acetate as only electron donor but utilizes a large number of linear short and long-chain fatty acids such as formiate, propionate, butyrate, palmitate, stearate or pelargonate. Other simple organic substrates as lactate and ethanol may also be degraded. Theoretically the organism has the capacity to oxidize its substrates completely to CO_2 . However the excretion of acetate was generally observed during butyrate degradation. The guanine plus cytosine content of DNA is 50.4 mol%. Presence of a c-type cytochrome and a sulfite reductase P582. No desulfoviridin.

Type strain. Strain BSD has been deposited in the Deutsche Sammlung von Mikroorganismen (DSM), Göttingen, F.R.G., under the number DSM 3669.

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