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Isolation and characterization of a thermophilic benzoate-degrading, sulfate-reducing bacterium, *Desulfotomaculum thermobenzoicum* sp. nov.

Masaharu Tasaki [1], Yoichi Kamagata [2], Kazunori Nakamura [2], and Eiichi Mikami [2]

[1] Institute of Technology Shimizu Corporation, 4–17, Etchujima 3-chome, Koto-ku, Tokyo 135, Japan
[2] Fermentation Research Institute, Agency of Industrial Science and Technology, Tsukuba, Ibaraki 305, Japan

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Abstract. A new thermophilic sulfate-reducing bacterium, strain TSB, that was spore-forming, rod-shaped, slightly motile and gram-positive, was isolated from a butyrate-containing enrichment culture inoculated with sludge of a thermophilic methane fermentation reactor. This isolate could oxidize benzoate completely. Strain TSB also oxidized some fatty acids and alcohols. SO_4^{2-}, SO_3^{2-}, $S_2O_3^{2-}$ and NO_3^- were utilized as electron acceptors. With pyruvate or lactate the isolate grew without an external electron acceptor and produced acetate. The optimum temperature for growth was 62°C. The $G + C$ content of DNA was 52.8 mol%. This isolate is described as a new species, *Desulfotomaculum thermobenzoicum*.

Key words: *Desulfotomaculum thermobenzoicum* – Anaerobic benzoate oxidation – Spore formation – Thermophile – Sulfate reduction

Benzoate is one of the most important intermediates in the anaerobic digestion of natural or chemically synthesized aromatic compounds. Several syntrophic bacteria are known as benzoate degraders; *Syntrophus buswellii* was the first isolate that was found to utilize benzoate in coculture with a hydrogen scavenging methanogen or sulfate reducer (Mountfort et al. 1984). Recently, a bacterium (strain HQGö 1) which is able to utilize hydroquinone nonsyntrophically was found to degrade benzoate in defined mixed culture (Szewzyk and Schink 1989). Some species of sulfate-reducing bacteria can also oxidize benzoate with SO_4^{2-} as external electron acceptor (Widdel 1987). However, thermophilic syntrophic or sulfate-reducing bacteria capable of utilizing benzoate have not yet been isolated. In this report, we describe a novel type of thermophilic sulfate-reducing bacterium belonging to *Desulfotomaculum* that can oxidize benzoate with complete oxidation.

Offprint requests to: Y. Kamagata

Materials and methods

Source of organism

Strain TSB was isolated in pure culture from an enrichment inoculated with sludge from a methane fermentation reactor. The reactor has been treating wastewater from a kraft pulp production process at 55°C for approximately a year.

Media and conditions for cultivation

The preparation of basal medium was based on the method of Widdel and Pfennig (1981). The medium contained (g/l): KH_2PO_4, 0.2; NH_4Cl, 0.5; $MgCl_2 \cdot 6H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.15. After autoclaving, 2.5 g $NaHCO_3$, 0.36g $Na_2S \cdot 9H_2O$, 1 ml vitamins solution, 1 ml trace elements solution and 50 mg yeast extract as organic supplement were added to the cooled medium under anaerobic conditions. Trace elements solution was supplemented with 3.0 mg $Na_2SeO_3 \cdot 5H_2O$ and 4.0 mg $Na_2WO_4 \cdot 2H_2O$ per 1000 ml. The pH of the medium was adjusted to 7.2 with 1 *N* HCl solution. The medium was distributed to sterilized screw cap tubes (20 ml) or bottles (50 ml), which were completely filled.

Neutralized substrates and electron acceptors such as sulfate were added to the tube from the stock solutions just prior to inoculation. Na_2SO_4, Na_2SO_3, $Na_2S_2O_3$, $NaNO_3$ and $S^{(0)}$ were used for the test of electron acceptors. All stock solutions were sterilized by membrane filtration (0.2 μm pore size). To check the utilization of H_2, 50 ml serum bottles containing 20 ml medium with H_2/CO_2 (4:1) as a gas phase were used. The butyl rubber stoppers were fixed with aluminum seals. All cultures were incubated at 56°C. For enrichment, 10 mM butyrate (Na) as electron donor and carbon source, and 10 mM sulfate (Na_2SO_4) as electron acceptor were used.

Isolation

A pure culture was obtained by repeated application of the agar dilution method (Widdel and Pfennig 1982).

To check for purity, the isolate was inoculated into media with 0.1% yeast extract plus 0.1% Bacto-peptone (DIFCO), glucose (2 mM), sucrose (1 mM), or H_2 plus CO_2 as substrates with and without sulfate. After incubation the media were examined microscopically.

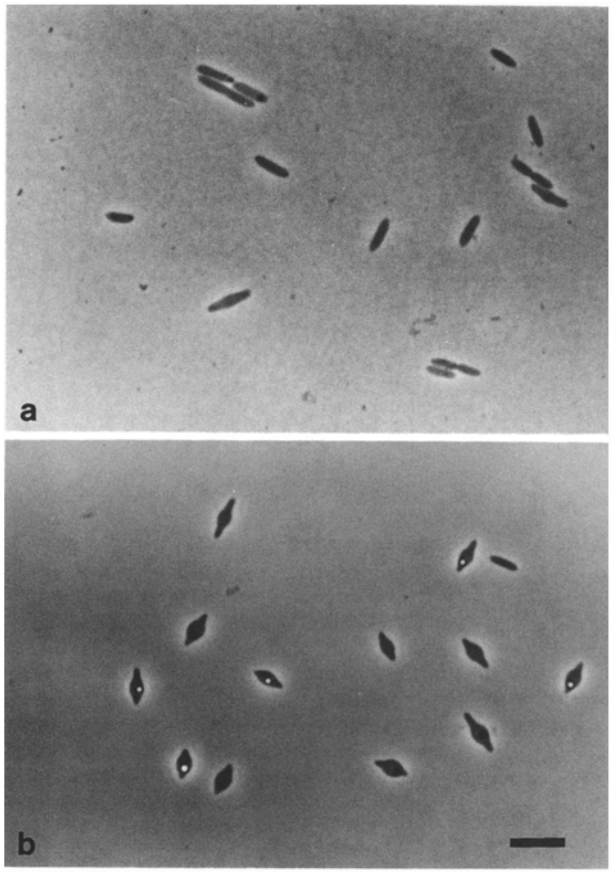

Fig. 1 a, b. Photomicrograph of strain TSB (*Bar* equals 10 µm). Phase contrast micrograph of cells grown on butyrate (**a**), and benzoate (**b**)

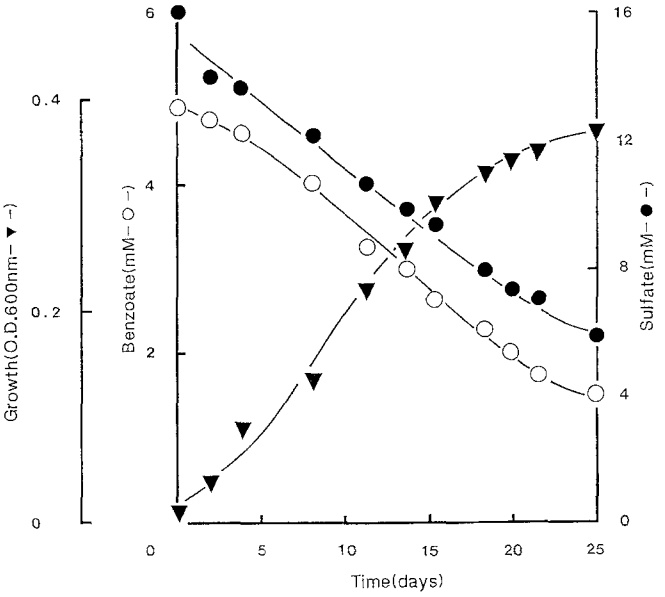

Fig. 2. Degradation of benzoate by strain TSB. 500 ml medium bottles containing 300 ml medium with N_2/CO_2 (4:1) as a gas phase were used. Cultivation was performed at 56° C. *Symbols:* ○---○; benzoate, ●---●; sulfate, ▼---▼; growth ($OD_{600\,nm}$)

Determinations

Growth was monitored spectrophotometrically at 600 nm. Substrates and products were measured by gas chromatography (PEG-6000 15% on Flusin P 60/80; glass column. ID 1.5 mm × 3 m; column temperature, 160° C with a FI-detector or by HPLC (Shimadzu-gel SCR-101H, 7.9 × 300 mm; eluent, acidified water; alternatively, Gasukuro Kogyo Inertsil ODS 5 µm, 4.6 × 250 mm; eluent, methanol/water (60/40) adjusted pH to 2.6 with H_3PO_4) with RI and UV (210 nm) detectors. Sulfate was determined by ion chromatography (Yokogawa electric corporation; column, SAX1-205; carrier, 4 mM Na_2CO_3 + 4 mM $NaHCO_3$; scavenger, 15 mM H_2SO_4; detector, electrical conductivity detector). The DNA base composition was determined by measuring deoxyribonucleosides using HPLC with a UV-detector (Tamaoka and Komagata 1984).

Results

Enrichment and isolation

Sludge from a thermophilic digestor was inoculated into the medium containing butyrate and sulfate (5% inoculum, v/v) and incubated at 56° C. After several transfers, slightly motile and rod-shaped bacteria were predominant in the culture. 10 mM Butyrate was degraded within two weeks with concomitant consumption of sulfate. Most of the colonies which appeared in the agar-dilution cultures were lens-shaped and dark-brown-colored. A butyrate-using, sulfate-reducing bacterium named strain TSB was isolated by repeated application of the isolation procedure.

Morphology

After growth of the isolate on butyrate, straight or slightly curved rods (1.5 − 2.0 × 5 − 8 µm) with pointed ends (Fig. 1 a) were observed. The bacteria were slightly motile. Cells grown on benzoate were almost spindle-shaped rods (Fig. 1 b). Spores were evidenced both by microscopy (Fig. 1 a, b) and growth after heat shock (15 min at 95° C). Gram-stain showed positive and gram-type test (Gregersen 1978) showed positive. Desulfoviridin was not detected by the *Postgate test* (Postgate 1979).

Determination of the DNA base ratio of the isolate by HPLC yielded a content of 52.8 mol% guanine + cytosine.

Growth conditions and nutrition

Strain TSB grew at 40° C to 70° C, the optimum temperature being at 62° C. The pH range was between 6.0 and 8.0 with an optimum at pH 7.2. The degradation of benzoate by strain TSB is shown in Fig. 2.

The isolate required organic nutrients such as yeast extract. With Bacto-peptone instead of yeast extract, it grew much more slowly.

In the presence of benzoate, strain TSB was able to use sulfate, sulfite, thiosulfate or nitrate as electron acceptor, although the growth with thiosulfate or nitrate was slow. Sulfur was not utilized.

Different substrates were tested as electron donors in the presence of sulfate (Table 1). The isolate utilized some

fatty acids such as formate, propionate, butyrate, valerate and caproate, but acetate was not utilized. Ethanol, propanol, butanol, lactate and pyruvate were also utilized. Slower growth was obtained on 1,2-propanediol or 1,3-propanediol. The isolate was capable of growth on H_2 and CO_2 as sole carbon and energy source with production of acetate. Lactate and pyruvate were utilized for growth without electron acceptor, and were fermented to acetate.

Stoichiometry of benzoate and butyrate oxidation

The stoichiometry of benzoate and butyrate oxidation was determined for strain TSB. Results are shown in Table 2. Although benzoate was completely oxidized to CO_2, acetate was produced from butyrate. Sulfite and thiosulfate were not detected. The molar ratios between substrate oxidized and SO_4^{2-} consumed coincided with the following equations.

Benzoate

$$C_6H_5COO^- + 3.75\,SO_4^{2-} + 4\,H_2O \rightarrow 7\,HCO_3^- + 3.75\,HS^- + 2.25\,H^+ \quad (1)$$

$$\Delta G^{0'} = -165.8 \text{ kJ/reaction.}$$

Butyrate

$$CH_3CH_2CH_2COO^- + 1.5\,SO_4^{2-} \rightarrow CH_3COO^- + 2\,HCO_3^- + 0.5\,H^+ + 1.5\,HS^- \quad (2)$$

$$\Delta G^{0'} = -84.0 \text{ kJ/reaction.}$$

The $\Delta G^{0'}$ values (for pH 7.0) were calculated from the data of Thauer et al. (1977).

Equation (2) of butyrate-oxidation was in agreement with that by *Desulfobacterium autotrophicum* (HRM2) as observed by Schauder et al. (1986).

Table 1. Organic compounds tested as electron donors and carbon sources in the presence of sulfate. Concentrations (mM) in the medium are given in parentheses

Utilized

H_2 plus CO_2
Formate[a](20), formate(20) + acetate(5),
Propionate(10), butyrate(10), valerate(5),
Caproate(2), ethanol(10), propanol(10),
Butanol(10), 1,2-propanediol[a](10),
1,3-Propanediol[a](10), crotonate(10),
Lactate(10), pyruvate(5), fumarate(10),
Malate[a](10), benzoate(5),
Pyruvate(10) without SO_4^{2-}, lactate(10) without SO_4^{2-}.

Tested but not utilized

Acetate(10), methanol(10), acrylate(10),
Succinate(10), glucose(2), fructose(2),
Sucrose(1), phenol(5),
o-, *m*-, *p*-Hydroxybenzoate(5).

Each culture was incubated at 56° C for 2 weeks.
After cultivation, remaining substrates were measured by HPLC.
[a] Utilized slowly

Discussion

Physiological aspects

It has been suggested that many aromatic compounds are degraded via benzoate. Benzoate could be detected as an intermediate of phenol or chlorophenol degradation by anaerobic consortia (Knoll and Winter 1989; Kobayashi et al. 1989; Zhang et al. 1990). In defined syntrophic cocultures or axenic cultures, benzoate is an intermediate

Table 2. Results of stoichiometric measurements with *Desulfotomaculum thermobenzoicum* strain TSB on benzoate or butyrate as electron donor and carbon source, and sulfate as electron acceptor

Substrate given (sulfate given) [mmol/l]		Substrate utilized (sulfate utilized) [mmol/l]	Acetate excreted [mmol/l]	Cell dry weight formed [mg/l]	Substrate consumed for cell material[a] [mmol/l]	Substrate oxidized by sulfate reduction [mmol/l]	mol SO_4^{2-} consumed per mol substrate oxidized	Growth yield: g dry weight per mol substrate oxidized
Benzoate	2 (10)	1.8 (6.2)	0.1	23.2	0.13	1.67	3.71	13.9
	4 (15)	3.4 (10.0)	0.2	37.8	0.21	3.19	3.13	11.8
Butyrate	5 (10)	5.0 (7.3)	4.7	20.2	0.17	4.83	1.51	4.18
	10 (20)	10.0 (13.5)	9.6	38.5	0.32	9.68	1.39	3.98
	20 (40)	20.0 (28.8)	19.2	58.8	0.49	19.51	1.48	3.01

[a] Substrate consumed for cell material was calculated by the following equations:
$17\,C_6H_5COO^- + HCO_3^- + 71\,H_2O \rightarrow 30(C_4H_7O_3) + 18\,OH^-$; thus, 0.0055 mmol benzoate are required for 1.0 mg of cell dry weight
$17\,CH_3CH_2CH_2COO^- + 12\,HCO_3^- + 19\,H_2O \rightarrow 20(C_4H_7O_3) + 29\,OH^-$; thus, 0.0083 mmol butyrate are required for 1.0 mg of cell dry weight

Table 3. Properties of various *Desulfotomaculum* species[a]

	D. thermobenzoicum	*D. sapomandens*	*D. geothermicum*	*D. thermoacetoxidans*
Range of temperature	40–70	20–43	37–56	45–65
Optimum temperature	62	38	54	55–60
mol% $G+C$	52.8	48	50.4	49.7
Growth ($+SO_4^{2-}$)				
H_2+CO_2	+	−	+	+
Formate	+	+	+	+
Acetate	−	+	−	+
Propionate	+	−	+	+
Butyrate	+	+	+	+
Ethanol	+	+	+	−
Lactate	+	−	+	+
Pyruvate	+	+	ND[b]	+
Benzoate	+	+	−	−
Fructose	−	−	+	ND
Lactate without SO_4^{2-}	+	ND	ND	+
Pyruvate without SO_4^{2-}	+	−	ND	+
As e^- acceptor;				
SO_4^{2-}	+	+	+	+
SO_3^{2-}	+	+	+	−
$S_2O_3^{2-}$	+	+	+	+
NO_3^-	+	−	−	ND
S^0	−	+	−	−

[a] Data from Cord-Ruwisch and Garcia (1985); Daumas et al. (1988); Min and Zinder (1990)
[b] Not determined.

in the degradation of 3-hydroxybenzoate or 3-chlorobenzoate. 3-Hydroxybenzoate was dehydroxylated to benzoate by strain KN032 in coculture with an H_2-consumer (Tschech and Schink 1986). Strain DCB-1 dechlorinated 3-chlorobenzoate to benzoate stoichiometrically when 3-chlorobenzoate was added to the pyruvate medium (Shelton and Tiedje 1984).

Some sulfate-reducing bacteria are known to oxidize benzoate; *Desulfonema magnum* (Widdel et al. 1983), *Desulfobacterium phenolicum* (Bak and Widdel 1986), *D. catecholicum* (Szewzyk and Pfennig 1987), *D. anilini* (Schnell et al. 1989), *Desulfococcus multivorans* (Widdel and Pfennig 1984), *Desulfosarcina variabilis* (Widdel and Pfennig 1984), *Desulfotomaculum sapomandens* (Cord-Ruwisch and Garcia 1985) and *Desulfomonile tiedjei* (DeWeerd et al. 1990) can utilize benzoate, but all isolates are mesophiles.

Only two species, *Desulfotomaculum geothermicum* (Daumas et al. 1988) and *D. thermoacetoxidans* (Min and Zinder 1990), are thermophilic fatty acid degrading sulfate-reducing bacteria, but neither bacteria can use benzoate. *D. thermoacetoxidans* is able to oxidize acetate completely, while strain TSB is not. Fructose which cannot be degraded by strain TSB can be degraded with or without electron acceptor by *D. geothermicum*. To our knowledge, the present study is the first description of the isolation of a thermophilic benzoate-degrading sulfate-reducing bacterium.

Strain TSB is a completely oxidizing type of bacterium, as shown by the stoichiometry of benzoate oxidation. However, with fatty acids, TSB produced acetate. In the case of incompletely oxidizing sulfate reducers, butyrate is usually oxidized to acetate as the entire oxidation product (Eq. 3, Widdel 1987).

$$CH_3CH_2CH_2COO^- + 0.5\,SO_4^{2-} \rightarrow 2\,CH_3COO^- + 0.5\,HS^- + 0.5\,H^+ . \quad (3)$$

However, strain TSB oxidizes 1 mol butyate to 1 mol acetate, and consumes 1.5 mol sulfate (Eq. 2). These findings are in good agreement with butyrate oxidation by *Desulfobacterium autotrophicum* which is also a complete oxidizer (Schauder et al. 1986). Therefore, it is likely that strain TSB activates butyrate by CoA transfer from acetyl-CoA formed from butyryl-CoA by β-oxidation. As a result, 1 mol butyrate is oxidized to 1 mol acetate and 2 mol CO_2 with consumption of 1.5 mol sulfate.

Taxonomy

We propose to place strain TSB in the genus *Desulfotomaculum*, because the isolate is a spore-forming rod-shaped sulfate-reducing bacterium. Strain TSB and all other *Desulfotomaculum* species are spore-forming, rod-shaped, slightly motile, and have no desulfoviridin. Among *Desulfotomaculum* species, there are three thermophilic ones, *D. nigrificans* (Werkman and Weaver 1927), *D. geothermicum* and *D. thermoacetoxidans*. Strain TSB differs from these other strains with respect to the range of substrates utilized (Table 3). In particular, strain TSB utilizes benzoate which the other strains cannot oxidize. Therefore, these differences justify to establish a new species, *Desulfotomaculum thermobenzoicum*.

Desulfotomaculum thermobenzoicum

Thermo.benzo'i.cum. Gr.adj.*thermos* hot; N. L. *benzoicum* pertaining to benzoate. *Desulfotomaculum thermobenzoicum* a sausage-shaped organism that reduces sulfur compounds and oxidizes benzoate under thermophilic conditions.

Rod shaped cells were 1.5–2 μm in diameter and 5–8 μm in length, single or in pairs. Spore forming and slightly motile.

The following substrates can be utilized: Benzoate, H_2+CO_2, formate, propionate, butyrate, valerate, caproate, ethanol, propanol, butanol, 1,2-propanediol, 1,3-propanediol, crotonate, lactate, pyruvate, fumarate and malate. Pyruvate and lactate are degraded without sulfate. Good growth occurs on benzoate, alcohols, butyrate and H_2 plus CO_2.

Electron acceptors are sulfate, sulfite, thiosulfate and nitrate. Sulfur cannot be used as electron acceptor.

Organic supplements (50 mg/l yeast extract) are required for growth. Addition of NaCl is not necessary.

The temperature range for growth is 40°–70°C, with an optimum at 62°C. The pH range is 6.0–8.0, with an optimum at 7.2.

Desulfoviridin not present. The DNA base ratio is 52.8 mol% $G+C$ (by HPLC).

D. thermobenzoicum has been enriched and isolated from the sludge of the thermophilic methane fermentation reactor treating wastewater from a kraft pulp production process.

Strain TSB has been deposited as the type strain of *Desulfotomaculum thermobenzoicum* (DSM 6193) in the Deutsche Sammlung von Mikroorganismen.

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