Physiological Characteristics of a New Thermophilic Obligately Chemolithotrophic *Thiobacillus* Species, *Thiobacillus tepidarius*

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We describe a new *Thiobacillus* species which is a gram-negative, motile, rod-shaped organism having polar flagella. The optimum growth temperature is 43 to 45°C, and the optimum pH range is 6.0 to 7.5. This organism is obligately chemolithotrophic and autotrophic and has ribulose biphosphate carboxylase activity. It is able to oxidize thiosulfate, trithionate, tetrathionate, hexathionate, heptathionate, sulfur, and sulfide, but is not able to use sulfite, thiocyanate, or dithionate for growth. In batch culture it converts thiosulfate to tetrathionate during or before growth. It has both rhodanese and thiosulfate-oxidizing enzyme activities. It does not grow anaerobically with nitrate or nitrous oxide on either thiosulfate or tetrathionate. The guanine-plus-cytosine content of its deoxyribonucleic acid is 66.6 mol%, and it contains ubiquinone Q-8 in its respiratory chain. The organism is named *Thiobacillus tepidarius* sp. nov. The type strain is strain DSM 3134, which has been deposited in the Deutsche Sammlung von Mikroorganismen, Göttingen, Federal Republic of Germany.

Although thermophilic, acidophilic strains belonging to the genus *Thiobacillus* have been reported in the past, no thermophilic species are currently known to be in culture collections, and no species has been properly described. We isolated a novel obligately chemolithotrophic, moderately thermophilic *Thiobacillus* species that is distinct from all species described previously. It differs from previously reported thermophiles in growing only around neutral pH and is unlike any other organism previously described except perhaps *Sulfomonas* sp. strain no. 3 of Czurda (1).

We named this organism *Thiobacillus tepidarius*, and it is 1 of the 13 species of *Thiobacillus* recognized in *Bergey's Manual of Systematic Bacteriology*, 1st ed. (D. P. Kelly and A. P. Harrison, in press).

MATERIALS AND METHODS

Isolation of the organism. Samples of water from the inflow of the Great Bath (in the Roman baths at Bath, Avon, United Kingdom) having a temperature of 43°C and a pH of 7.3 were inoculated into liquid medium containing either thiosulfate (20 mM) or methylammonium chloride (10 mM) or both and were incubated in 250-ml flasks shaken in air at 50°C. The most successful culture, which was obtained by growing the organism on methylammonium plus thiosulfate, was subcultured into medium containing methylammonium and 5mM NaHCO₃. The resulting culture was then plated onto thiosulfate agar medium and incubated at 50°C in gas jars with an enhanced CO₂ atmosphere (5%, vol/vol). Single-colony isolations were made, and the two most promising isolates were inoculated into liquid media containing either thiosulfate or methylammonium with or without NaHCO₃. At this stage no growth was obtained on methylammonium, but growth on thiosulfate was good. One of the single-colony isolates was studied further and characterized.

Culture conditions. *Thiobacillus tepidarius* was isolated by using liquid medium containing (per liter) 4.0 g of K₂HPO₄, 4.0 g of KH₂PO₄, 0.4 g of NH₄Cl, 0.8 g of MgSO₄·7H₂O, and 10 ml of a trace metal solution (8). To vary the pH in growth experiments in batch cultures, the ratio of the phosphates

was altered, but the same overall molar concentration of phosphates was maintained. The phosphates were sterilized separately and aseptically mixed with the other medium components after autoclaving and cooling. We made agar slants and plates containing the medium described above supplemented with 10 g of Bacto-agar (Difco Laboratories) per liter and 2 ml of saturated aqueous bromocresol purple per liter. The energy source supplied was either 20 mM Na₂S₂O₃·5H₂O or 10 mM K₂S₄O₆. Once purified, the organism was maintained on agar slants containing tetrathionate medium.

Agar medium supplemented with either Oxoid nutrient broth (1.3 g/liter) or thiosulfate or both was used for plating dilutions of cultures to check for contaminants. To determine heterotrophic potential, the following agar media were used: Lab M nutrient agar (2.8%, wt/vol) and Oxoid nutrient broth (0.325%, wt/vol) containing Bacto-agar (1.5%, wt/vol) with and without glucose (0.5%, wt/vol).

Growth experiments. Inoculum cultures (100 ml) for experiments were grown routinely on 20 mM thiosulfate medium that was inoculated from thiosulfate slants and shaken in 250-ml Erlenmeyer flasks. When NaHCO₃ was added as the CO₂ supply, the cultures were grown in Quickfit flasks fitted with Suba Seal vaccine stoppers. Inocula (10%, wt/vol) from these fully grown cultures were added to 100 ml of test medium and shaken at the appropriate temperature on a Gallenkamp orbital incubator. Samples (2 to 5 ml) were removed at intervals for analysis. Growth was monitored by measuring culture absorbance at 440 nm, which was converted to dry weight (in grams per liter) by referring to a calibration curve prepared for suspensions of organisms dried to constant weight at 105°C. Thiosulfate, tetrathionate, and other polythionates were assayed by the cyanolysis method (5).

Anaerobic growth was tested at 43°C in 100- or 150-ml bottles completely filled with medium (pH 7.0) supplemented with 33 mM KNO₃.

pH-controlled growth experiments. Batch cultures (1 liter) were grown in an LH Engineering series 500III fermentor. Initially, the pH was controlled to 7.0 by using automatic titration with Na₂CO₃; the culture was stirred at 750 rpm and aerated with 100 ml of air per min. The aeration and stirrer

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TABLE 1. Effect of initial pH on the growth of *T. tepidarius* at 50°C^a

Culture	Initial pH	Final pH	% Of initial Na ₂ S ₂ O ₃ (20 mM) remaining at the end of growth as:		Specific growth rate (h ⁻¹)
			S ₂ O ₃ ²⁻	S ₄ O ₆ ²⁻	
1	5.0	5.4	54.6	21.5	NG ^b
2	5.5	5.2	0	77.3	0.046
3	5.6	4.9	0	62.3	0.053
4	5.7	4.9	1.8	67.1	0.058
5	5.7 ^c	4.8	13.8	58.0	0.046
6	6.3	4.8	0.7	49.5	0.058
7	6.4	5.0	0	47.5	0.066
8	7.0 ^c	6.5	7.1	1.6	0.043
9	7.0	5.1	4.9	21.6	0.039
10	7.7	5.3	4.2	8.3	0.020
11	8.0	7.4	96.7	6.8	NG
12	8.5	7.6	96.5	2.8	NG
13	6.9 ^d	5.2	0	61.0	0.060

^a Except where indicated, cultures were grown on 20 mM Na₂S₂O₃ by using inoculum cultures grown initially at pH 7.0. The inoculum for culture 1 came from culture 4. The inocula for cultures 5 and 8 came from cultures 4 and 9 respectively. The inoculum for culture 13 came from culture 9.

^b NG, No growth.

^c The initial thiosulfate concentration was 5 mM.

^d Tetrathionate was supplied at a concentration of 10 mM in the absence of thiosulfate.

rates were increased to 300 ml/min and 1,000 rpm, respectively, when we found that these rates increased the growth rates at the higher temperatures tested. Once the temperature optimum had been determined, the culture temperature was constant while the growth rates were measured at a number of pH values.

Preparation of cell extracts. Cultures were harvested by centrifugation, washed once with 0.055 M phosphate buffer (pH 8.0), centrifuged at 23,000 × *g*, and then suspended in 0.1 M PIPES [piperazine-*N*, *N'*-bis (2-ethanesulfonic acid)] buffer (pH 7.0). This cell suspension was then passed twice through a French pressure cell at 1.4 × 10⁸ Pa and centrifuged at 38,000 × *g* for 45 min. The resultant supernatant was used to assay for rhodanese and thiosulfate-oxidizing enzymes.

Enzyme assays. The method used for the rhodanese assay was based on a previously described method (6), as was the assay used for thiosulfate-oxidizing enzyme (3). Ribulose biphosphate carboxylase was assayed by the whole-cell procedure (7). All enzyme assays were carried out at 43 to 45°C.

DNA base composition. The guanine-plus-cytosine (G+C) content of the deoxyribonucleic acid (DNA) was determined by the buoyant density method and was carried out by C. S. Dow.

Ubiquinone analysis. The ubiquinone fraction of the organism was isolated by acetone extraction (2) of a bacterial suspension that had been passed twice through the French pressure cell. The purified ubiquinone fraction (in hexane) was reduced almost to dryness by rapid rotary evaporation at 55°C. The residue was dissolved in ethanol (2 ml), and the ubiquinone was identified by comparative thin-layer chromatography. The marker compounds used were ubiquinone Q-10 (Sigma Chemical Co.) and ubiquinone Q-8 isolated from *Thiobacillus denitrificans* by the same procedure. Chromatograms were run with benzene on Gelman ITLC SAF or Merck DC Alufolien Kieselgel 60F₂₅₄ plates. Both of these contained a fluorescent dye which enabled direct

observation of ubiquinones as dark spots under ultraviolet light (254 nm). The *R_f* values obtained were 0.78 and 0.59 for ubiquinone Q-8 and 0.38 and 0.15 for ubiquinone Q-10 on ITLC SAF and DC Alufolien Kieselgel 60F₂₅₄ plates, respectively.

RESULTS

Morphological and physiological characteristics. *T. tepidarius* cells are motile, rod shaped, and about 0.3 by 0.8 μm, have polar flagellation, and contain storage polyphosphate inclusions. This organism is able to grow autotrophically on a wide range of reduced sulfur compounds, including sulfur, sulfide, thiosulfate, trithionate, tetrathionate, hexathionate, and heptathionate, but is not able to grow on dithionate, sulfite, or thiocyanate. Sulfur is deposited in colonies on thiosulfate agar but not in aerobic liquid cultures. A microscopic examination of organisms from such colonies revealed significant pleiomorphism (apparently due mainly to variable amounts of intracellular inclusions); such pleiomorphism was not seen in organisms grown in liquid cultures. *T. tepidarius* is not able to grow heterotrophically or as a mixotroph. Autotrophic growth appears to be explained by the presence of ribulose biphosphate carboxylase, the activity of which was 0.2 μmol of CO₂ fixed per min per mg (dry weight) in the whole-cell assay. Rhodanese and thiosulfate-oxidizing enzyme activities of 0.2 to 0.8 μmol of S₂O₃²⁻ used per min per mg of protein were found in crude extracts. Although *T. tepidarius* can reduce nitrate to nitrite, it is not able to grow anaerobically with either nitrate or nitrous oxide.

Growth experiments. *T. tepidarius* grew on thiosulfate in media having initial pH values of 5.5 to 7.7 at 50°C (Table 1). Before growth started, the thiosulfate was always quantitatively converted to tetrathionate, and there was a concurrent increase in pH. Once the pH had fallen to about 5.0, growth stopped. No growth occurred in medium at pH 5.0, although some oxidation of thiosulfate was detected. Some cultures (Table 1, cultures 4, 10, and 11) were analyzed for trithionate, but none was detected as an intermediate product.

Addition of glucose (1 mM), yeast extract (0.02%), or nutrient broth (0.13%) had little effect on the growth rate of *T. tepidarius* on thiosulfate at pH 7.0 and did not increase biomass. The organism was not able to grow in a liquid

TABLE 2. Temperature range for growth of *T. tepidarius* on thiosulfate (20 mM) in batch cultures initially at pH 6.6

Temp (°C)	Final pH	% Of initial thiosulfate remaining at the end of incubation as:		Specific growth rate (h ⁻¹)
		S ₂ O ₃ ²⁻	S ₄ O ₆ ²⁻	
15	7.2	9.0	77.0	NG ^a
20	4.4	0	35.2	0.014
25	6.1	0	45.2	0.024
30	3.9	0.5	29.6	0.048
37	5.1	0	39.2	0.063
43	4.7	0	40.5	0.055
50	4.5	0	37.2	0.063
52 ^b	ND ^c	62.5	31.0	0.004
55	6.6	74.7	12.1	NG
60 ^d	6.6	107.3	0	NG

^a NG, No growth.

^b The inoculum culture was grown at 43°C.

^c ND, Not determined.

^d The inoculum culture was grown at pH 7.7.

nutrient broth culture without thiosulfate or at 43°C on yeast extract (0.02%), succinate (10 mM), glucose (10 mM), glutamate (10 mM), ribose (10 mM), fructose (10 mM), sucrose (10 mM), citrate (10 mM), acetate (20 mM), glycolate (30 mM), formate (40 mM), methylammonium chloride (50 mM), nutrient agar, or nutrient agar supplemented with glucose.

Using batch culture methods and media at pH 6.6 to 6.8, we determined the temperature range for growth (Table 2). The limits were 20 to 52°C, with a broad optimum range of 37 to 50°C. Oxidation of thiosulfate to tetrathionate was possible at 15°C (without growth) but not at 55 or 60°C.

Additional studies were carried out by using a fermentor with automatic pH control so that batch cultures could be maintained under constant environmental conditions for precise determination of optimum temperature and pH values for growth. In these studies we used tetrathionate as the substrate since production of tetrathionate from thiosulfate results in a pH increase which would have complicated the control of pH unnecessarily. The optimum temperature for growth (Fig. 1) was 43 to 45°C. Cultures controlled at this optimum temperature were used to measure growth rates over a range of pH values (Fig. 1). The pH optimum covered a broad range (pH 6.0 to 7.5).

Trithionate (6.5 mM) was supplied to a fully grown tetrathionate culture at 45°C and pH 7.2. This substrate disappeared from the medium with a half-life of 30 min and without any detectable sulfur, thiosulfate, or tetrathionate being produced.

Anaerobic growth on tetrathionate was not observed although about one-half of the nitrate supplied was converted to nitrite.

Molar growth yields. When grown at a dilution rate of 0.4 h⁻¹ in a substrate-limited chemostat culture, *T. tepidarius* gave yields of 10.2 and 17.2 g (dry weight per mol of substrate oxidized) on thiosulfate and tetrathionate, respectively (Wood and Kelly, submitted for publication).

DNA base composition. Triplicate estimations by the buoyant density method indicated that the G+C content of the DNA was 66.6 ± 0.5 mol%.

Ubiquinone content. The mobility of the ubiquinone isolated from *T. tepidarius* indicated that it was ubiquinone Q-8 (based on the mobilities of the ubiquinone Q-8 and Q-10 markers).

DISCUSSION

The thermophile described above belongs in the genus *Thiobacillus* since it is a gram-negative, motile, rod-shaped organism which obtains energy by oxidizing reduced inorganic sulfur compounds. It is similar to *Thiobacillus thioparus*, *Thiobacillus neapolitanus*, *Thiobacillus thiooxidans*, and *Thiobacillus denitrificans* in that it is obligately chemolithotrophic and autotrophic and contains ubiquinone Q-8 (4), but it differs from all of the other species currently known to be in pure culture in that it is thermophilic. The G+C content of its DNA (66.6 mol%) indicates that it is significantly different genetically from the type species, *T. thioparus* (62 mol%) and from *T. neapolitanus* (56 mol%), although because of its ability to oxidize and grow on sulfur compounds it closely resembles *T. neapolitanus*. We propose the name *Thiobacillus tepidarius* sp. nov. for this new thermophile.

Description of *Thiobacillus tepidarius*. The description of *T. tepidarius* (tep.i.dar'i.us. L.n. *tepidarium* a warm bath fed by natural thermal water; M.L. adj. *tepidarius* warm-bathing) given below is based on one strain (the type strain).

FIG. 1. Temperature and pH profiles for autotrophic growth of *T. tepidarius* sp. nov. on tetrathionate. The specific growth rates are shown for a range of temperatures with aeration of 100 ml/min and stirring at 750 rpm (○) or with aeration of 300 ml/min and stirring at 1000 rpm (●) at pH 7.0 and for a range of pH values (□) at 43°C.

Cells are gram-negative, motile, rod shaped, and 0.2 to 0.4 by 0.6 to 1.0 μm, with polar flagella that are three to five times the length of the cell. Cells contain storage polyphosphate inclusions. This organism is obligately chemolithotrophic and autotrophic, using the oxidation of thiosulfate, trithionate, tetrathionate, hexathionate, heptathionate, sulfur, and sulfide for growth. Thiosulfate is initially quantitatively converted to tetrathionate before growth proceeds. Carbon dioxide is fixed by means of ribulose biphosphate carboxylase. On agar medium containing thiosulfate, colonies are creamy white, opaque, and up to 2 mm in diameter. The temperature for optimum growth is 43 to 45°C. The pH for optimum growth is pH 6.0 to 7.5. Nitrate is reduced to nitrite, but growth does not occur with either nitrate or nitrous oxide. The DNA contains 66.6 mol% G+C. Ubiquinone Q-8 is present in the respiratory chain. The organism was isolated from water at the Roman baths, Bath, Avon, United Kingdom. The type strain has been deposited with the Deutsche Sammlung von Mikroorganismen, Göttingen, Federal Republic of Germany, as strain DSM 3134.

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