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Thermothrix thiopara: Growth and Metabolism of a Newly Isolated Thermophile Capable of Oxidizing Sulfur and Sulfur Compounds

DANIEL K. BRANNAN AND DOUGLAS E. CALDWELL*

Department of Biology, University of New Mexico, Albuquerque, New Mexico 87131

Thermothrix thiopara is isolated from hot sulfur springs. It occurs in situ at a temperature of 72°C, a pH of 7.0, and an HS^- concentration of 17.4 μmol/liter (0.8 ppm). The organism was capable of autotrophic growth. Sulfite, sulfur, and polythionates were formed and subsequently degraded to sulfate during growth with thiosulfate as the sole energy source. Thiosulfate was oxidized by the polythionate pathway, and the stoichiometry of growth on thiosulfate was determined. The organism was also capable of heterotrophic growth in amino acids and simple sugars. A source of reduced sulfur (methionine, glutathione) was required for heterotrophic growth. Growth occurred aerobically or anaerobically with nitrate as a terminal oxidant. Both nitrous oxide and dinitrogen were produced. At 73°C the maximum autotrophic growth rate in batch culture using thiosulfate was 0.56 generation per h. Under the same conditions in continuous culture, washout occurred at a dilution rate of 0.3 to 0.4 per h, corresponding to a cellular growth rate of 0.43 to 0.58 generation per h. This was nearly three times the growth rate for *Thiobacillus denitrificans*. *T. thiopara* is gram negative. It was also found to be both lysozyme and penicillin susceptible. As a result, this organism cannot be considered an archaebacterium.

Thermodynamic considerations suggest that the ancient atmosphere of the Earth was thermal and reducing (36). Autotrophic thermophiles such as *Methanobacterium thermoautotrophicum* and *Sulfolobus acidocaldarius* have been discovered in remanent environments that are today considered extreme. Such organisms have been referred to as archaebacteria and are considered to be predecessors of gram-negative and gram-positive bacteria (12, 30). *Thermothrix thiopara* is also a thermophilic autotroph and may be related to *M. thermoautotrophicum* or *S. acidocaldarius*.

Because procaryotes lack the complex sexual barriers that protect gene pools (species) in higher organisms, the record of early biochemical evolution in mesophiles may have been obscured due to transformation, transduction, and conjugation. However, thermophily may provide a recombination barrier between primitive bacteria, which evolved in thermal environments, and modern bacteria, which evolved at lower temperatures. Further studies of autotrophic thermophiles are thus important to understand both the early evolution of life as well as the chemical evolution of Earth.

Descriptions of thermophilic autotrophs (4) and thermophilic sulfur-oxidizing bacteria growing at neutral pH (7, 34) are sparse. *T. thiopara*, the subject of this report, is a unique sulfur-oxidizing bacterium growing at neutral pH and high temperature (65 to 75°C). It is found in sulfide-containing hot springs as long filaments which are often encrusted with elemental sulfur. It is apparently the first facultatively autotrophic and facultatively anaerobic thermophile to be described (9). In contrast, the thiobacillus-like thermophiles are aerobic and facultatively chemolithotrophic (5, 22, 40). *Bacillus stearothermophilus* is heterotrophic and facultatively anaerobic (13); *Thermus* spp. are obligately aerobic and heterotrophic (8). *Thermoplasma acidophilum* is heterotrophic (10); *S. acidocaldarius* is facultatively autotrophic and aerobic (6).

This study describes improved methods for autotrophic cultivation of *T. thiopara*. Denitrification and the stoichiometry of growth on thiosulfate are described. *T. thiopara* is also found to have a higher growth rate than a similar mesophile, *Thiobacillus denitrificans*. This suggests that high temperature could be an optimum rather than an extreme environment.

MATERIALS AND METHODS

Organism and culture conditions. *T. thiopara*, which was previously described (9), was grown heterotrophically on nitrate broth (Difco Laboratories, Detroit, Mich.) and autotrophically on a thiosulfate mineral salts medium (TXB) composed of (in grams per

liter of distilled deionized water): $Na_2S_2O_3 \cdot 5H_2O$, 3.0; NaH_2CO_3, 2.0; NH_4Cl, 1.0; KNO_3, 2.0; $MgSO_4 \cdot 7H_2O$, 0.5; KH_2PO_4, 2.0; and $FeSO_4 \cdot 7H_2O$-ethylenediaminetetraacetic acid chelate, 0.02. Trace elements (milligrams per liter) were: $MnCl_2 \cdot 4H_2O$, 1.8; $ZnSO_4 \cdot 7H_2O$, 0.44; H_3BO_4, 2.9; $CuSO_4$, 0.1; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.37; and $CoCl_2 \cdot 6H_2O$, 0.81. *T. thiopara* was grown mixotrophically on TXB plus yeast extract (0.5 g/liter). A pH indicator (0.3 g of bromothymol blue and 0.05 g of methyl red in 1 liter of 0.1 N NaOH) was added (4 ml/liter), and the pH of the solution containing bicarbonate was adjusted to 6.8 (indicator color yellow) with 1 N HCl before autoclaving. The trace elements, iron solution, and phosphate were autoclaved separately and added to the remaining salts after cooling. Media with elemental sulfur (1%) or sulfide (1 mg/liter) in place of thiosulfate were also used. The ability of *T. thiopara* to use organic substrates was tested by supplementing the mineral salts solution without thiosulfate with the organic compound. *T. thiopara* was aerobically grown in screw-cap flasks. Capped flasks were significant at the 95% confidence limit in maintaining aerobic conditions (4.3 ± 0.3 ppm of O_2), whereas uncapped flasks contained less oxygen (3.2 ± 0.3 ppm). *T. thiopara* was anaerobically grown in serum vials sealed with neoprene stoppers and purged with argon gas. Cultures were grown in a New Brunswick rotary shaker at 200 rpm and 73 ± 1°C. Growing cells were maintained by daily transfers or stored at 4°C for no longer than 5 days. The viability of the cells decreased rapidly if the cells were allowed to remain longer than 24 h at 73°C. Cells stored at 4°C were nonviable after 10 days.

Growth studies. A 10% inoculum of a log-phase culture was used for growth studies. Growth rate in log-phase batch culture was determined as a function of temperature on TXB medium. Cultures were then grown at the optimum temperature and sampled at various time intervals to determine cell number, pH change, thiosulfate remaining, sulfide, sulfite, polythionates, sulfur, and sulfate formed. Direct cell counts were made with a Petroff-Hausser bacterial counter.

Continuous culture studies on TXB medium were carried out in a Bioflo model C30 bench-top chemostat (New Brunswick Scientific Co., New Brunswick, N.J.) with a culture volume of 350 ml, provided with agitation (400 rpm), aeration, and temperature control (72 ± 2°C). The culture pH remained constant (6.8 ± 0.2) during steady states. Wall growth was minimized by coating the culture vessel with 5% (vol/vol) dichlorosilane (Dow Corning, Midland, Mich.) in chloroform. Continuous cultures were started from batch cultures in early stationary phase. The dilution rate in continuous culture was varied from 0.08 to 0.4 h^{-1}. Steady state was considered to have been reached at each dilution rate after three volume changes and when the absorbancy at 460 nm (A_{460}) remained constant over the time required for two culture doublings. Biomass was estimated from A_{460}, using a dry weight-absorbance calibration curve (A_{460} = 0.1 was equivalent to 80 mg [dry weight] per liter).

Uptake and respiration of glucose. *T. thiopara* was grown on glucose (0.5%) plus yeast extract (0.05%) (GYE) or on TXB medium to midlog phase, harvested, and resuspended in fresh medium. The cells were then brought to 72°C, and the experiment was initiated by adding 0.042 mg of D-[*U*-^{14}C]glucose (specific activity, 0.72 μCi/mmol) at a concentration of 0.5%. Samples were taken over a 5-h period, filtered (0.45-μm membrane filters; Millipore Corp., Bedford, Mass.), and washed with phosphate-buffered saline (1.36 g of KH_2PO_4, 2.13 g of K_2HPO_4, and 8.5 g of NaCl per liter of distilled deionized water, pH 7.0). The washed filters were placed in Biofluor scintillation cocktail. Evolved carbon dioxide was trapped in phenethylamine scintillation fluor. Samples were counted in a Searle Delta 300 scintillation counter.

Uptake of radiolabeled carbon dioxide. *T. thiopara* grown on TXB was harvested at 10,000 × *g* for 10 min, washed, and suspended (0.5 g [wet weight] in 50 ml) in TXB without bicarbonate. The suspension was warmed to 72°C, and 100 μg of $NaH_2{}^{14}CO_3$ (10 μCi) in 1 ml of water was added. Two-milliliter portions were taken at specific time intervals and added to equal amounts of 2 N HCl. After evaporation to dryness, scintillation fluor was added and the samples were counted.

Gas chromatography. Gas samples were analyzed with a Varian model 3700 gas chromatograph. The Porapak Q in series with Porapak R column of Whilhite and Hollis was used (39). The following operating conditions were used: carrier gas, helium at 30 lb/in^2; flow rate, 40 ml/min; column temperature, 100°C; detector, thermal conductivity type operated at 150°C with 35-mA current; injector temperature, 100°C; sample size, 100 μl. Samples were identified and quantitated by using 1,000-ppm standards.

Chemical analyses. Thiosulfate, sulfite, and polythionates were measured titrimetrically with a slightly modified method of Koh and Taniguchi (19). Sulfide was determined by the method of Pachmayr (7), sulfur by the Barlett and Skoog procedure (3), and sulfate by barium sulfate precipitation (2). Nitrite was assayed by the Griess-Ilosvay colorimetric method (20). Dissolved oxygen was determined by a slightly modified Winkler titration and correcting for the presence of thiosulfate (16).

RESULTS

T. thiopara grew either heterotrophically or autotrophically. Two to five days, however, was required to adapt heterotrophically grown cells to autotrophic growth and vice versa. Therefore, heterotrophically (UNM B-100) and autotrophically (UNM B-142) grown cells were maintained lyophilized and frozen on glass beads (31) in the University of New Mexico Culture Collection.

Morphology of *T. thiopara* was variable depending on culture conditions. During subculture on autotrophic medium, the filamentous form was gradually lost and short chains of cells formed that later broke into both motile and nonmotile rod-shaped unicells.

Growth studies. To develop a suitable autotrophic medium, the concentrations of thiosulfate and nitrate that gave maximal cell yield (measured as optical density at 460 nm) were

determined. These were 1.0 and 1.2 g/liter respectively. Sulfide inhibited growth at concentrations greater than 5 mg/liter.

Growth of *T. thiopara* in TXB medium plus yeast extract was followed by determination of viable cell counts and optical density (Fig. 1). The population, as determined by viable cell counts, entered stationary phase after 4 h. It briefly remained in this phase for 4 h, followed by logarithmic death. The optical density, however, continued to increase and did not reach stationary phase until 8 h of incubation.

Growth of *T. thiopara* on TXB at various temperatures showed a maximum cellular growth rate of 0.56 generation (gen) h^{-1} at 73°C (Fig. 2). The growth rate of *T. thiopara* on nitrate broth was previously found to be 0.4 gen h^{-1} at 70 to 73°C (9). In continuous culture on TXB at 73°C, washout occurred between dilution rates of 0.3 and 0.4 h^{-1}. This μ_{max} is equivalent to a cellular growth rate of 0.43 to 0.58 gen h^{-1}.

Oxidation of thiosulfate to sulfate during growth is seen in Fig. 3. During log and stationary phase, sulfite, sulfur, and polythionate intermediates were formed and degraded to the final product, sulfate, with a decrease in pH. Approximately 0.5 mol of thiosulfate was oxidized to 1 mol of sulfate. Sulfide was not detected; under the experimental conditions of high temperature and neutral pH, it may have rapidly been converted to sulfur by chemical processes (32). *T. thiopara* grew on precipitated powdered sulfur, and its growth was accompanied by a drop in pH and the formation of sulfate (4.0 ppm of SO_4^{2-}/h per 10^6 cells). *T. thiopara* could use either thiosulfate or elemental sulfur for energy and demonstrated no lag period when transferred from one to the other.

Heterotrophic growth of *T. thiopara*. *T. thiopara* did not grow on glucose or amino acids unless a reduced form of sulfur such as glutathione, methionine, or thiosulfate was available (Table 1). *T. thiopara* grown on glucose plus yeast extract accumulated and respired glucose at rates of 208 and 82 μmol/h per g of cells (dry weight), respectively. When grown autotrophically on TXB, *T. thiopara* accumulated 80 and respired 10 μmol of glucose/h per g of cells.

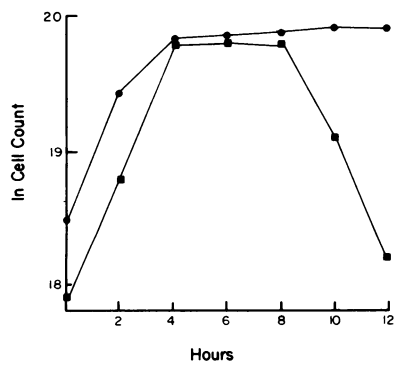

FIG. 1. *Growth of T. thiopara on TXB plus yeast extract (1 g/liter) at 73°C showing ln total cell count (●), and ln viable cell number (■) determined by most probable number.*

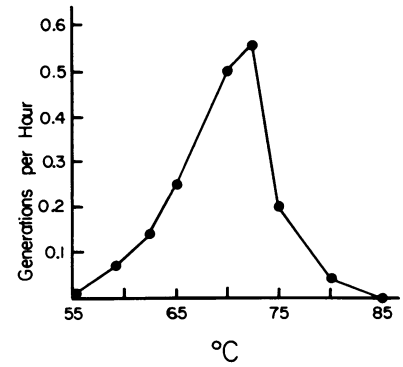

FIG. 2. *Growth rate of T. thiopara on TXB as a function of temperature from 55 to 85°C.*

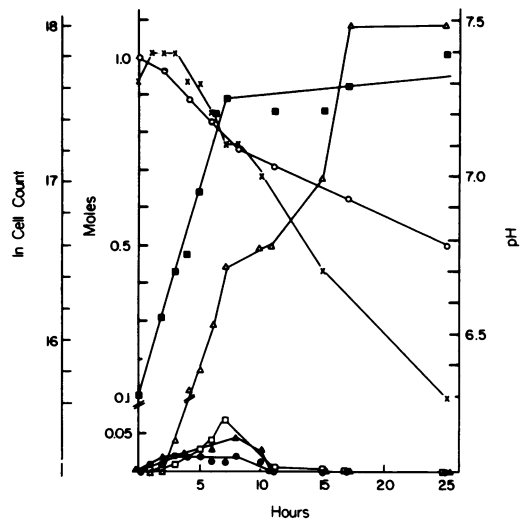

FIG. 3. *Oxidation of thiosulfate (○) to sulfate (△) accompanied by a drop in pH (×) and with formation of the intermediates sulfur (□), sulfite (●), and polythionates (▲) during growth of T. thiopara (■) on TXB at 73°C.*

Anaerobic respiration. Anaerobic oxidation of organic compounds with nitrate as a terminal electron acceptor occurred with production of dinitrogen (1.3×10^{-2} μmol/h per g of cells) and nitrous oxide (2.0×10^{-3} μmol/h per g of cells). Nitrite was also detected. Ammonia was not detected even after addition of 2 N NaOH.

Growth did not occur anaerobically in autotrophic media when either sulfide, sulfur, or thiosulfate was supplied as the electron donor and nitrate served as the electron acceptor.

Uptake of radiolabeled carbon dioxide. Carbon dioxide fixation by *T. thiopara* is shown in Fig. 4. The average fixation rate of $^{14}CO_2$ was 3.4×10^{-1} μmol/min per g of cells (wet weight).

DISCUSSION

Time was required to adapt autotrophically grown *T. thiopara* to heterotrophic growth and vice versa. Facultatively autotrophic *Thiobacillus* spp. also require an adaptation period between heterotrophic and autotrophic growth (27). This period has been explained as the time required for induction, repression, or derepression of enzymes characteristic of the heterotrophic or autotrophic metabolism (24).

In batch culture, exponential growth occurred for two to four generations, followed by a 3- to 5-h stationary phase and then logarithmic death. Stationary phase occurred after 4 h. After approximately 8 h, total counts remained constant and the viable counts declined logarithmically. Thiosulfate was not depleted and the pH dropped from 7.3 to 6.3. As a result, death must be attributed to either the accumulation of a toxic product(s) or the drop in pH rather than to nutrient depletion.

TABLE 1. *Aerobic growth of T. thiopara at 73°C on organic substrates in the presence of various sulfur sources*

Substrate (5 g/liter)	Growth on given sulfur source[a]			
	SO_4^{2-} (0.4 g/liter)	$S_2O_3^{2-}$ (0.5 g/liter)	Methionine (0.5 g/liter)	Glutathione (0.5 g/liter)
Amino acids[b]	−	+	+	+
Glucose	−	+	+	+

[a] Growth as determined by turbidity and microscopic observation of living bacteria (motility).
[b] Mixture of glutamate, aspartate, and serine.

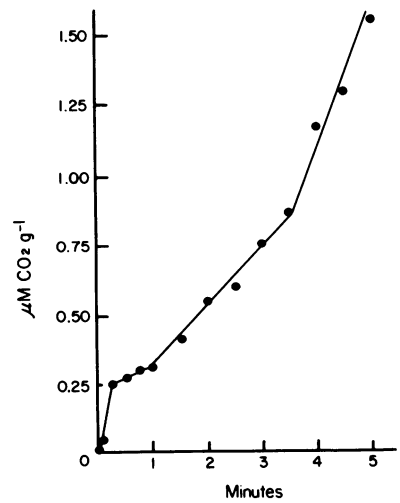

FIG. 4. *Fixation of $^{14}CO_2$ by T. thiopara, using thiosulfate as the sole source of energy.*

The growth rate of *T. thiopara* in log-phase batch culture and the maximum specific growth rate in continuous culture on thiosulfate at 37°C were nearly three times the values for *Thiobacillus denitrificans*, the mesophilic analog of *T. thiopara* (17). Thermophiles attain maximum energy efficiency (growth/growth plus maintenance) at their optimum growth temperature (1). Thus there are two possibilities: *T. thiopara* may be more efficient than chemosynthetic mesophiles, explaining its rapid growth rate, or it may grow more rapidly but less efficiently. The former explanation suggests that thermal environments are not extreme but rather optimum for chemosynthetic metabolism. The later suggests that thermal environments are extreme, resulting in rapid growth but inefficient metabolism. Continuous culture studies are in progress to determine whether there are increased cellular maintenance requirements at high temperature in *Thermothrix thiopara*, a thermophile, as compared with *Thiobacillus denitrificans*, a mesophile.

The formation of sulfite, sulfur, and polythionate intermediates from thiosulfate, and their subsequent oxidation to sulfate by *T. thiopara*, agrees with the polythionate pathway for thiosulfate oxidation (32, 35). As could be predicted (18), 1 mol of thiosulfate was oxidized to 2 mol of sulfate by *T. thiopara*. The organism, like *Thiobacillus* spp., did not oxidize all the available thiosulfate (15). This is not attributed to a pH effect because neutralization does not permit complete oxidation of thiosulfate.

T. thiopara deposited sulfur when grown on thiosulfate. Sulfur may arise from the sulfane sulfur group of thiosulfate, from polythionates that react with sulfide, or from the extracellular decomposition of pentathionate to tetrathionate (32). Whether these processes are purely chemical or biologically catalyzed is unclear. This is in contrast to the *Beggiatoaceae* and *Thiorhodaceae*, which biologically catalyze intracellular sulfur deposition when grown with sulfide (37).

Thiobacillus spp. are often considered the major geochemical agents for oxidizing reduced sulfur compounds (38). The activity of thermophilic species is usually limited to temperatures below 55°C, and organic supplements are fre-

quently required for growth (4, 11, 22, 40). *T. thiopara* oxidized elemental sulfur at 73°C and did not require organic supplements for autotrophic growth. In regions where temperatures exceed 65°C, such as sulfotaras and thermal springs, thermophilic sulfur oxidizers such as the genera *Sulfolobus* and *Thermothrix* predominate and are the major geochemical agents.

Thermothrix thiopara, like *Thiobacillus intermedius* (32), was incapable of growth on organic compounds unless supplied with a reduced sulfur source (Table 1). *T. thiopara* apparently cannot synthesize the necessary enzymes for heterotrophic growth if sulfate is the sulfur source. Organic forms of reduced sulfur supply an available sulfur source and may also serve as carbon and energy sources (24, 33).

Heterotrophically or autotrophically grown *T. thiopara* accumulated and respired glucose. Autotrophically grown cells metabolized glucose at one-third the rate of heterotrophically grown cells. Presumably this was due to repression of glucose catabolism when grown autotrophically on thiosulfate (25). Unlike *Thiobacillus intermedius* (26), however, complete repression did not occur since autotrophically grown cells were capable of low rates of glucose respiration. The ability to assimilate preformed organic compounds from the environment gives the organism a selective advantage over autotrophs without this ability (23).

Studies on neutral thermal springs (70 to 90°C) have demonstrated autotrophic carbon dioxide fixation in situ (7). *T. thiopara* is the first organism grown in pure culture that accounts for this fixation. It fixed carbon dioxide in the laboratory, using thiosulfate as the energy source, and therefore may contain thermostable ribulose diphosphate carboxylase (RuDPcase). Based on its molecular weight and quaternary structure, RuDPcase has been dichotomized into T- and O-type enzymes, with the O-type enzyme considered more primitive (21). It is assumed that the ancestral gene for RuDPcase was first established in anaerobic bacteria (28, 29). The carboxylase for *Rhodospirillum rubrum*, a facultatively anaerobic facultative autotroph, is the simplest yet described and may represent an ancestral form of the enzyme (29). It is significant in this respect that *Thiobacillus denitrificans*, a facultatively anaerobic chemolithotroph, contains RuDPcase of intermediate size between *R. rubrum* and obligately aerobic chemolithotrophs (28, 29). Because *T. thiopara* is also a facultatively anaerobic, facultative autotroph, it too may contain an O-type RuDPcase. Studies of the CO_2 fixation mechanism are in progress.

T. thiopara occurs in an environment presumed to have existed during at least one stage of archaen ecology. *Sulfolobus acidocaldarius*, also a thermophilic sulfur bacterium, is considered to be one of the archaebacteria. However, electron microscopy shows that *T. thiopara* posseses a gram-negative cell wall (9). It is also susceptible to both lysozyme and penicillin. Thus it probably contains peptidoglycan and cannot be considered an archaebacterium. However, further studies of its physiology and ecology may augment knowledge of evolution in autotrophs and help to explain the chemical evolution of Earth.

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