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Mixotrophic and Autotrophic Growth of *Thiobacillus acidophilus* on Glucose and Thiosulfate

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Mixotrophic growth of the facultatively autotrophic acidophile *Thiobacillus acidophilus* on mixtures of glucose and thiosulfate or tetrathionate was studied in substrate-limited chemostat cultures. Growth yields in mixotrophic cultures were higher than the sum of the heterotrophic and autotrophic growth yields. Pulse experiments with thiosulfate indicated that tetrathionate is an intermediate during thiosulfate oxidation by cell suspensions of *T. acidophilus*. From mixotrophic growth studies, the energetic value of thiosulfate and tetrathionate redox equivalents was estimated to be 50% of that of redox equivalents derived from glucose oxidation. Ribulose 1,5-bisphosphate carboxylase (RuBPCase) activities in cell extracts and rates of sulfur compound oxidation by cell suspensions increased with increasing thiosulfate/glucose ratios in the influent medium of the mixotrophic cultures. Significant RuBPCase and sulfur compound-oxidizing activities were detected in heterotrophically grown *T. acidophilus*. Polyhedral inclusion bodies (carboxysomes) could be observed at low frequencies in thin sections of cells grown in heterotrophic, glucose-limited chemostat cultures. Highest RuBPCase activities and carboxysome abundancy were observed in cells from autotrophic, CO_2-limited chemostat cultures. The maximum growth rate at which thiosulfate was still completely oxidized was increased when glucose was utilized simultaneously. This, together with the fact that even during heterotrophic growth the organism exhibited significant activities of enzymes involved in autotrophic metabolism, indicates that *T. acidophilus* is well adapted to a mixotrophic lifestyle. In this respect, *T. acidophilus* may have a competitive advantage over autotrophic acidophiles with respect to the sulfur compound oxidation in environments in which organic compounds are present.

The acidophilic thiobacilli are capable of autotrophic growth in extremely acidic environments. Their ability to oxidize various inorganic sulfur compounds is of fundamental interest because of the extreme acid tolerance of some of the periplasmic enzymes involved in these reactions. The biochemical activities of these bacteria are also of considerable economic importance. For example, oxidation of metal sulfides by *Thiobacillus ferrooxidans* is applied on a large scale for the biological leaching of metal ores (19). However, the pathways involved in the oxidation of sulfur compounds by the acidophilic thiobacilli are still poorly understood (for a review, see reference 22a).

Studies into the physiological mechanisms involved in growth and substrate oxidation by the obligately autotrophic *Thiobacillus* species are hampered by the low growth yields of these organisms. Facultative autotrophs provide an attractive alternative model system for physiological studies. The facultative autotroph *T. acidophilus* is capable of heterotrophic growth on glucose and various other simple organic compounds (7, 22). Autotrophic growth can be supported by a variety of inorganic sulfur compounds (7, 15, 20) and formate (J. T. Pronk, P. de Bruijn, J. P. van Dijken, P. Bos, and J. G. Kuenen, Arch. Microbiol., in press). The organism has been used to study mechanisms involved in ΔpH maintenance under acidic growth conditions (15, 16, 28, 29). Its physiological characteristics also make *T. acidophilus* an attractive model organism to study the enzymology of acidophilic sulfur compound oxidation (22a).

Facultatively autotrophic acidophiles such as *T. acidophilus* are not only interesting because of their suitability as model organisms. The presence of acidophilic heterotrophs can increase the performance of metal-leaching operations (26), probably by preventing the accumulation of toxic organic compounds (9, 22, 26).

We have recently studied the mixotrophic growth of *T. acidophilus* on glucose and the C_1 compounds formate and formaldehyde (Pronk et al., Arch. Microbiol., in press). As observed with other facultative autotrophs, mixotrophic growth yields were higher than the sum of the heterotrophic and autotrophic growth yields. Mason and Kelly (14) reported that mixotrophic growth of *T. acidophilus* on glucose and tetrathionate also led to an increase of growth efficiency. However, their mixotrophic chemostat studies were limited to one glucose/tetrathionate ratio. Therefore, no quantitative comparison could be made of the energetics of the mixotrophic utilization of inorganic sulfur compounds and C_1 compounds.

The aim of the present study was to investigate the energetics of mixotrophic growth of *T. acidophilus* on mixtures of glucose and thiosulfate or tetrathionate. Furthermore, attention was paid to the regulation of sulfur compound oxidation and inorganic carbon metabolism during mixotrophic and autotrophic growth.

MATERIALS AND METHODS

Organism and maintenance. *T. acidophilus* DSM 700 was obtained from the Deutsche Sammlung von Mikroorganismen as a liquid culture on glucose. A sample was plated on mineral medium (pH 3.5) supplemented with glucose and 0.8% agarose. A single colony was inoculated in 200 ml of mineral medium plus glucose (20 mM). The resulting culture

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was made 10% (vol/vol) with dimethyl sulfoxide and stored at −70°C in 1-ml aliquots. These frozen samples were used as inocula for continuous-culture studies.

Mineral medium. Mixotrophic chemostat cultures of *T. acidophilus* were fed with a mineral medium containing the following, per liter of demineralized water: $(NH_4)_2SO_4$, 3.0 g; KH_2PO_4, 0.15 g; K_2HPO_4, 0.19 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; Na_2SO_4, 1.4 g; $CaCl_2 \cdot 2H_2O$, 0.26 g; $FeSO_4 \cdot 7H_2O$, 11 mg; $ZnSO_4 \cdot 7H_2O$, 0.9 mg; $MnCl_2 \cdot 2H_2O$, 2.0 mg; $CoCl_2 \cdot 6H_2O$, 0.6 mg; $CuSO_4 \cdot 5H_2O$, 0.6 mg; $NaMoO_4$, 0.8 mg; H_3BO_3, 2.0 mg; KI, 0.2 mg; EDTA, 30 mg; nitrilotriacetic acid, 5 mg; silicon antifoaming agent (BDH Chemicals, Poole, Dorset, United Kingdom), 25 μl; and variable amounts of sodium thiosulfate or potassium tetrathionate. Prior to the addition of $MgSO_4$ and $CaCl_2$, the mineral medium was adjusted to pH 7.5 with 5 M KOH and autoclaved at 120°C. $MgSO_4$, $CaCl_2$, and glucose were sterilized separately at 110°C. Heterotrophic cultures were grown in the same mineral medium (without thiosulfate), adjusted to pH 3.0 with H_2SO_4.

Growth conditions. Continuous cultivation was performed in Applikon laboratory fermentors with a working volume of 1 liter. The pH was automatically titrated with 2 M KOH. The cultures were continuously gassed with water-saturated air (1 liter min^{-1}) and stirred at 800 rpm. The dissolved oxygen concentration in the cultures was monitored with a steam-sterilizable Clark-type electrode. Chemostat cultures were grown at a minimum dissolved oxygen concentration of 75% air saturation, at 30°C, at pH 3.0, and at a dilution rate of 0.05 h^{-1}. Biomass concentrations in the chemostat cultures were linearly proportional to the concentrations of the growth-limiting substrates in the reservoir media.

Control of culture purity. The purity of chemostat cultures was routinely checked by phase-contrast microscopy and by plating on mineral medium plus glucose, solidified with 0.8% (wt/vol) agarose. Also, immunofluorescence microscopy with specific antisera against *T. acidophilus* was performed as described by Muyzer et al. (18).

Analytical procedures. (i) Dry-weight determination. The dry weight of cell suspensions was determined by filtrating aliquots over nitrocellulose filters (pore diameter, 0.45 μm; Schleicher and Schüll, Dassel, Federal Republic of Germany). The cells were washed three times with demineralized water and dried to constant weight at 70°C.

(ii) Protein determination. The protein content of whole cells was assayed with a modified biuret method: cells were harvested from continuous cultures, washed with demineralized water, and suspended to a concentration of approximately 2.5 mg (dry weight) ml^{-1}. The concentrate was boiled in 1 M KOH for 10 min and subsequently cooled on ice. $CuSO_4 \cdot 5H_2O$ was then added to a final concentration of 25 mM. After 5 min, the mixture was centrifuged in an Eppendorf bench-top centrifuge (13,000 × *g*) for 2 min. The A_{550} of the supernatant was measured. The protein concentration in cell extracts was determined by the method of Bradford (2). In both assays, bovine serum albumin (fatty acid-free; Sigma Chemical Co., St. Louis, Mo.) was used as a standard.

(iii) Organic carbon determination. A Beckman model 915B Tocamaster total organic carbon analyzer was used to determine the carbon content of whole cultures and culture supernatants. The carbon content of the bacteria was obtained from the difference. Cell suspensions were acidified with H_3PO_4 prior to analysis to expel carbon dioxide accumulated inside the cells.

(iv) Substrate determinations. Since thiosulfate interfered with the GOD-PAP method (Boehringer, Mannheim, Federal Republic of Germany), glucose concentrations in media and culture supernatants were measured with a commercial hexokinase–glucose 6-phosphate dehydrogenase kit (Boehringer Mannheim test combination no. 676543). Thiosulfate and tetrathionate were determined by the method of Sorbö (25). The analyses were carried out at room temperature. Separate calibration curves were made for thiosulfate and tetrathionate.

Measurement of substrate-dependent oxygen consumption. Respiration rates of cells were assayed polarographically with a Clark-type oxygen electrode (Yellow Springs Instruments Inc., Yellow Springs, Ohio). Cells from carbon-limited chemostat cultures were assayed directly in the culture fluid or after appropriate dilution in mineral medium without a carbon source (pH 3.0). When cell suspensions were diluted with mineral medium or culture supernatant, the observed oxygen uptake rates were linearly proportional to the biomass concentration (data not shown). Calculations were made on the basis of an oxygen concentration of 236 μM in air-saturated water at 30°C. The values presented here have been corrected for the (low) endogenous respiration rates.

RuBPCase. Cell extracts for Ribulose 1,5-bisphosphate carboxylase (RuBPCase) assays were prepared as described previously (22). RuBPCase was assayed by the method of Beudeker et al. (1).

Electron microscopy. Preparation of culture samples for electron microscopy was done by the method of Handley et al. (8). Ultrathin sections were studied in a Philips EM 201 electron microscope.

Chemicals. Ribulose 1,5-bisphosphate was obtained from Sigma, and $[^{14}C]NaHCO_3$ (2.11 TBq mol^{-1}) was from Amersham International PLC. Sodium thiosulfate pentahydrate was obtained from J. T. Baker Chemicals, Deventer, The Netherlands. Anhydrous potassium tetrathionate was obtained from Fluka AG, Buchs, Switzerland. All other chemicals were reagent grade and obtained from commercial sources.

RESULTS

Autotrophic and mixotrophic growth on thiosulfate. Mixotrophic growth of *T. acidophilus* was studied in substrate-limited chemostat cultures grown at a dilution rate of 0.05 h^{-1} and at pH 3.0. At this pH, thiosulfate is unstable at millimolar concentrations (12). The residual thiosulfate and tetrathionate concentrations in the chemostat cultures were lower than the detection limit of the cyanolysis assay (approximately 10 μM). At these low concentrations, chemical decomposition of thiosulfate was negligible (data not shown). The thiosulfate concentration in the reservoir medium, which was adjusted to pH 7.5, did not change during the experiments (data not shown).

Attempts to grow *T. acidophilus* autotrophically on thiosulfate at $D = 0.05$ h^{-1} were unsuccessful, in accordance with the observation of Mason et al. (15) that the maximum growth rate of *T. acidophilus* on thiosulfate was below 0.05 h^{-1}. However, steady-state cultures could be obtained at $D = 0.03$ h^{-1}. The observed biomass yield at this dilution rate was 6.0 g mol of $thiosulfate^{-1}$. This yield is in good agreement with a yield of 5.5 g mol^{-1} found at $D = 0.025$ h^{-1} by Mason et al. (15). No effect on the biomass yields was observed when the cultures were sparged with air containing 5% (vol/vol) carbon dioxide, indicating that the cultures were not carbon limited.

In contrast to autotrophic cultures, which washed out at

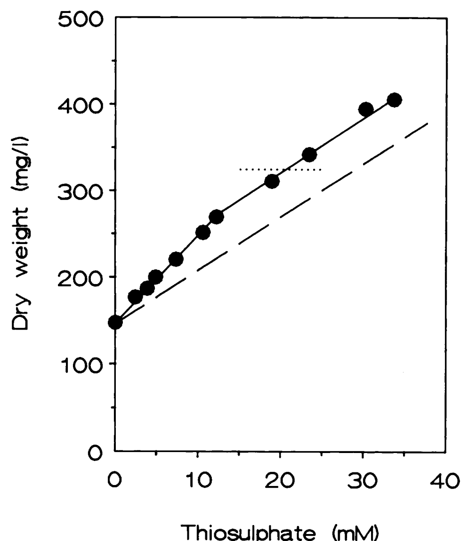

FIG. 1. Effect of increasing concentrations of thiosulfate in the reservoir medium on biomass concentrations in mixotrophic, substrate-limited chemostat cultures of *T. acidophilus* (2.5 mM glucose; D = 0.05 h^{-1}; pH 3.0; T = 30°C). The dotted line indicates the theoretical upper limit of carbon conversion during heterotrophic growth on glucose (5). The slope of the dashed line indicates the growth yield in autotrophic, thiosulfate-limited chemostat cultures (D = 0.03 h^{-1}).

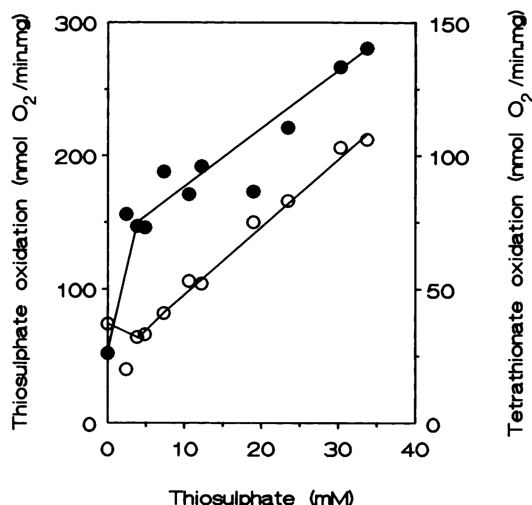

FIG. 2. Effect of increasing concentrations of thiosulfate in the reservoir medium on rates of sulfur compound oxidation by cells from mixotrophic chemostat cultures (2.5 mM glucose; D = 0.05 h^{-1}; pH 3.0; T = 30°C). Substrate-dependent oxygen uptake by cell suspensions was assayed with a Clark-type oxygen electrode. Symbols: ●, oxidation of thiosulfate (200 μM); ○, oxidation of tetrathionate (200 μM).

dilution rates above 0.03 h^{-1}, mixotrophic utilization of thiosulfate was observed at a dilution rate of 0.05 h^{-1}. Thiosulfate was completely oxidized to sulfate at this dilution rate up to a molar ratio of thiosulfate/glucose of 14.

Addition of thiosulfate to the reservoir medium of glucose-limited chemostat cultures resulted in an increase of the biomass concentration in the cultures. At thiosulfate/glucose ratios below 5, the biomass density in the cultures increased linearly with the influent thiosulfate concentration (Fig. 1). At thiosulfate/glucose ratios above 5, the increase of the biomass concentration corresponded with the autotrophic growth yield on thiosulfate (Fig. 1).

The influent glucose and thiosulfate concentrations of the chemostat cultures did not significantly influence the carbon and protein contents of the biomass, which remained at 49 ± 1 and 69 ± 2%, respectively.

Oxidation of thiosulfate. Cells of *T. acidophilus* pregrown in heterotrophic, glucose-limited chemostat cultures exhibited significant rates of thiosulfate-dependent oxygen uptake (22) (Fig. 2). Thiosulfate-dependent oxygen uptake rates increased during mixotrophic growth on glucose and thiosulfate (Fig. 2). Thiosulfate oxidation by cell suspensions from mixotrophic chemostat cultures exhibited a typical biphasic pattern. After an initial rapid oxygen uptake, during which approximately 0.25 mol of oxygen was consumed per mol of thiosulfate, oxygen uptake continued at a lower rate. The oxygen uptake rates in the second phase corresponded to the rates of tetrathionate oxidation by the cell suspensions. The explanation of this phenomenon becomes evident from Fig. 3. During the first phase of thiosulfate oxidation, a near-quantitative conversion to tetrathionate occurred, in accordance with the observed biphasic oxygen uptake patterns.

Mixotrophic growth of *T. acidophilus* on glucose and thiosulfate led to an increase of the tetrathionate-dependent oxygen uptake rates of cell suspensions (Fig. 2). The latter oxygen uptake rates were almost identical to the rates required for complete oxidation of thiosulfate via tetrathionate in the chemostat cultures. In contrast, the maximum rates of thiosulfate-dependent oxygen uptake by cell suspensions were much higher than the actual thiosulfate oxidation rates observed in the chemostat cultures.

Also, the rates of substrate-dependent oxygen uptake with the inorganic sulfur compounds trithionate, sulfide, and elemental sulfur increased during mixotrophic growth on glucose and thiosulfate (data not shown).

Mixotrophic utilization of tetrathionate. As discussed above, experiments with cell suspensions suggested that tetrathionate is an obligatory intermediate during the oxida-

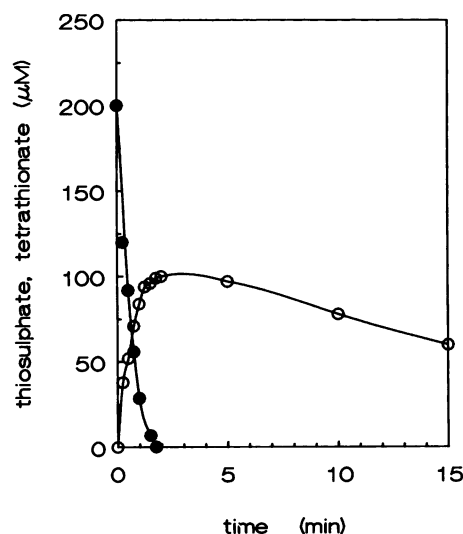

FIG. 3. Oxidation of thiosulfate by *T. acidophilus*, pregrown in a mixotrophic chemostat culture (2.5 mM glucose, 10 mM thiosulfate; D = 0.05 h^{-1}; pH 3.0; T = 30°C). At t = 0, 200 μM thiosulfate was added to an aerated cell suspension (0.25 g [dry weight] $liter^{-1}$; pH 3.0; T = 30°C). Symbols: ●, thiosulfate; ○, tetrathionate.

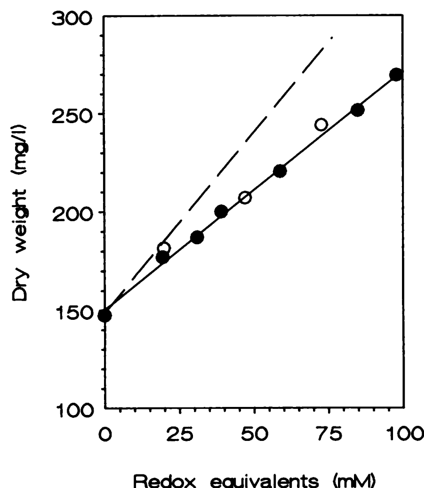

FIG. 4. Comparison of energetic value of redox equivalents derived from oxidation of thiosulfate and tetrathionate by *T. acidophilus*, grown in mixotrophic chemostat cultures (2.5 mM glucose; $D = 0.05\ h^{-1}$; pH 3.0; $T = 30°C$). The concentrations of the inorganic sulfur compounds in the reservoir medium are given as the amount of electrons derived from their complete oxidation to sulfate (i.e., concentrations of thiosulfate and tetrathionate were multiplied by 8 and 14, respectively). The dashed line represents results from mixotrophic cultures grown on glucose and formate (Pronk et al., Arch. Microbiol., in press). Symbols: ●, mixotrophic growth on glucose and thiosulfate; ○, mixotrophic growth on glucose and tetrathionate.

tion of thiosulfate by *T. acidophilus*. Only one of the eight electrons available from the complete oxidation of thiosulfate to sulfate is derived from the initial formation of tetrathionate. If tetrathionate is indeed an obligatory intermediate during thiosulfate oxidation by *T. acidophilus*, it can be expected that the energetic value of the redox equivalents derived from thiosulfate and tetrathionate oxidation will be similar.

In a recent study, Mason et al. (15) reported that the molar growth yield of *T. acidophilus* in tetrathionate-limited, autotrophic chemostat cultures was 2.3-fold higher than the molar growth yield in thiosulfate-limited chemostat cultures. This would imply that the energetic value of tetrathionate redox equivalents is 1.3-fold higher than those derived from thiosulfate oxidation. This conclusion is in apparent contradiction with our conclusions regarding the role of tetrathionate as an intermediate during thiosulfate oxidation. To investigate the energetic value of tetrathionate redox equivalents, mixotrophic utilization of glucose and tetrathionate was studied.

The addition of tetrathionate to the reservoir medium of glucose-limited chemostat cultures led to an increase of the biomass yields. The increase of the biomass yield per mole of redox equivalents was identical to that observed with thiosulfate as an energy source (Fig. 4).

RuBPCase activities and polyhedral bodies. Many (facultatively) autotrophic bacteria that use the Calvin cycle for CO_2 fixation contain typical polyhedral inclusion bodies. Since in all species studied these organelles have been demonstrated to contain active RuBPCase (EC 4.1.1.39), they are commonly referred to as carboxysomes (3). It has recently been reported that elemental sulfur-grown *T. acidophilus* cells also contain polyhedral inclusion bodies (13). However, there are no data in the literature on the regulation of carboxysome synthesis in *T. acidophilus*.

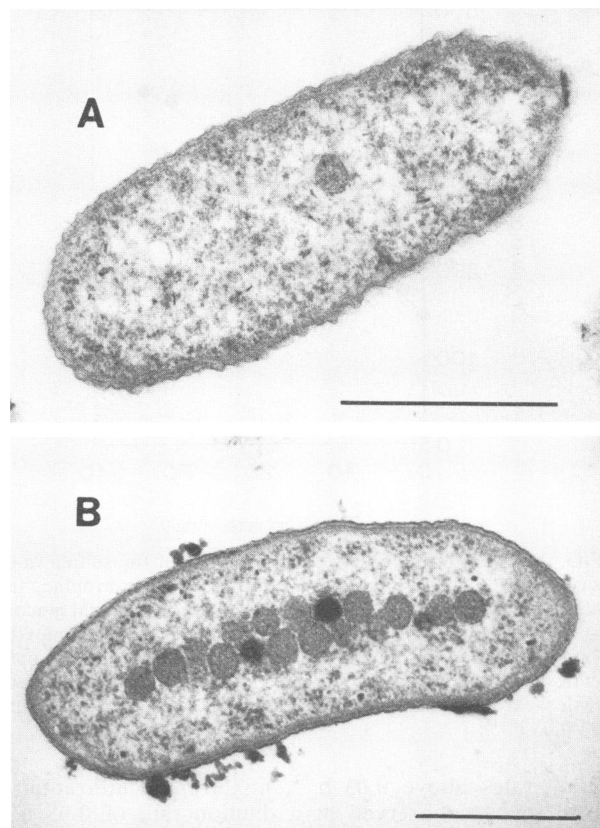

FIG. 5. Electron microscopic photographs of thin sections of *T. acidophilus* cells grown under various growth conditions in chemostat cultures. Bars, 0.5 μm. (A) Heterotrophic chemostat culture (5 mM glucose; $D = 0.05\ h^{-1}$; $T = 30°C$; pH 3.0). Longitudinal cell section showing one occasional carboxysome. (B) Autotrophic, CO_2-limited chemostat culture (20 mM thiosulfate; $D = 0.03\ h^{-1}$; $T = 30°C$; pH 3.0). Typical longitudinal cell section.

The RuBPCase activity of 43 nmol min^{-1} mg of $protein^{-1}$ in cell extracts of thiosulfate-limited, autotrophic chemostat cultures was sufficient to account for the observed rate of inorganic carbon fixation in the cultures (31 nmol min^{-1} mg of $protein^{-1}$). Polyhedral inclusion bodies were abundant in cells from thiosulfate-limited chemostat cultures (data not shown). However, the organelles were not observed in all sections. This can be explained from the fact that they were typically located in the center of the cells (Fig. 5). The mean diameter of the organelles, measured in various thin sections of fixed cells, was 100 ± 10 nm. Both RuBPCase activities in cell extracts and the abundancy of polyhedral bodies increased when autotrophic cultures were grown under CO_2 limitation (Table 1, Fig. 5).

As reported previously (22), *T. acidophilus* retained significant activities of RuBPCase during heterotrophic growth in glucose-limited chemostat cultures (Table 1). In addition to this, polyhedral inclusion bodies similar to those in autotrophic cultures could be observed at a low frequency in thin sections (Fig. 5). Mixotrophic growth on glucose and thiosulfate led to an increase of the RuBPCase activities in cell extracts (Table 1). Furthermore, polyhedral inclusion bodies were more abundant than in cells from heterotrophic chemostat cultures (data not shown).

TABLE 1. Activities of RuBPCase in cell extracts of *T. acidophilus* grown in chemostat cultures (pH 3.0, 30°C) under various growth limitations

Growth-limiting substrate	Growth rate (h^{-1})	RuBPCase (nmol mg of protein min^{-1})
Glucose (5 mM)	0.05	5
Glucose (5 mM), $S_2O_3^{2-}$ (20 mM)	0.05	10
Glucose (2.5 mM), $S_2O_3^{2-}$ (34 mM)	0.05	29
$S_2O_3^{2-}$ (20 mM)[a]	0.03	43
CO_2[b]	0.03	78

[a] Culture sparged with air (1 liter min^{-1})

[b] Thiosulfate-grown chemostat culture (S_R = 20 mM), sparged (1 liter min^{-1}) with air containing 0.0125% CO_2. Although CO_2 was limiting, all thiosulfate was oxidized to sulfate. The molar growth yield on thiosulfate was 70% of that of a thiosulfate-limited autotrophic culture.

DISCUSSION

Energetics of mixotrophic growth. During mixotrophic growth of facultatively autotrophic bacteria, CO_2 assimilation is in most cases strictly regulated (4, 6). Under heterotrophic growth conditions, or at low ratios of inorganic and organic substrates, CO_2-assimilating activity is not expressed. In such cultures, the inorganic substrate is used to enhance heterotrophic carbon assimilation. As a result, the cell yields on the organic substrate increase up to the theoretical limit of organic carbon assimilation (88% conversion of glucose carbon into biomass [5]). A further increase of the inorganic substrate concentration does not lead to a situation of energy excess, since under such conditions autotrophic CO_2-assimilating capacity is induced. As demonstrated by Gommers et al. (5), this situation is encountered with *T. versutus* (growth on acetate and thiosulfate [6]) and *Pseudomonas oxalaticus* (growth on acetate and formate [4]).

At first sight, the same situation seems to apply for *T. acidophilus* grown mixotrophically on glucose and thiosulfate. Also in this case, the relation between the thiosulfate/glucose ratio and the biomass yields was biphasic (Fig. 1). At thiosulfate/glucose ratios of <5, autotrophic CO_2 fixation did not seem to occur, since the growth yields were higher than the sum of the heterotrophic and autotrophic growth yields (Fig. 1). Autotrophic CO_2 fixation set in before the theoretical limit of glucose assimilation was reached, as judged from the fact that the further increase of the biomass yields paralleled the autotrophic growth yield on thiosulfate (Fig. 1).

When *T. acidophilus* is grown mixotrophically on formate and glucose, autotrophic growth sets in when glucose assimilation reaches its theoretical maximum (Pronk et al., Arch. Microbiol., in press). The difference between mixotrophic growth on glucose and formate and results in the present study may be found in the fact that redox equivalents from formate can enter the electron transport chain at the level of NADH (Pronk et al., Arch. Microbiol., in press). Redox equivalents from thiosulfate probably enter the electron transport chain at the level of cytochrome *c*. With the latter substrate, reversed electron transport is therefore required for NAD or NADP reduction. The fact that, as judged by the growth pattern shown in Fig. 1, maximum assimilation of glucose is not reached with thiosulfate as an additional energy source may therefore reflect a shortage of low potential redox equivalents for biosynthesis.

Cell extracts from heterotrophic cultures and from mixotrophic cultures grown at low ratios of thiosulfate to glucose contained significant RuBPCase activities (Table 1). Theoretically, this enzyme could be involved in actual in vivo CO_2 assimilation. However, if these RuBPCase activities in cell extracts were indicative of in vivo CO_2 assimilation, a strictly biphasic curve as shown in Fig. 1 would not be expected. The apparent contradiction between growth yields and RuBPCase activities in cell extracts suggests that RuBPCase synthesis is not the only site of regulation of autotrophic CO_2 assimilation. For example, in vivo RuBPCase activity might be regulated by intracellular metabolite concentrations. Alternatively, another step in inorganic carbon metabolism (CO_2 uptake, phosphoribulokinase) may be the site of control of CO_2 fixation in vivo.

When it is postulated that, indeed, at low ratios of thiosulfate/glucose, thiosulfate was used exclusively to increase the efficiency of glucose assimilation, a quantitative comparison can be made of the energetic value of the redox equivalents derived from the oxidation of glucose and thiosulfate. At thiosulfate/glucose ratios below 5, the increase of the biomass concentration as a result of thiosulfate addition was 9.79 g mol of thiosulfate^{-1} or 1.22 g mol of redox equivalents^{-1} (Fig. 1). The molar growth yield of *T. acidophilus* in glucose-limited chemostat cultures grown at D = 0.05 h^{-1} and pH 3.0 was 59 g mol of glucose^{-1} or 2.46 g mol of redox equivalents^{-1}. Thus, the energetic value of thiosulfate redox equivalents was only $(1.22/2.46) \times 100 = 50\%$ of that of the redox equivalents from glucose oxidation.

The energetic value of tetrathionate redox equivalents did not differ significantly from those derived from thiosulfate (Fig. 4). We have recently also studied the mixotrophic growth of *T. acidophilus* on glucose and formate (Pronk et al., Arch. Microbiol., in press). The energetic value of formate redox equivalents was 75% of that of glucose redox equivalents, or 50% higher than those from thiosulfate and tetrathionate (Fig. 4). The low growth yields of *T. acidophilus* in heterotrophic cultures (22) may be caused by a low efficiency of the proton-translocating respiratory chain. In *T. acidophilus*, formate redox equivalents may enter the respiratory chain at the level of NAD (Pronk et al., Arch. Microbiol., in press). If it is assumed that active uptake of formate is coupled to the inward translocation of one proton, the relative efficiency of formate and thiosulfate redox equivalents is compatible with H^+/O ratios of 4 and 2 for NADH and thiosulfate, respectively.

Thiosulfate metabolism. The transient accumulation of tetrathionate during thiosulfate oxidation (Fig. 3) indicated that tetrathionate can be an intermediate of thiosulfate oxidation by *T. acidophilus*. The near-quantitative conversion of thiosulfate to tetrathionate observed in these experiments suggests that this pathway plays a major role in thiosulfate oxidation. In this respect, thiosulfate metabolism in *T. acidophilus* is similar to that of the acidophiles *T. ferrooxidans* (24) and *T. thiooxidans* (21). Also, the identical energetic values of thiosulfate and tetrathionate calculated from growth yields of mixotrophic chemostat cultures are consistent with oxidation of thiosulfate via tetrathionate.

Tetrathionate is much more stable in acidic environments than thiosulfate. The very high maximum rates of the conversion of thiosulfate to tetrathionate may allow the organism to compete successfully with the chemical decomposition of thiosulfate. Further metabolism of tetrathionate is probably initiated by a hydrolytic cleavage, yielding sulfate and S_3-sulfane monosulfonic acid (10, 22a).

In addition to an increase of the growth efficiency (Fig. 1), simultaneous utilization of glucose and thiosulfate led to an increase of the maximum growth rate at which thiosulfate

could be utilized as an energy source, compared with the maximum growth rate of autotrophic, thiosulfate-limited cultures. These factors may be advantageous in the competition of *T. acidophilus* with obligate autotrophs in environments in which both inorganic sulfur compounds and organic substrates are available.

The high specific activities and the possibility to grow dense mixotrophic cultures make *T. acidophilus* an attractive model organism to study the enzymology of sulfur compound metabolism in acidic environments.

Carboxysomes and RuBPCase activities. Polyhedral inclusion bodies are widespread among autotrophic procaryotes. In all species containing the organelles which have been examined, the inclusion bodies have been shown to contain active RuBPCase (3). Although we have not demonstrated the presence of RuBPCase in the organelles, the morphological similarities with carboxysomes from other thiobacilli (3, 11) and the apparent coordinate regulation of RuBPCase activities and inclusion body abundancy suggest that the *T. acidophilus* inclusion bodies are carboxysomes. Attempts to isolate carboxysomes from *T. acidophilus* by a procedure described for *T. neapolitanus* (11) were unsuccessful.

With the exception of *T. denitrificans*, carboxysomes have been detected in all obligately autotrophic *Thiobacillus* species studied (3). In the facultatively autotrophic thiobacilli, carboxysomes are less ubiquitous. The organelles have been detected in the facultative autotrophs *T. intermedius* and *T. acidophilus*, but not in *T. versutus* and *T. novellus* (13). Regulation of carboxysome synthesis has been studied in batch cultures of *T. intermedius*. Both RuBPCase activity and carboxysomes were observed after autotrophic growth on thiosulfate, but not in cultures which had been grown heterotrophically on yeast extract (23). In contrast to these observations, carboxysomes could be observed at low frequencies in heterotrophically grown cells of *T. acidophilus* (Fig. 5).

The regulation of RuBPCase activity in *T. acidophilus* is less strict than in other facultatively autotrophic thiobacilli. The retention of significant RuBPCase levels during heterotrophic growth (22) (Table 1) may reduce the time required for adaptation to autotrophic growth. Also, the retention of sulfur compound-oxidizing capacity under heterotrophic growth conditions suggests that *T. acidophilus* is well adapted to growth in rapidly changing environments.

As observed in other thiobacilli (1, 3), RuBPCase activities and carboxysome abundancies in cell sections appeared to be correlated. This observation suggests a physiological role of carboxysomes in CO_2 assimilation by *T. acidophilus*. Several physiological functions of carboxysomes have been proposed in the literature, including protection of RuBPCase from oxygen and a function as RuBPCase storage bodies (3). Its metabolic versatility (7, 22) makes *T. acidophilus* well suited for further studies into the regulation and function of carboxysome synthesis.

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