Energetics of mixotrophic and autotrophic C₁-metabolism by *Thiobacillus acidophilus*

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Abstract. Although the facultatively autotrophic acidophile *Thiobacillus acidophilus* is unable to grow on formate and formaldehyde in batch cultures, cells from glucose-limited chemostat cultures exhibited substrate-dependent oxygen uptake with these C₁-compounds. Oxidation of formate and formaldehyde was uncoupler-sensitive, suggesting that active transport was involved in the metabolism of these compounds. Formate- and formaldehyde-dependent oxygen uptake was strongly inhibited at substrate concentrations above 150 and 400 μM , respectively. However, autotrophic formate-limited chemostat cultures were obtained by carefully increasing the formate to glucose ratio in the reservoir medium of mixotrophic chemostat cultures. The molar growth yield on formate ($Y = 2.5 \text{ g} \cdot \text{mol}^{-1}$ at a dilution rate of 0.05 h^{-1}) and RuBPCase activities in cell-free extracts suggested that *T. acidophilus* employs the Calvin cycle for carbon assimilation during growth on formate. *T. acidophilus* was unable to utilize the C₁-compounds methanol and methylamine. Formate-dependent oxygen uptake was expressed constitutively under a variety of growth conditions. Cell-free extracts contained both dye-linked and NAD-dependent formate dehydrogenase activities. NAD-dependent oxidation of formaldehyde required reduced glutathione. In addition, cell-free extracts contained a dye-linked formaldehyde dehydrogenase activity. Mixotrophic growth yields were higher than the sum of the heterotrophic and autotrophic yields. A quantitative analysis of the mixotrophic growth studies revealed that formaldehyde was a more effective energy source than formate.

Key words: *Thiobacillus acidophilus* — Acidophiles — Mixotrophic growth — Chemostat cultures — Formate metabolism — Formaldehyde metabolism

as a contaminant of a ferrous iron-grown culture of the obligate autotroph *T. ferrooxidans* (Guay and Silver 1975). Substrates for autotrophic growth of *T. acidophilus* include elemental sulphur, thiosulphate and tetrathionate (Guay and Silver 1975; Norris et al. 1986), but not ferrous iron.

T. acidophilus has been used as a model organism to study the energetics of growth in acidic environments (Matin et al. 1982; Matin and Matin 1982; Zychlinski and Matin 1983a, b). It has been suggested that heterotrophic acidophiles like *T. acidophilus* play an important role in natural bioleaching populations by scavenging toxic organic compounds (Tsuchiya et al. 1974; Nerkar et al. 1977; Pronk et al. 1990).

A number of simple organic compounds can be used as carbon sources for heterotrophic growth of *T. acidophilus*. However, most small organic acids can not be used as carbon sources for growth in batch cultures (Guay and Silver 1975; Pronk et al. 1990). Toxicity of weak acids in acidic environments has been observed with other acidophilic microorganisms and can be explained by their accumulation in the cytoplasm, leading to a dissipation of the transmembrane pH gradient (Ingledew 1982; Alexander et al. 1987). For example, pyruvate can not be used as a carbon source for heterotrophic growth of *T. acidophilus* in batch cultures due to substrate inhibition. However, this substrate can support growth in carbon-limited chemostat cultures (Pronk et al. 1990).

Although autotrophic growth of neutrophilic thiobacilli on formate has been reported, the acidophilic *Thiobacillus* species are generally considered to be unable to use this compound (Kelly and Harrison 1989). However, we have found that cell suspensions of *T. acidophilus* pregrown on glucose readily oxidize formate. Therefore, the ability of *T. acidophilus* to use formate and other C₁-compounds as energy sources for mixotrophic and autotrophic growth was reinvestigated.

Material and methods

Organism and maintenance

Thiobacillus acidophilus DSM 700 was obtained from the Deutsche Sammlung von Mikroorganismen (Braunschweig, FRG) as a liquid

Thiobacillus acidophilus is a facultatively autotrophic, acidophilic bacterium. The organism was first isolated

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culture on glucose. A sample was plated on mineral medium (pH 3.5) supplemented with glucose and solidified with 0.8% agarose. Agarose was sterilized separately to prevent acid hydrolysis. A single colony was inoculated in 200 ml mineral medium plus glucose (20 mM). The resulting culture was made 10% (v/v) with dimethylsulphoxide and stored at -70°C in 1 ml aliquots. These frozen samples were used as inocula for continuous culture studies.

Mineral medium

T. acidophilus was grown in a synthetic medium containing per litre of demineralized water: $(\text{NH}_4)_2\text{SO}_4$, 3.0 g; KH_2PO_4 , 0.3 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; Na_2SO_4 , 1.4 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.26 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 11 mg; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.9 mg; $\text{MnCl}_2 \cdot 2\text{H}_2\text{O}$, 2.0 mg; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.6 mg; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.6 mg; NaMoO_4 , 0.8 mg; H_3BO_3 , 2.0 mg; KI, 0.2 mg; EDTA, 30 mg; nitrilo-triacetic acid, 5 mg and silicon antifoaming agent (BDH Chemicals, Poole, Dorset, UK), 25 μl . The medium was adjusted to pH 3.0 with 1 M H_2SO_4 and autoclaved at 120°C . Solutions of formic acid and glucose were sterilized separately at 110°C and added to the autoclaved mineral medium. Formaldehyde solutions were prepared by hydrolysis of paraformaldehyde in 10 mM KOH (10 min at 100°C) and filter-sterilized before addition to the autoclaved mineral medium.

Growth conditions

Continuous cultivation was performed in Applikon laboratory fermenters with a working volume of 1 l. The pH was automatically titrated with 1 M KOH. The cultures were continuously gassed with water-saturated air ($1\text{ l} \cdot \text{min}^{-1}$) 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 dissolved oxygen concentration of above 75% of air saturation, at 30°C , at pH 3.0 and at a dilution rate of 0.05 h^{-1} . Since biomass concentrations in the chemostat cultures were linearly proportional to the substrate concentrations in the reservoir media, it was concluded that the cultures were carbon- and energy-limited.

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% (w/v) agarose. Additionally, immuno-fluorescence microscopy with specific antisera against *T. acidophilus* was performed as described by Muyzer et al. (1987).

Analytical procedures

Dry weight determination. The dry weight of cell suspensions was determined by filtrating aliquots over nitrocellulose filters (pore diameter $0.45\text{ }\mu\text{m}$, Schleicher and Schüll, Dassel, FRG). The cells were washed three times with demineralized water and dried to constant weight at 70°C .

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 resuspended to a concentration of approximately $2.5\text{ mg dry weight} \cdot \text{ml}^{-1}$. The concentrate was boiled in 1 M KOH for 10 min and subsequently cooled on ice. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 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 rpm) for 2 min. The absorbance of the supernatant was measured at 550 nm. The protein content of cell-free extracts was determined by the method of Bradford (1976). In both assays, bovine serum albumin (fatty acid free, Sigma, St. Louis, MO, USA) was used as a standard.

Determination of organic carbon content

A Beckman model 915B Tocamaster total organic carbon analyser 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 in order to expel carbon dioxide accumulated inside the cells.

Substrate determinations

Glucose concentrations in media and culture supernatants were measured with the GOD-PAD method (Boehringer, Mannheim, FRG). The formate concentrations in the reservoir media were determined according to Lang and Lang (1972), formaldehyde was assayed according to Nash (1953).

Measurement of substrate-dependent oxygen consumption

Respiration rates of cells were assayed polarographically with a Clark-type oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH, USA). Cells from carbon-limited chemostat cultures were assayed directly in the culture fluid or after dilution with mineral medium without carbon source (pH 3.0). Calculations were made on the basis of an oxygen concentration of 0.24 mM in air-saturated mineral medium at 30°C . The values presented here have been corrected for the (low) endogenous respiration rates.

Preparation of cell-free extracts

Cells were harvested from chemostat cultures by centrifugation (10,000 g, 10 min) and washed with a buffer containing 100 mM sodium acetate and 5 mM magnesium nitrate (pH 5.0). This buffer proved to be optimal for the extraction of formate dehydrogenase activity. For convenience, the same buffer was used for formaldehyde dehydrogenase assays. In this case, no difference was observed with activities of extracts prepared in Tris-HCl buffer (0.1 M, pH 8.0). Cells were resuspended in the same buffer to a concentration of approximately $10\text{ mg dry weight} \cdot \text{ml}^{-1}$ and disrupted by sonication in an MSE 150 W sonifier (12 bursts of 30 s with intermittent cooling). Intact cells and debris were removed by centrifugation (40,000 g, 15 min). The clear supernatants were used as cell-free extracts.

Enzyme assays

The reduction of NAD and DCPIP were monitored spectrophotometrically at 340 and 600 nm, respectively. Enzyme activities were calculated using extinction coefficients of $6.3\text{ mM}^{-1}\text{ cm}^{-1}$ for NADH and $20.6\text{ mM}^{-1}\text{ cm}^{-1}$ for DCPIP (Armstrong 1964) and expressed as U (mg protein^{-1}). (1 U = 1 $\mu\text{mol electron acceptor reduced} \cdot \text{min}^{-1}$.) In all enzyme assays, the enzyme activity was linearly proportional to the amount of cell-free extract added. Enzyme assays were performed at 30°C .

Formate dehydrogenase, NAD-dependent

The reaction mixture (1 ml) contained: potassium phosphate buffer (pH 7.0), 100 μmol ; MgSO_4 , 10 μmol ; Triton X-100, 2 mg; NAD, 2 μmol ; and cell-free extract. The reaction was started by the addition of 10 μmol potassium formate.

Formate dehydrogenase, dye-linked

The reaction mixture (1 ml) contained: potassium phosphate buffer (pH 7.0), 100 μmol ; MgSO_4 , 10 μmol ; Triton X-100, 2 mg; phenazine methosulphate (PMS), 0.3 μmol ; dichlorophenolindophenol (DCPIP), 0.2 μmol ; and cell-free extract. The reaction was started by the addition of 10 μmol potassium formate.

Formaldehyde dehydrogenase, NAD-dependent

The reaction mixture (1 ml) contained: potassium phosphate buffer (pH 7.0), 100 μmol ; MgSO_4 , 10 μmol ; Triton X-100, 2 mg; reduced glutathione (GSH), 2 μmol ; NAD, 2 μmol ; and cell-free extract. The reaction was started by the addition of 5 μmol formaldehyde.

Formaldehyde dehydrogenase, dye-linked

The reaction mixture was identical to that employed for assaying dye-linked formate dehydrogenase. The reaction was started by the addition of 5 μmol formaldehyde.

Ribulose 1,5-bisphosphate carboxylase (RuBPCase)

Cell-free extracts for RuBPCase assays were prepared as described by Pronk et al. (1990). RuBPCase was assayed according to Beudeker et al. (1980).

Chemicals

Ribulose 1,5-bisphosphate was obtained from Sigma Chemical (St. Louis, MO, USA), [^{14}C]- NaHCO_3 (2.11 TBq $\cdot \text{mol}^{-1}$), from Amersham International PLC. Agarose (medium electroendosmosis) and formic acid (p.a. quality) were from Merck, Darmstadt, FRG. All other chemicals were reagent grade and obtained from commercial sources.

Results

Oxidation of C_1 -compounds by *Thiobacillus acidophilus*

In batch cultures, *T. acidophilus* can not grow on formate as a sole source of energy (Pronk et al. 1990). However, cells from glucose-limited chemostat cultures exhibited high rates of formate-dependent oxygen uptake. At a formate concentration of 100 μM , the pH optimum for formate oxidation by intact cells was approximately 3.0. This pH optimum is comparable with the pH optima for the oxidation of various reduced sulphur compounds by *T. acidophilus* (results not shown). In all oxygen uptake studies, the stoichiometry of formate and oxygen consumption was as expected (2:1), indicating that the ob-

Fig. 1. Double reciprocal plot of formate oxidation versus rate of formate-dependent oxygen uptake by *Thiobacillus acidophilus*. The oxygen uptake rate (v) is expressed as $\mu\text{mol O}_2 \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$. *T. acidophilus* was pregrown in a glucose-limited chemostat culture ($D = 0.05 \text{ h}^{-1}$, pH 3.0, $T = 30^\circ\text{C}$, $S_R = 10 \text{ mM}$).

served oxygen uptake rates were not caused by stimulation of endogenous respiration.

At substrate concentrations below 150 μM , formate oxidation by cell suspensions obeyed Monod kinetics (Fig. 1). The apparent K_s and $V_{\max}$ for formate were 57 μM and $0.37 \mu\text{mol O}_2 \cdot \text{min}^{-1} \cdot (\text{mg dry weight})^{-1}$, respectively. At formate concentrations higher than 150 μM , substrate inhibition occurred (Fig. 1). The oxidation rates observed with formate as a substrate were significantly higher than with the inorganic sulphur compounds thiosulphate ($V_{\max} = 70 \text{ nmol O}_2 \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$) and tetrathionate ($V_{\max} = 50 \text{ nmol O}_2 \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$).

In addition to formate, *T. acidophilus* could also respire formaldehyde. Cells from glucose-limited chemostat cultures exhibited formaldehyde-dependent respiration rates of $35\text{--}40 \text{ nmol O}_2 \cdot \text{min}^{-1} \cdot (\text{mg dry weight})^{-1}$ at a formaldehyde concentration of 200 μM . Substrate inhibition was observed at formaldehyde concentrations above 400 μM . The pH optimum for formaldehyde oxidation was approximately pH 3.0. The kinetics of formaldehyde oxidation were not studied in detail.

Oxidation of formate and formaldehyde by cell suspensions was inhibited by the protonophores 2,4-dinitrophenol (Fig. 2) and carbonyl cyanide *m*-chlorophenylhydrazine (data not shown). In contrast, tetrathionate-dependent oxygen uptake was not inhibited by uncouplers (Fig. 2).

Heterotrophically grown cells of *T. acidophilus* did not exhibit significant oxygen uptake rates with methylamine and methanol.

Autotrophic growth on C_1 -compounds

In a facultative autotroph like *T. acidophilus*, it may be expected that the oxidation of formate and formaldehyde

Fig. 2. Effect of 2,4-dinitrophenol on substrate-dependent oxygen uptake rates of *T. acidophilus*. Oxygen uptake rates are expressed as a percentage of the rates observed in the absence of 2,4-dinitrophenol. $\blacktriangle$ Tetrathionate (100 μM ; 100% = 45 $\text{nmol O}_2 \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$). $\bullet$ Formate (100 μM , 100% = 209 $\text{nmol O}_2 \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$). $\blacksquare$ Formaldehyde (200 μM ; 100% = 35 $\text{nmol O}_2 \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$).

Fig. 3. Effect of increasing concentrations of formic acid in the reservoir medium on the biomass concentrations in glucose-limited chemostat cultures of *T. acidophilus* (5 mM glucose, $D = 0.05 \text{ h}^{-1}$, pH 3.0, $T = 30^\circ\text{C}$). The dashed line indicates the growth yield in autotrophic, formate-limited chemostat cultures, the dotted line indicates the theoretical maximum growth yield on glucose as a carbon source ($130 \text{ g} \cdot \text{mol}^{-1}$; Gommers et al. 1988).

can provide the metabolic energy for mixotrophic and autotrophic growth. For mixotrophic growth of *T. acidophilus* on glucose and formate, the formate concentration in the reservoir medium was chosen in such a way that the rate of formate addition to the culture did not exceed its formate-oxidizing capacity. This was routinely calculated from formate-dependent oxygen consumption rates of culture samples.

When mixotrophic chemostat cultures of *T. acidophilus* grown on glucose and formate were switched to a medium containing formic acid as a sole energy source, stable autotrophic formate-limited cultures were obtained. The use of formic acid in the reservoir media does not lead to accumulation of mineral salts in the cultures, since all substrate is converted into carbon dioxide and water. Formate-limited chemostat cultures with influent formic acid concentrations of 75–250 mM, resulting in biomass concentrations of up to $0.6 \text{ g} \cdot \text{l}^{-1}$. The molar growth yield of *T. acidophilus* in formate-limited chemostat cultures was $2.5 \text{ g} \cdot (\text{mol formate})^{-1}$. This growth yield is similar to the growth yields of other bacteria that employ the Calvin cycle for carbon assimilation during growth on formate (Dijkhuizen et al. 1977a; van Verseveld and Stouthamer 1978; Kelly et al. 1979). Molar growth yields of 7–8 $\text{g} \cdot (\text{mol formate})^{-1}$ have been reported for bacteria that employ the serine pathway of formate assimilation (Rokem et al. 1978). The growth yields observed in the present study therefore strongly suggest that *T. acidophilus* employs the Calvin cycle for carbon assimilation during formate-limited growth.

Analogous to the growth experiments with formate, it was possible to grow substrate-limited chemostat cultures on glucose and formaldehyde. We have not investigated growth of *T. acidophilus* on formaldehyde as a sole source of energy.

The carbon and energy sources used in mixotrophic and autotrophic chemostat cultures did not significantly influence the carbon and protein content of the biomass, which remained at $48 \pm 1\%$ and $67 \pm 2\%$, respectively. The residual concentrations of glucose, formaldehyde and formate in the chemostat culture were below the detection limits of the analytical procedures used.

Attempts to induce methanol-oxidizing activity by growth on mixtures of glucose and methanol were unsuccessful. This is in accordance with previous reports that *T. acidophilus* is unable to utilize methanol as a sole energy source in batch cultures (Pronk et al. 1990).

Mixotrophic growth on C_1 -compounds

Addition of formic acid to the reservoir medium of glucose-limited chemostat cultures resulted in an increase of the biomass density of the cultures. At formate to glucose ratios up to 19, the biomass density increased linearly with the formate concentration in the reservoir medium. At these formate to glucose ratios, the cell yields of the cultures were higher than the sum of the autotrophic and heterotrophic growth yields (Fig. 3). At formate to glucose ratios above 19, the increase of the biomass density corresponded with the autotrophic growth yield of *T. acidophilus* on formate (Fig. 3).

T. acidophilus exhibits a low growth yield during growth in glucose-limited chemostat cultures ($Y_{\text{max}} = 69 \text{ g} \cdot \text{mol}^{-1}$; Pronk et al. 1990). The actual growth yield at a dilution rate of 0.05 h^{-1} ($59 \text{ g} \cdot \text{mol}^{-1}$) was lower due to maintenance effects (Pronk et al. 1990). In bacteria, the theoretical upper limit of carbon conversion during growth on glucose is 88% (Gommers et al. 1988), equivalent to a molar growth yield of $130 \text{ g} \cdot (\text{mol glu-})$

Fig. 4. Comparison of the efficiency of formate and formaldehyde as energy sources for mixotrophic growth of *T. acidophilus*. Mixotrophic chemostat cultures were grown at a fixed glucose concentration of 5 mM in the reservoir medium. The concentrations of formate and formaldehyde are expressed as the amount of electrons derived from their complete oxidation to CO_2 . Growth conditions: $D = 0.05 \text{ h}^{-1}$, pH 3.0, $T = 30^\circ\text{C}$. ● Cultures grown on glucose and formate; ○ cultures grown on glucose and formaldehyde

case) $^{-1}$. The biomass density observed at a formate to glucose ratio of 19 corresponded almost exactly with this theoretical value (Fig. 3).

Also the addition of formaldehyde to the reservoir media of glucose-limited chemostat cultures led to an increase of the biomass yields. On a molar basis, this yield increase was more than twice that observed with formate as the auxiliary energy source (Fig. 4).

Enzymology of C_1 -metabolism

T. acidophilus grown in glucose-limited chemostat cultures exhibited high rates of formate-dependent oxygen uptake (Table 1). Neither the V_{max} nor the K_s values calculated from oxygen uptake experiments changed significantly when the cells were grown mixotrophically on glucose and formate or in autotrophic, formate-limited chemostat cultures (Table 1). Formate-oxidizing activity was not repressed in heterotrophic, nitrogen-limited chemostat cultures (Table 1). Apparently the formate-oxidizing activity was not influenced by the presence of organic substrates in the culture supernatant. Also thiosulphate-limited, autotrophic cultures of *T. acidophilus* exhibited high formate-oxidizing activities. These data suggest that formate oxidation by *T. acidophilus* is constitutive and not subject to regulation.

Cell-free extracts prepared from chemostat cultures of *T. acidophilus* contained both NAD-dependent and dye-linked formate dehydrogenase activities (Table 2). No activity was observed with NADP as an electron acceptor. The activity with the artificial electron-accepting couple PMS-DCPIP was approximately sixfold higher than with DCPIP alone. In all extracts tested, the

Table 1. Kinetic parameters of formate-dependent oxygen uptake by cell suspensions of *Thiobacillus acidophilus* from carbon-limited chemostat cultures. Growth conditions: $D = 0.05 \text{ h}^{-1}$, pH 3.0, $T = 30^\circ\text{C}$

Carbon and energy source	Growth limitation	K_s [μM]	V_{max} [$\text{nmol O}_2 \cdot \text{min}^{-1} \text{mg}^{-1}$]
Glucose [10 mM]	glucose	57	366
Glucose [20 mM]	nitrogen ^a	54	334
Glucose [5 mM]/formate [100 mM]	glucose/formate	58	291
Formate [250 mM]	formate	51	305

^a The residual glucose concentration in the culture was 3.8 mM

activities of formate dehydrogenase assayed with PMS-DCPIP were comparable with the NAD-dependent activities. Both enzyme activities exhibited maximum activity at pH 7.

In contrast to the formate-dependent oxygen uptake rates observed with intact cells (Table 1), NAD- and dye-linked formate dehydrogenase activities in cell-free extracts were dependent on the culture conditions (Table 2). NAD- and dye-linked formate dehydrogenase activities in cell-free extracts prepared from heterotrophic chemostat cultures were roughly one order of magnitude too low to account for the rates of formate oxidation observed with cell suspensions (Tables 1 and 2). Attempts to increase the formate-oxidizing activities in extracts from heterotrophic cultures by changing the assay conditions or the procedures for cell-free extract preparation were unsuccessful. Activities of mixed cell-free extracts (combined extracts from heterotrophic and autotrophic chemostat cultures or extracts prepared from mixed cell suspensions) were additive, indicating that the different formate dehydrogenase activities were not due to the presence of soluble stimulatory or inhibitory compounds (data not shown). The experimental data suggest that the stability of formate dehydrogenase during cell-free extract preparation is positively influenced by growth conditions that require formate dehydrogenase activity. We have no satisfactory explanation for this phenomenon.

Formaldehyde-dependent oxygen uptake rates by cell suspensions increased only slightly to approximately $55 \text{ nmol O}_2 \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ during growth of *T. acidophilus* on mixtures of glucose and formaldehyde. NAD-dependent oxidation of formaldehyde by cell-free extracts required the presence of reduced glutathione. The rates of formaldehyde-dependent NAD reduction in the absence of added GSH were approximately 30-fold lower than in its presence. GSH could not be replaced by β -mercaptoethanol or dithiothreitol. No activity was observed with NADP as an electron acceptor. Cell-free extracts also contained a dye-linked formaldehyde dehydrogenase activity (Table 2). Formaldehyde-dependent reduction of DCPIP could not be detected in the absence of PMS.

In contrast to the formaldehyde-dependent oxygen uptake rates observed with intact cells, NAD-dependent

Table 2. Enzymes of C_1 -metabolism in cell-free extracts of *T. acidophilus* pregrown in chemostat cultures ($D = 0.05 \text{ h}^{-1}$, $\text{pH} = 3.0$, $T = 30^\circ\text{C}$). Enzyme activities are expressed as $\text{U (mg protein)}^{-1}$

Enzyme activity	Growth limitation			
	Heterotrophic [10 mM glucose]	Mixotrophic [5 mM glucose] [35 mM formaldehyde]	Mixotrophic [5 mM glucose] [100 mM formate]	Autotrophic [125 mM formate]
Formate dehydrogenase NAD-dependent	0.03	0.50	0.81	0.96
Formate dehydrogenase dye-linked	0.03	0.61	0.97	0.78
Formaldehyde dehydrogenase NAD/GSH-dependent	0.20	1.9	0.27	0.19
Formaldehyde dehydrogenase dye-linked	0.11	0.10	n.d.	0.13
Ribulose-1,5-bisphosphate carboxylase	0.005	0.013	0.014	0.053

n.d.: not determined

formaldehyde dehydrogenase activities in cell-free extracts increased substantially when cells were grown on mixtures of glucose and formaldehyde (Table 2). The dye-linked formaldehyde dehydrogenase activity did not vary significantly with the culture conditions (Table 2).

Cell-free extracts prepared from carbon-limited, heterotrophic chemostat cultures of *T. acidophilus* contained significant activities of RuBPCase (ribulose-1,5-bisphosphate carboxylase, EC 4.1.1.39), the key enzyme of the Calvin cycle for CO_2 fixation (Table 2, Pronk et al. 1990). The activity of RuBPCase in cell-free extracts of cells grown in mixotrophic cultures increased with the formate to glucose ratio (Table 2). Cell-free extracts prepared from cells grown autotrophically on formate contained RuBPCase activities of $53 \text{ nmol} \cdot \text{min}^{-1} \cdot (\text{mg protein})^{-1}$. This corresponds well with the activity of approximately $50 \text{ nmol} \cdot \text{min}^{-1} \cdot (\text{mg protein})^{-1}$ that is required to realize the carbon fixation rates observed in the autotrophic chemostat cultures.

Discussion

Ecophysiological significance of C_1 -compound utilization

Autotrophic or mixotrophic utilization of formate by acidophilic bacteria has not been reported previously. Due to the toxicity of formate and formaldehyde, substrate-limited growth conditions were required for utilization of these compounds by *Thiobacillus acidophilus*. It is well-known that formaldehyde is toxic at millimolar concentrations (Attwood and Quayle 1984). Formate is even more toxic to *T. acidophilus*, as demonstrated by the substrate inhibition of formate-dependent oxygen uptake (Fig. 1). A further demonstration of the toxicity of formate was encountered during mixotrophic growth experiments. When the rate of formate addition to a chemostat culture only slightly exceeded its formate-oxidizing capacity, this resulted in cell lysis and washout (data not shown). Autotrophic cultures on formate could only be obtained by careful manipulation of the influent formate concentration.

The ability to oxidize formate is expressed constitutively in *T. acidophilus* (Tables 1, 2). Furthermore, the

dilution rate of 0.05 h^{-1} used throughout the present study is higher than the maximum growth rate of *T. acidophilus* on the reduced sulphur compounds thiosulphate and tetrathionate. This suggests that the ability to oxidize these C_1 -compounds is of physiological significance for the organism.

Little is known about the fluxes of organic carbon in the natural environment of the acidophilic thiobacilli. If formate and formaldehyde are generated either biologically or non-biologically, the ability of *T. acidophilus* to metabolize these toxic compounds is a prerequisite for survival. This could also explain the constitutive nature of formate oxidation.

Enzymology of formate and formaldehyde metabolism

The uncoupler sensitivity of formate and formaldehyde-dependent oxygen uptake by *T. acidophilus* (Fig. 2) suggests that the uptake of these compounds is energy-dependent. Energy-dependent formaldehyde uptake has recently also been reported in the RuMP-type methylotroph T15 (Bussineau and Papoutsakis 1988) and *Paracoccus denitrificans* (Köstler and Kleiner 1989). Since protonophore uncouplers do not abolish ΔpH in *T. acidophilus* (Matin et al. 1982), the uncoupler sensitivity also implicates involvement of the electrical component of the proton motive force in both uptake processes.

It has often been assumed in the literature that passive diffusion of non-dissociated formic acid across the cytoplasmic membrane is by definition an energy-requiring process, equivalent to the inward translocation of one proton (Dijkhuizen et al. 1977b; van Verseveld and Stouthamer 1978; Papoutsakis et al. 1981). However, this assumption neglects the fact that the proton associated with the carboxyl moiety is consumed again during formate oxidation. Passive diffusion of formate can only exert a negative influence on the membrane potential when the rate of formate influx exceeds the rate of formate oxidation. The latter situation does not occur in substrate-limited chemostat cultures. Thus, in case of energy-dependent formate transport, the formate anion has to be symported with more than one proton. This is likely to be the case in *T. acidophilus*.

Both NAD-dependent and dye-linked formate dehydrogenase activities could be detected in cell-free extracts of *T. acidophilus* (Table 2). Some purified NAD-dependent bacterial formate dehydrogenases can also use dyes as artificial electron acceptors (Müller et al. 1978). Although the formate dehydrogenase activities in different cell-free extracts varied substantially, the ratio of the NAD-dependent and dye-linked activities was always close to unity (Table 2). Moreover, the two formate dehydrogenase activities in cell-free extracts of *T. acidophilus* had identical pH optima. We have no evidence to suggest that different enzymes were responsible for the NAD-dependent and dye-linked activities.

Cell-free extracts of *T. acidophilus* grown on mixtures of glucose and formaldehyde contained high activities of an NAD-dependent, GSH-requiring formaldehyde dehydrogenase (Table 2). This type of formaldehyde dehydrogenase is widespread among bacteria and yeasts (Attwood and Quayle 1984). The increase of the formaldehyde dehydrogenase activity as a result of mixotrophic growth on glucose and formaldehyde (Table 2) was not paralleled by the formaldehyde-dependent oxygen uptake rates of cell suspensions. Apparently, the amount of formaldehyde dehydrogenase was not limiting the rate of formaldehyde oxidation by cell suspensions. Instead, the uncoupler sensitivity of the latter process suggests that formaldehyde transport may be the rate-limiting step. The dye-linked formaldehyde dehydrogenase activity did not increase during mixotrophic growth on glucose and formaldehyde and is therefore probably of minor importance for in vivo formaldehyde oxidation.

Energetics of mixotrophic growth on C₁-compounds

In most facultatively autotrophic microorganisms, inorganic carbon fixation is strictly regulated during mixotrophic growth. When the concentration of the inorganic substrate is low relative to the concentration of the organic substrate, RuBPCase is not synthesized and the inorganic substrate is used exclusively for heterotrophic carbon assimilation. As a result, a larger fraction of the organic substrate can be converted into biomass. With increasing concentrations of the inorganic substrate, the cell yields on the organic substrate increase until the theoretical limit of carbon conversion is reached (Gommers et al. 1988). A further increase of the inorganic substrate concentration would lead to a situation of energy excess. However, this does not occur because in such situations the capacity to assimilate CO₂ is induced. As demonstrated by Gommers et al. (1988), this situation is encountered with *Thiobacillus versutus* (growth on glucose and thiosulphate; Gottschal and Kuenen 1980) and *Pseudomonas oxalaticus* (growth on acetate and formate; Dijkhuizen and Harder 1979).

The same pattern seems to apply for mixotrophic utilization of formate by *T. acidophilus*. Also in this case the relation between the ratio of formate to glucose and the biomass concentration was biphasic (Fig. 3). In the first phase, autotrophic CO₂ fixation seemed not to occur, since the growth yields were higher than the sum of the

heterotrophic and autotrophic growth yields. Only when maximum assimilation of glucose was reached (Gommers et al. 1988), CO₂ fixation set in, as judged from the fact that the further increase of the cell yields paralleled the autotrophic growth yield on formate (Fig. 3). However, the RuBPCase activities in cell-free extracts were in apparent contradiction with this conclusion. Since the enzyme was present in cells grown heterotrophically and mixotrophically at low formate to glucose ratios, it could be argued that autotrophic CO₂ assimilation occurred under these growth conditions (Table 2). However, if the RuBPCase activities in cell-free extracts would reflect in vivo Calvin cycle activity, a strictly biphasic curve (Fig. 3) would not be expected. One of the possible explanations is that other key enzymes of autotrophic CO₂ assimilation are more strictly regulated than RuBPCase. Alternatively, in vivo RuBPCase activity may be regulated at other levels than enzyme synthesis.

When indeed, at low formate to glucose ratios, formate 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 formate. At formate to glucose ratios below 19, the increase in biomass concentration as a result of formate addition was 3.67 g · mol⁻¹ or 1.84 g · (mol redox equivalents)⁻¹ (Fig. 3). The molar growth yield of *T. acidophilus* in glucose-limited chemostat cultures grown at the same dilution rate was 59 g · mol⁻¹ or 2.46 g · (mol redox equivalents)⁻¹. Thus, the energetic value of the formate redox equivalents is only (1.84:2.46) × 100% = 75% of that of the redox equivalents from glucose oxidation. This difference is too large to be explained satisfactorily by substrate level phosphorylation occurring during glucose catabolism (data not shown). However, this calculation does not take into account the energy requirement of formate transport. For example, with an assumed the H⁺/O ratio of 4 for glucose and formate (this value is consistent with the low growth yields of *T. acidophilus*), the apparent H⁺/O ratio for formate would only be 3 in the case of an HCOOH/H⁺ symport mechanism. This could explain the lower energetic value of formate redox equivalents.

The increase of the biomass concentration as a result of formaldehyde addition to glucose-limited chemostat cultures was 8.9 g · (mol formaldehyde)⁻¹ (Fig. 4). Analogous to the calculations mentioned above, the energetic value of formaldehyde redox equivalents was estimated to be approximately 90% of that of glucose redox equivalents. The energetic value of formaldehyde redox equivalents was higher than the value of formate redox equivalents (Fig. 4). Also this observation can be explained in terms of energy requirement for transport of these C₁-compounds. For example, if both formate and formaldehyde transport occur via symport mechanisms with one proton, an H⁺/O ratio of 4 for both substrates would result in apparent H⁺/O ratios for formate and formaldehyde oxidation of 3 and 3.5, respectively [net translocation of 3 mol H⁺ (mol formate)⁻¹ and 7 mol H⁺ (mol formaldehyde)⁻¹].

The low growth yields of *T. acidophilus* in glucose-limited chemostat cultures can not be explained from energy-dependent maintenance of the intracellular pH (Pronk et al. 1990). The present study suggests that the low growth yields of the organism are due to low stoichiometries of respiration-linked proton translocation. Unfortunately, the acidophilic nature of *T. acidophilus* prevented the use of direct proton translocation assays (Mitchell and Moyle 1967) to confirm or renounce this conclusion.

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