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Carbon dioxide fixation by *Thiobacillus versutus*: apparent absence of a CO_2-concentrating mechanism in organisms grown under carbon-limitation in the chemostat

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1. SUMMARY

Thiobacillus versutus responds to both CO_2-limitation and increase in chemostat dilution rate under thiosulphate-limitation by increasing ribulose bisphosphate carboxylase specific activity. It has no high affinity CO_2-concentrating mechanism like that shown in *Synechococcus,* and may depend on diffusional uptake of CO_2/HCO_3^-.

2. INTRODUCTION

All the thiobacilli use the Calvin cycle to effect autotrophic fixation of carbon dioxide at the expense of energy from the oxidation of inorganic sulphur compounds. Ribulose bisphosphate carboxylase (RuBisCO) is constitutive in the obligately chemolithoautotrophic species, but can be completely repressed during heterotrophic growth in facultative species like *T. versutus* [1–8]. Partial repression of RuBisCO has been shown in the obligate species during growth in the presence of high concentrations of carbon dioxide [2,3]. *T. versutus* has previously been shown to exhibit a requirement for CO_2 at concentrations far above that present in normal air in order to grow on thiosulphate in chemostat culture, and to produce raised levels of RuBisCO under conditions of carbon limitation [5]. It is significant that while the obligate autotroph, *T. neapolitanus* can grow with much lower concentrations of CO_2 than *T. versutus,* both organisms contain similar activities of RuBisCO when grown under various conditions of CO_2 supply [1,4–9], reaching 17% of the total cell protein during CO_2-limited growth of *T. neapolitanus* [3]. Extensive studies have been made of CO_2 uptake and concentration by photosynthetic cells, which may take up both bicarbonate and CO_2, and in some cases have been proved to possess 'CO_2-concentrating mechanisms' which maintain high intracellular CO_2 concentrations [10–13].

Recently, *T. neapolitanus* was shown to possess a CO_2-concentrating system driven by a metabolic-energy-dependent uptake system [14]. This produced intracellular CO_2 concentrations up to 1500 times that of the solution external to the cells [14]. Our aim was to test whether the facultative *T. versutus* also exhibited any ability to concentrate inorganic carbon when grown under conditions of

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either sufficiency or limitation in the chemostat. For comparison, *Synechococcus*, which is known to have a concentrating system, was also studied.

3. MATERIALS AND METHODS

3.1. Organisms and cultivation

T. versutus was maintained and grown at 30 °C in chemostat culture as described previously [5,14]. Chemostat cultures (one litre culture volume) were supplied with about 50 mM thiosulphate as energy substrate and maintained at pH 7.8 by automatic titration with 2 M NaOH. Aeration was with air + CO_2 mixtures passed at about 100 ml min^{-1} through the cultures, and gas transfer to solution was assisted by a magnetically-driven double-paddle stirrer (LH Engineering) running at 750 rpm.

Synechococcus PCC 7942 was grown in shake flask culture at 30 °C in BG-11 medium [15] supplemented with 12 mM $NaHCO_3$ and aerated either 5% (v/v) CO_2 in air. Continuous illumination (108 $\mu E\ m^{-2}\ s^{-1}$) was provided by fluorescent lamps. For culture under CO_2-limiting conditions, cultures were grown in the same way but without aeration. *Synechococcus* PCC 7942 was also grown in continuous culture as described previously [16].

3.2. Growth and thiosulphate oxidation by T. versutus

Biomass production and steady state concentrations and consumption rates in the chemostat were measured as described previously [16]. The ability of organisms taken directly from the chemostat to oxidize thiosulphate under conditions of excess supply was estimated as follows: samples (20 ml) of culture were placed in 250 ml conical flasks containing 1 ml 200 mM thiosulphate at 30 °C; duplicate samples (0.5 ml) were removed into the KCN-buffer mixture for cyanolysis assay [18] immediately and at 15 min intervals for 2 h.

3.3. Determination of total CO_2 uptake

T. versutus. Organisms were harvested from the chemostat vessel by centrifuging and resuspended to give suspensions containing about 1 mg dry wt ml^{-1} in phosphate buffer (44 mM Na_2HPO_4 + 11 mM KH_2PO_4). This suspension (0.4 ml) was incubated at 30 °C in a small glass tube and supplemented with 0.05 ml 0.2 M thiosulphate, followed within 30 s by 0.5 ml $NaH^{14}CO_3$ (giving final concentrations of 2–500 μM). After 20, 40 and 60 s, samples (0.15 ml) were removed and rapidly filtered on to 25 mm diameter membrane filters (Whatman, 0.65 μm or Nucleopore 0.6 μm pore size), and washed with 5 ml unlabelled bicarbonate. The membranes were allowed to air dry for 30 min in scintillation vials, then were counted in 10 ml LKB Optiphase 'Safe' scintillant using a Beckman LS 7000 series spectrometer.

Synechococcus PCC 7942 from batch and chemostat cultures were harvested and resuspended in BG-11 medium to give about 1 mg dry wt ml^{-1}. Suspensions were stirred at 30 °C in the light for 5 min then 0.18 ml aliquots placed in Eppendorf microcentrifuge tubes and supplemented with 0.02 ml of various solutions of $NaHCO_3$ to give the required final concentrations. After 30 s the contents of the tubes were filtered on to Whatman GF/F glass microfibre filters, washed with 5 ml unlabelled bicarbonate then with 1 ml 2 M NaOH on 20% (v/v) methanol. Filters were placed in 3 ml Beckman EP scintillation fluid and counted in an LKB Mini-Beta counter.

3.4. Assay of RuBisCO in T. versutus

RuBisCO was measured by a modification of the permeabilized whole-cell assay [5,7]. Samples (containing 0.3–0.4 mg dry wt) taken directly from steady state chemostat cultures were centrifuged in glass centrifuge tubes (4 ml), the supernatant removed, and the cell pellet resuspended in 0.2 ml 5% (v/v) Triton X-100. After 10 min, 0.45 ml of the carboxylase assay mixture was added. This contained (mM): 'Tris'-HCl buffer, pH 8.0 (77); $MgCl_2$ (25); reduced glutathione (2); and sodium ^{14}C-bicarbonate (44) containing about 300 cpm ^{14}C per nmol. The suspension was then vortex-mixed and incubated at 30 °C for 10 min. RuBP (0.15 ml) was added to give an initial concentration of 3 mM and incubation continued. Samples (0.2 ml) were removed after 5, 10 and 15 min and mixed with 0.1 ml 70% H_3PO_4 to discharge excess bicarbonate. Control incubations

without RuBP were also run. ^{14}C-fixed was measured by adding 10 ml Optiphase 'Safe' scintillant and counting in the Beckman spectrometer.

4. RESULTS AND DISCUSSION

Steady state chemostat cultures of *T. versutus* were maintained in three different growth conditions: with thiosulphate-limitation at two growth rates and under carbon dioxide limitation (Table 1). Under thiosulphate limitation, halving the dilution rate lowered the growth yield, as expected [19], and resulted in a drop of two-thirds in the specific activity of RuBisCO. Halving the concentration of CO_2 supplied to the culture caused a 70% fall in the growth yield and resulted in about half the input thiosulphate remaining unused. RuBisCO increased fourfold in activity compared to the thiosulphate-limited culture at the same growth rate (Table 1).

Table 1

Growth yields, thiosulphate oxidation rates and RuBisCO activities in *Thiobacillus versutus* grown under thiosulphate (steady states 1 and 2) or carbon dioxide (steady state 3) limitation in the chemostat

Steady state number	1	2	3
CO_2 supplied (%v/v, in air)	1.0	1.0	0.5
Aeration rate (ml min^{-1})	100	100	90
Dilution rate (h^{-1})	0.073	0.037	0.036
Culture volumes passed before determining steady state values	11.1	6.3	2.8
Thiosulphate supplied (mM)	46.6	64.9	57.8
Residual thiosulphate (mM)	0	0	32.0
Steady state biomass (mg l^{-1})	370	446	114
Growth yield (g dry wt mol^{-1})	7.9	6.9	2.0
RuBisCO activity (nmol CO_2 fixed (min·mg dry wt)$^{-1}$)	50.5 (±12.6)	15.9 (±2.8)	60.5 (±7.8)
Rate of thiosulphate consumption by the chemostat culture ($q_{thiosulphate}$; mmol (h·g dry wt)$^{-1}$)	9.2	5.4	8.2

The specific rate of thiosulphate oxidation in the CO_2-limited culture was 50% higher than that under thiosulphate-limitation at the same growth rate. There was thus considerable 'energy-spilling' thiosulphate oxidation. The maximum rates of thiosulphate oxidation by samples removed directly from the thiosulphate-limited chemostats were about double the rates occurring in the steady state cultures (11.4 and 21.6 mmol thiosulphate/hour/mg dry wt for cells growing at $D = 0.037$ and 0.073 h^{-1}, respectively). The CO_2-limited culture was obviously oxidizing thiosulphate at the maximum rate of which it was capable, as there was excess thiosulphate in the culture.

Using *Synechococcus* as a control organism, known have a CO_2-concentrating mechanism under CO_2-limitation, short-term uptake of $^{14}CO_2$ by both it and *T. versutus,* grown under CO_2- or energy-limitation, was measured (Fig. 1). The cyanobacterium exhibited a greatly increased rate of short-term fixation after growth with limiting CO_2. Its apparent K_m for $^{14}CO_2$ fixation (computed by the Eadie-Hofstee procedure) was 8 μM. In contrast, *T. versutus* showed no $^{14}CO_2$ fixation under these conditions. This result can be contrasted with *T. neapolitanus,* which exhibited a very active CO_2-concentrating system, especially after culture under CO_2-limitation [14]. The complete failure of *T. versutus* to effect short-term CO_2-fixation with low concentrations of CO_2 is wholly consistent with its inability to grow without high levels of dissolved CO_2. *T. neapolitanus* clearly maintains a high intracellular CO_2 concentration by active uptake, while to attain the same concentration *T. versutus* must have a high concentration in the external environment.

We conclude that CO_2-fixation by *T. versutus* is rate-limited either by diffusion of CO_2 and bicarbonate into the organisms, or because any transport system is of low affinity and possibly of low reaction velocity. Comparing the response to growth rate and CO_2 concentration of RuBisCO activity in *T. versutus* and *T. neapolitanus* show that both respond to CO_2-limitation by increasing RuBisCO three to five fold. *T. neapolitanus,* however, showed a slight decrease (to 72%) of RuBisCO

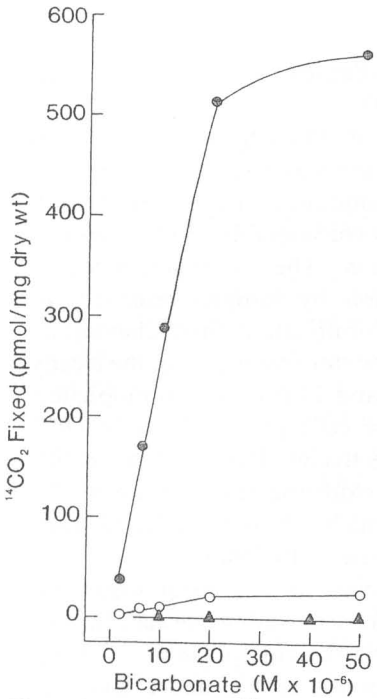

Fig. 1. Incorporation of $^{14}CO_2$ by *Synechococcus* PCC 7942, previously grown with limiting (●) or unlimited (○) CO_2, and by *Thiobacillus versutus* grown in CO_2-limited chemostat culture (▲). ^{14}C-uptake was measured after 30 s (*Synechococcus*) or 60 s (*Thiobacillus*) incubation with $^{14}CO_2$.

when the dilution rate for growth was increased from 0.1 to 0.27 h^{-1} [20]. This contrast with *T. versutus,* which increased RuBisCO activity at increased growth rate (Table 1), indicates that the availability of CO_2 inside the cell is the main factor determining the maximum growth rate of *T. versutus.* Ability to take up CO_2 could clearly also be a factor in determining the outcome of competition between these two thiobacilli [3,17].

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