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EXPERIMENTAL ARTICLES

Major Factors Affecting Isocitrate Lyase Activity in *Rhodobacter capsulatus* B10 under Phototrophic Conditions

E. P. Petushkova[1] and A. A. Tsygankov

Institute of Basic Biological Problems, Russian Academy of Sciences, Pushchino, Moscow oblast, Russia

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Abstract—External factors affecting the activity of isocitrate lyase (ICL) in *Rhodobacter capsulatus* B10 grown under controlled photoheterotrophic anaerobic conditions were investigated. The activity of this enzyme was found to depend on the history of the inoculum and on the growth phase on acetate medium. Intracellular degradation of ICL under unfavorable conditions was shown. However, transition of the growing culture from acetate to lactate did not result in active degradation of the enzyme. When transferred to acetate, *Rba. capsulatus* could grow without the lag phase and did not exhibit ICL activity, suggesting another anaplerotic pathway in *Rba. capsulatus* cells. Since emergence of the ICL activity in the cells grown on acetate results in an increase in its growth rate, the glyoxylate bypass plays an important role in acetate metabolism of *Rba. capsulatus*.

Keywords: *Rhodobacter capsulatus,* acetate assimilation, isocitrate lyase, glyoxylate cycle, anaplerotic pathway, glycogen.

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Anaplerotic pathways, which make it possible for the cells to replenish the oxaloacetate pool when the intermediates of the cycle are used for biosynthesis, are a prerequisite for bacterial growth on acetate as the sole carbon source. One of these pathways is the glyoxylate bypass, with isocitrate lyase (EC 4.1.3.1) and malate synthase (EC 4.1.3.2) as the key enzymes. The purple nonsulfur bacterium *Rhodobacter capsulatus* B10 possesses the structural gene (open reading frame RRC01711) of isocitrate lyase (ICL), the key enzyme of the glyoxylate cycle (http://www.ergo-light.com). The literature data on the ICL activity in *Rba. capsulatus in vivo* are, however, controversial. Some publications report complete absence of the activity of this enzyme [1, 2], while others confirm its presence [3–6]. Different authors obtained different results even for the same strain of this species, specifically, strain B10. We have previously shown the activity of the key enzyme of the glyoxylate cycle (ICL) if *Rba. capsulatus* B10 grown in batch culture in the light on minimal medium with acetate as the sole organic substrate. ICL was shown to degrade rapidly in the cell-free extracts without protease inhibitors [7]. However, even in *Rba. capsulatus* grown in a photobioreactor under controlled conditions, significant fluctuations in the ICL activity were observed, up to its complete absence.

The goal of the present work was to determine the external factors affecting the ICL activity in *Rba. capsulatus* B10 grown under standardized photoheterotrophic anaerobic conditions.

MATERIALS AND METHODS

The nonsulfur purple bacterium *Rhodobacter capsulatus* B10 was grown anaerobically under light (94.5 W/m^2) in the Ormerod medium [8] with 20 mM acetate or 40 mM lactate supplemented with thiamin (1 mg/l). Cultivation was carried out at 30°C and pH 7 (pH was maintained by automatic addition of 0.5 M H_3PO_4) in a controlled photobioreactor using coaxial cylinders [9]. In order to exclude the effect of the previous history of the inoculum on the results, culture of *Rba. capsulatus* B10 was used that was not grown with acetate as the sole carbon source. In order to exclude the presence of other carbon sources, the inoculum was grown until the stationary phase occurring after complete exhaustion of lactate. Prior to inoculation, the medium was bubbled with argon (100 ml/min, 20 min). The photobioreactor was filled with the medium to 1 l (including the 10% inoculum), so that the volume of the gas phase was minimal (~0.1 l). After inoculation, in order to maintain anaerobic conditions without significant disturbance of the gas balance of the medium (including CO_2 removal), argon (20 ml/min) was blown over the medium. This mode of cultivation was chosen because preliminary experi-

[1] Corresponding author; e-mail: ekaterina-petushkova@rambler.ru

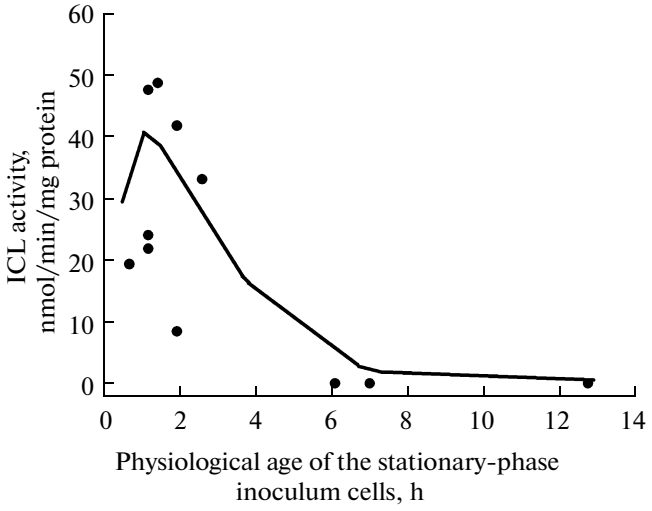

Fig. 1. The maximal ICL activity of the *Rba. capsulata* culture grown with acetate depending on the age of the stationary-phase cells of the inoculum grown in lactate-containing medium (h).

ments with batch cultures in sealed vials without CO_2 removal revealed ICL activity at various growth phases. In order to decrease the number of investigated factors, the role of CO_2 was therefore considered a separate problem requiring special research.

The growth rate was monitored as optical density (OD_{630}) determined using the OD sensor built into the bioreactor.

Determination of the ICL activity was carried out in 100 mg of the wet biomass washed with 50 mM Tris-HCl buffer (pH 7.5), resuspended in 1 ml of 100 mM Tris-HCl buffer (pH 7.5), and supplemented with dithiothreitol (1 mg/ml) and an Invitrogen Roch protease inhibitor (catalogue no. 11 836 153 001) in the concentration recommended by the manufacturer. Sonication for 30 s at 4°C (UZG13-0.1-0.063, 30–35 W) resulted in disintegration of over 90% of the cells (according to concentration of released protein). Large fragments were removed by centrifugation (Eppendorf AG 22331, 12 000 *g*, 2 min). The supernatant was used for analysis. Isocitrate lyase activity was determined spectrophotometrically under aerobic conditions according to Dixon and Kornberg [10]. The reaction mixture (1 ml) contained the following: 100 mM Tris-HCl buffer (pH 7.5), 5 mM $MgSO_4 \cdot 7H_2O$, 3.5 mM phenylhydrazine (pH 7.5), 2 mM isocitrate, and 50 μl of the cell-free extract (5–7 mg/ml protein). The reaction was initiated by addition of isocitrate. Formation of glyoxylate–phenylhydrazone was detected at 324 nm (ε = 11.5 mM^{-1} cm^{-1}). The enzymatic reaction was carried out at 30°C. The amount of the substrate (nmol) converted during 1 min/1 mg of protein was used as an activity unit. Protein was determined by the Lowry method [11]. The concentration of glycogen released from the cells was determined by glucose oxidase method after hydrolysis (6 h in 4 N HCl at 100°C) [12].

For determination of lactate and acetate in the medium, the cells were removed by centrifugation (Eppendorf AG 22331, 12 000 *g*, 2 min). The supernatant was frozen and stored in a freezer prior to analysis. Acetate concentration in the supernatant was determined after acidification to pH ≤ 3 with 30% H_3PO_4 without preliminary gas chromatographic treatment [13]. Lactate concentration was determined by the enzymatic method [14].

Bacteriochlorophyll *a* was determined spectrophotometrically by adsorption of the acetone–methanol extracts at 772 nm (ε = 75 mM^{-1} cm^{-1}) as described in [15].

The data presented are the averages of three to five independent experiments within a 95% confidence interval and the results of the typical experiments.

RESULTS AND DISCUSSION

No reliably reproducible results on ICL activity were obtained in batch cultures of *Rba capsulatus* B10 grown under photoheterotrophic conditions at constant pH with acetate as the sole carbon source. That these scatter is caused by several reasons. One of them is that the ICL activity depended on the physiological state of the stationary-phase cells used as inocula for the acetate-grown cultures (Fig. 1). The ICL activity was highest in the culture inoculated with the cells from the first 2 h of the stationary phase (Fig. 1). The maximal ICL activity (measured at the onset of the stationary phase of the experimental culture) decreased with increasing age of the stationary-phase inoculum. In the case of 5-h stationary-phase cells, ICL activity was not detected in the experimental culture for 8–12 h. During the stationary phase, the content of enzymes and regulatory proteins in *Rba. capsulatus* cells probably decreases, so that ICL activity becomes detectable only 8–12 h or more after transition to active growth in acetate-containing media, as was observed in our experiments. A more accurate explanation of these findings requires, however, additional investigation.

The ICL activity of *Rba. capsulatus* B10 depended also on the growth phase of the culture (Fig. 2). It became detectable at the onset of the exponential growth phase and peaked in the early stationary phase. Prolonged incubation of the stationary-phase cultures resulted in a decrease in the ICL activity to levels below the sensitivity of the method.

A substantial decrease in the ICL activity during the stationary phase (Fig. 2) suggests that the proteases responsible for the rapid decline of the ICL activity in

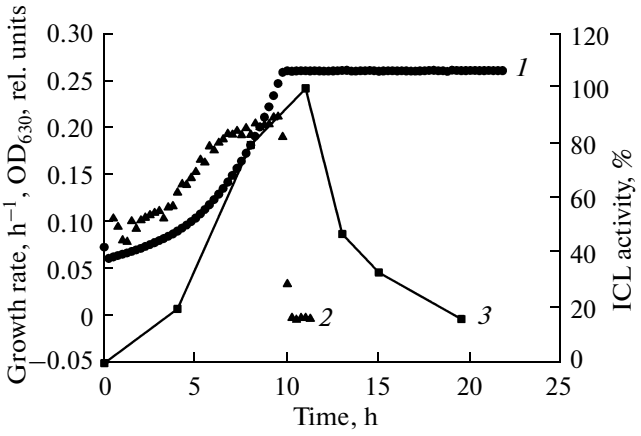

Fig. 2. The ICL activity of *Rba. capsulatus* depending on the growth phase of the cultures transferred from lactate to acetate: optical density (OD_{630}) (*1*); specific growth rate, h^{-1} (*2*); and ICL activity, % of the maximum (*3*).

cell-free extracts [7] are probably involved in decomposition of the enzymes not required during the stationary phase. This line of reasoning implies active ICL degradation in cells transferred from acetate as the sole carbon source to another substrate not requiring the glyoxylate bypass. In order to check this suggestion, medium with 30 mM lactate was added (one-third of the overall volume) to the acetate-grown culture of *Rba. capsulatus* immediately after its transition to the stationary phase, i.e., when the ICL activity was the highest and acetate was consumed (Fig. 3). During the first hour after addition of lactate, the ICL activity did not decrease, but rather increased somewhat (Fig. 3, curve *3*). Lines *4–6* of Fig. 3 show the theoretically calculated changes in the ICL activity, based on the supposition that ICL synthesis stopped at the origin of the curve, no active degradation of the enzyme occurred, and the enzyme was diluted due to growth of new cells not synthesizing ICL. Since the ICL activity remained at the initial level after 3 h of cultivation, while the calculation predicted its decrease to ~40% of the original level, our findings suggest additional ICL synthesis under these conditions. The ICL activity decreased below the level predicted by the curve *4* only after 3 h of cultivation, when the growth rate decreased (Fig. 3, curve *2*). At the onset of the stationary phase caused by exhaustion of lactate, the ICL activity decreased rapidly and completely disappeared after 6 h (Fig. 3, curve *3*). Thus, the intracellular proteases of *Rba. capsulatus* B10 probably degrade ICL only under unfavorable conditions, i.e., when the carbon source is consumed, but not upon the change of the cultivation conditions when this enzyme is not required for growth, as in the case of a change in the carbon source.

Importantly, the ICL activity of the cells transferred to acetate was not detected immediately (Fig. 2). The culture of *Rba. capsulatus* B10 grew, however, without a noticeable lag period. Growth on acetate as the sole carbon source without a functioning glyoxylate bypass is possible due to utilization of the intracellular storage compounds, which may be used to replenish the intermediates of the Krebs cycle. For example, glycogen is the main storage compound in *Rba. capsulatus* B10 [7]. Release of glucose from gly-

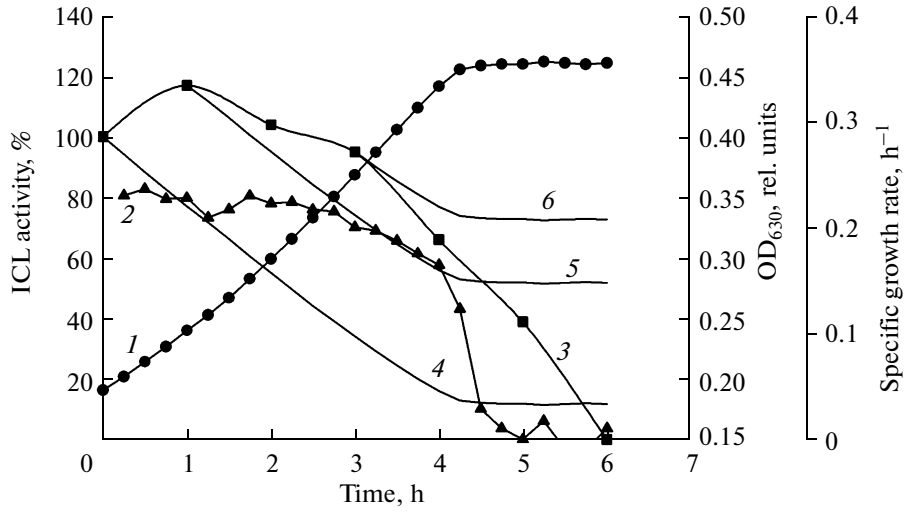

Fig. 3. The ICL activity of *Rba. capsulatus* acetate-grown cultures supplemented with 10 mM lactate: optical density (OD_{630}) (*1*); specific growth rate, h^{-1} (*2*); ICL activity, % of the maximum (*3*); and the calculated decrease in the ICL activity suggesting cessation of the ICL synthesis at the onset of the curve (100%) (*4, 5*, and *6*).

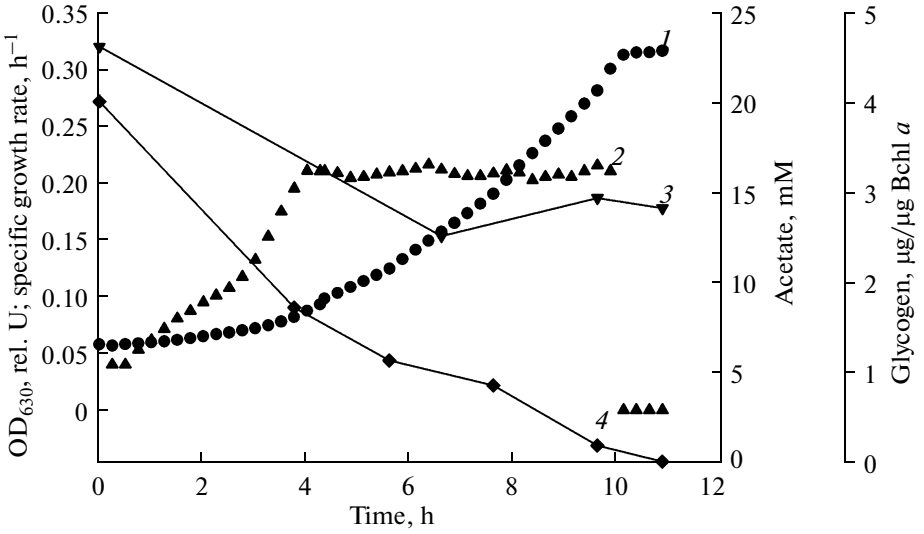

Fig. 4. The content of glycogen and acetate in the medium after transfer from lactate to acetate: optical density (OD_{630} (*1*); specific growth rate, h^{-1} (*2*); glycogen content in the cells (μg/μg Bchl *a*) (*3*); and acetate concentration in the medium, mM (*4*).

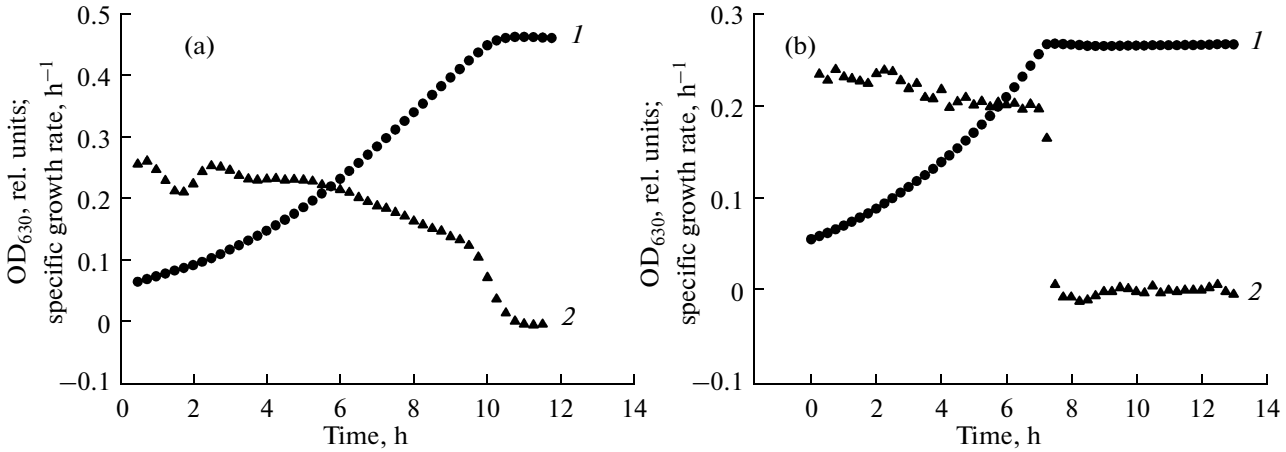

Fig. 5. Growth curves of *Rba. capsulatus* transferred from lactate to lactate (a) and from acetate to acetate (b): optical density (OD_{630} (*1*) and specific growth rate, h^{-1} (*2*).

cogen via the Entner–Doudoroff pathway [18] may result in pyruvate carboxylation by pyruvate carboxylase (EC 6.4.1.1); the gene (ORF RRC02720; http://www.ergo-light.com) and activity of this enzyme in *Rba. capsulatus* have been reported [5]. The oxaloacetate thus produced may replenish the pool of the Krebs cycle intermediates so that 2 mol acetate may be consumed per 1 mol glucose resulting from glycogen decomposition for functioning of the Krebs cycle. Additional alternative pathways for replenishing the oxaloacetate pool in the Krebs cycle are another possible explanation for growth in the presence of acetate [2].

In order to determine whether reserve compounds may be utilized to replenish the oxaloacetate pool in *Rba. capsulatus*, the medium with acetate was inoculated with the lactate-grown culture. The content of glycogen in the cells and acetate concentration in the medium were monitored (Fig. 4). The data shown on Fig. 4 are the results of the typical experiment. The initial concentration of glycogen in the cells varied (in five repeats) from 2.4 to 4.8 with the average of 3.8 μg/μg Bchl *a*. By the end of the exponential phase, glycogen content varied from 1.2 to 2.5 with an average of 1.9 μg/μg Bchl *a*. In all experiments, transition from the exponential growth phase to decelerated growth resulted in an ~10% increase in the intracellular glycogen content with its subsequent rapid decrease after transition to the stationary phase (Fig. 4). The average decrease in the intracellular glycogen content from the onset to the end of growth (10 h) was

1.82 ± 0.534 μg/μg Bchl *a*. Considering the cell concentration, this difference was ~2.8 μg glycogen per 1 ml of the medium or 15 μmol glucose (the monomer of glycogen) per 1 l. The concentration of acetate decreased during this period from 20 to 1.2 mM (Fig. 4). Thus, consumption of the intracellular storage compound (glycogen) in the course of transition from lactate to acetate utilization is insufficient for explanation of growth with acetate in the absence of the ICL activity. The *Rba. capsulatus* cells therefore possess other anaplerotic pathways, as was confirmed by other authors [2].

The glyoxylate bypass plays, however, an important role in growth of *Rba. capsulatus* on acetate. This conclusion follows from the fact that transfer of the culture from lactate to acetate resulted in an increase in its growth rate concurrent with the increase of the ICL activity (Fig. 2). The initial growth rate (during the first hour after the lag phase) measured in nine independent experiments was 0.084 ± 0.053 h^{-1}. The period of increasing growth rate varied form 3 to 5 h (3.5 h in Fig. 4). The maximal growth rate was 0.179 ± 0.0145 h^{-1}.

Importantly, both the lag phase and acceleration of growth were less pronounced when the culture was transferred from lactate to lactate (Fig. 5a) and completely absent in the case of transfer from acetate to acetate (Fig. 5b). In the first and second case, the average growth rates were 0.239 ± 0.014 and 0.197 ± 0.041 h^{-1} (the figures represent the results of a typical experiment). Considering the role of the differences in microbial growth rates for the competition in natural consortia, a significant increase in the growth rate in the course of adaptation of the culture to growth on acetate by the activation of the glyoxylate pathway indicates its important role for *Rba. capsulatus* B10.

Thus, we determined that *Rba. capsulatus* grown on acetate possesses the ICL activity, which depends on the previous history of the inoculum and on the phase of growth on acetate. Under conditions of exhaustion of the carbon source (the stationary growth phase), ICL is subject to the intracellular degradation. However, active degradation of this enzyme does not occur when the carbon source is changed from acetate to lactate. Ability of the cultures to grow on acetate without the lag phase in the subsequent transfers suggests the presence of another anaplerotic pathway in *Rba. capsulatus*. However, the presence of the ICL activity in the cells results in higher growth rates.

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