The regulation of poly- β -hydroxybutyrate metabolism in *Azospirillum brasilense* during balanced growth and starvation

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Activities of the enzymes which are involved in the poly- β -hydroxybutyrate (PHB) cycle, in both synthesis and degradation reactions, were assayed in crude extracts of *Azospirillum brasilense* cells containing different amounts of PHB. The enzymes of the PHB cycle, of both the synthesis and the degradation process, were more active in PHB-rich cells than in PHB-poor cells. During 96 h of starvation of cells suspended in phosphate buffer, enzymes of the PHB cycle were more active in PHB-rich cells. There was a peak of activity of hydroxybutyrate dehydrogenase (BOHB-DH), β -ketothiolase and thiophorase after 24 h of starvation, due to polymer degradation. During the following hours of starvation there was a decrease in the activity of these enzymes. After 24 h of starvation the activity of acetoacetyl-CoA reductase dropped to a minimum level, because the cells could not synthesize PHB under these conditions. The specific activities of BOHB-DH, β -ketothiolase and thiophorase were higher in *A. brasilense* cells which were grown under low oxygen tension and consequently accumulated high levels of PHB, than in cells grown under high oxygen tension, with a low PHB content. Similarities to the pathway of PHB biosynthesis and degradation and its control in *Azotobacter beijerinckii* are described.

Introduction

Poly- β -hydroxybutyrate (PHB) is synthesized and accumulated by *Azospirillum brasilense* Cd up to 70% of the cell dry weight. PHB synthesis is favoured under oxygen limitation and by a high C/N ratio towards the end of exponential growth in batch culture (Tal & Okon, 1985).

A cyclic mechanism for the biosynthesis and degradation of PHB in *Azotobacter beijerinckii* was proposed by Senior & Dawes (1973). They also proposed a regulatory scheme for the PHB cycle. Certain similarities were found in the cyclic pathway and its control between *Azotobacter beijerinckii* and *Hydrogenomonas eutropha* (Oeding & Schlegel, 1973). These two groups suggested that the PHB cycle is controlled by the redox state of the cell and by the concentration of CoA-SH, pyruvate and 2-oxoglutarate. Our work with *Azospirillum brasilense* (Tal *et al.*, 1990) suggested a similar control mechanism in this organism.

Azospirillum brasilense is a rhizosphere micro-organism with potential use as an inoculant for promot-

ing crop plant growth (Okon, 1985*a, b*). Tal & Okon (1985) showed that in the presence of stress factors, PHB-rich *A. brasilense* survived better than PHB-poor cells. This ability to survive adverse conditions is important in the rhizosphere. PHB degradation and its utilization as sole carbon and energy source by *A. brasilense* could be involved in favouring establishment, proliferation, survival and competition with other micro-organisms in the rhizosphere (Tal & Okon, 1985). It is therefore, important to study and examine in detail the PHB cycle in *A. brasilense*. This paper reports studies of the enzymes and intermediates which are involved in the cycle, and the mechanism of the regulation of synthesis and degradation of the polymer.

Methods

Chemicals. The following were obtained from Sigma: disodium succinate, DTT, Na₂EDTA, NAD⁺, NADP⁺, NADH, NADPH, CoASH, acetyl-CoA, hydroxybutyryl-CoA, 3-hydroxybutyrate sodium salt, hydroxybutyryl-CoA dehydrogenase, aceto-cetic acid and malic acid.

Bacterial strain and growth conditions. *Azospirillum brasilense* Cd (ATCC 29729) was used throughout. The following growth conditions were used.

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Abbreviations: BOHB-DH, D(-)-3-hydroxybutyrate dehydrogenase; CoA-SH, coenzyme A; PHB, poly- β -hydroxybutyrate.

(a) Cells were grown in batch culture (120 r.p.m.) at 32 °C for 36 h, in ammonia-malate mineral medium (Okon *et al.*, 1976) which, unless stated otherwise, contained 2.8 mM-NH₄Cl. The concentration of carbon source (malic acid) was 45 mM. The C/N ratio was therefore 46. Towards the end of the exponential growth stage (36 h), the cells were harvested and crude extract was prepared as described below. PHB accumulation is favoured under these conditions and its content in the cell is around 40% of the dry weight.

(b) Cells were grown as described in (a), except that NH₄Cl concentration in the medium was 14.8 mM (C/N = 8.7). The cells, in which PHB comprised around 5% of the dry weight, were collected after 36 h of growth and crude extract was prepared.

(c) Cells were grown as described in (a) (C/N = 46). After 36 h of growth the cells were harvested, using sterile techniques, centrifuged at 5000 g for 10 min and washed twice with sterile 0.06 M-potassium phosphate buffer, pH 6.8. After washing, the cells were resuspended in the same buffer up to the initial volume, and were maintained under these starvation conditions for an additional 96 h. During this period samples were taken every 24 h, and crude extracts were prepared.

(d) Cells were grown as described in (b) (C/N = 8.7) and were starved as described in (c). Crude extracts were prepared.

Crude extract preparation. Cultures of each treatment above were collected by centrifugation at 5000 g for 10 min and washed twice with the appropriate buffer, depending upon the enzyme of interest. The cells were then resuspended in approximately 5 vols of the particular buffer. All subsequent procedures were carried out at 4 °C. The bacterial suspension was disintegrated in a French pressure cell at 30000 p.s.i. (207 MPa). Crude extract was prepared by centrifuging the disintegrated cell suspension at 35 000 g for 20 min; the supernatant was decanted and used for enzyme assays.

Activity assays of PHB cycle enzymes in crude extracts. *Hydroxybutyrate dehydrogenase (BOHB-DH)* (EC 1.1.1.30). The assays of Senior & Dawes (1973) and Tal *et al.* (1990) were used, with minor modifications in the concentration of the reactants. Activity was measured in both the oxidation direction [$D(-)$ -3-hydroxybutyrate + NAD⁺ → acetoacetate + NADH] and the reduction direction [acetoacetate + NADH → $D(-)$ -3-hydroxybutyrate + NAD⁺], by following the change in absorbance at 340 nm at 36 °C. One unit of enzyme activity was defined as 1 μmol NAD⁺ reduced or NADH oxidized min⁻¹ (ϵ for NADH = 6.21×10^3 M⁻¹ cm⁻¹).

β -Ketothiolase (acetyl-CoA acetyltransferase) (EC 2.3.1.9). The assays of Senior & Dawes (1973), Oeding & Schlegel (1973) and Ruhr & Schlegel (1975) were used. The activity for the condensation reaction [2 acetyl-CoA → acetoacetyl-CoA + CoA-SH] was calculated as described above for BOHB-DH. The activity for the catalytic cleavage reaction [acetoacetyl-CoA + CoA-SH → 2 acetyl-CoA] was measured at 303 nm. The decrease in A_{303} measures the decrease in the Mg²⁺-enol complex of acetoacetyl-CoA. One unit was defined as the decrease in A_{303} min⁻¹.

Thiophorase (succinyl-CoA CoA transferase EC 2.8.3.5). The assays of Senior & Dawes (1973), Oeding & Schlegel (1973) and Ruhr & Schlegel (1975) were used. The activity for the reaction [acetoacetyl-CoA + succinate → acetoacetate + succinyl-CoA] was measured spectrophotometrically, at 303 nm, as in the cleavage reaction by β -ketothiolase.

Acetoacetyl-CoA reductase ($D(-)$ -hydroxybutyryl-CoA : NADH oxidoreductase) (EC 1.1.1.36). The assays of Senior & Dawes (1973) and Ritchie *et al.* (1971) were used. The activities for the reduction reaction [acetoacetyl-CoA + NAD(P)H → $D(-)$ - β -hydroxybutyryl-CoA + NAD(P)⁺] and the oxidation reaction [$D(-)$ - β -hydroxybutyryl-CoA + NAD(P)⁺ → acetoacetyl-CoA + NAD(P)H] were measured and calculated in the same way as for BOHB-DH.

Fig. 1. BOHB-DH activity in crude extracts of *A. brasilense* cells grown for 36 h at different NH₄Cl concentrations. Columns with the same letter are not significantly different.

For each enzyme, specific activity was defined as activity units per A_{280} unit.

Results

Activity of PHB cycle enzymes in crude extracts of bacteria grown with different NH₄Cl concentrations

BOHB-DH activity was measured in crude extracts of bacteria grown for 36 h in medium containing different concentrations of NH₄Cl (Fig. 1). The specific activity of the enzyme, which degrades monomers of PHB to acetoacetate, was highest in bacteria grown at the lowest NH₄Cl concentration (2.8 mM), and thus the highest C/N ratio (46); these cells had a high PHB level (around 40%, w/w). At higher NH₄Cl concentrations, where the cells had a lower level of PHB (up to 5%), the BOHB-DH activity was lower. Under nitrogen-fixation conditions the activity was also low.

β -Ketothiolase. β -Ketothiolase activity was measured in crude extracts of bacteria grown for 36 h in medium containing two different NH₄Cl concentrations (Table 1). The activity of NADH oxidase was measured prior to the addition of the substrate, disodium succinate, and served as a blank for the reaction in the condensation direction. The specific activity of β -ketothiolase in the condensation direction was higher in the (PHB-rich) bacteria grown at the lower concentration of NH₄Cl than in the (PHB-poor) cells grown at the higher concentration, whereas NADH oxidase specific activity was threefold higher in crude extracts of the latter cells. In this case, there was no significant additional β -ketothiolase activity after the addition of the substrate to the crude extract. That means there is low β -ketothiolase activity in crude extracts of PHB-poor cells.

The specific activity of β -ketothiolase in the catalytic

Table 1. β -Ketothiolase activity in the condensation and in the catalytic cleavage directions in crude extracts of *A. brasilense* cells grown with different NH_4Cl concentrations

Treatment	Specific activity (units per A_{280} unit)			
	Condensation		Catalytic cleavage	
	2.8 mM NH_4Cl	14.8 mM NH_4Cl	2.8 mM NH_4Cl	14.8 mM NH_4Cl
Preincubation without the substrate for 3 min	39*	122*	194	4.4
Incubation with the substrate	90	129	0.4	4.8
Net activity	51	7	—	0.4

* NADH oxidase blank (see text).

Table 2. Thiophorase activity in crude extracts of *A. brasilense* cells grown with different NH_4Cl concentrations

Treatment	Specific activity (units per A_{280} unit)	
	2.8 mM- NH_4Cl	14.8 mM- NH_4Cl
Preincubation for 1.5 min	3.3	5.7
Preincubation for an additional 2 min	3.3	0.1
Incubation with the substrate	8.8	0.2
Net activity	5.5	0.1

cleavage direction, in the same crude extracts as above, is also shown in Table 1. When the substrate was added to the crude extract of PHB-rich cells, which were grown at a low concentration of NH_4Cl , the activity immediately decreased from 194 to 0.4 units per A_{280} unit. This indicates that the enzyme is extremely inhibited in the presence of the substrate in crude extracts. It is possible that the activity recorded before adding the substrate is due to low internal concentrations of substrate in the crude extract. Incubation of the reaction mixture, including the substrate, for 2 min before the assay, led to no enzyme activity (0.4 units per A_{280} unit). No β -ketothiolase activity was recorded for PHB-poor cells.

Thiophorase. Thiophorase activity was measured in crude extracts of bacteria grown for 36 h at two NH_4Cl concentrations (Table 2). During 1.5 min preincubation without the substrate, some activity was recorded for crude extracts of cells grown under both low and high NH_4Cl concentrations. After 3.5 min of preincubation the blank activities remained constant and the substrate, disodium succinate, was added. The enzyme specific activity was much higher in crude extracts of the cells

Table 3. Acetoacetyl-CoA reductase activity in crude extracts of *A. brasilense* cells grown with different NH_4Cl concentrations

Treatment	Specific activity (units per A_{280} unit)	
	2.8 mM- NH_4Cl	14.8 mM- NH_4Cl
Preincubation for 2 min	1.5	2.5
Incubation with the substrate	3.8	3.4
Net activity	2.3	0.9

Table 4. Activity of PHB cycle enzymes in crude extracts of *A. brasilense* cells grown at different $p\text{O}_2$ values

Enzyme	Specific activity (units per A_{280} unit)		
	0.024 mM O_2	0.096 mM O_2	0.024 mM O_2
BOHB-DH	52	14	13
β -Ketothiolase (condensation)	41	39	11
Acetoacetyl-CoA reductase	7	4	3

grown at the lower concentration of NH_4Cl , which had accumulated a high level of PHB. When the substrate was added at the beginning of the preincubation period, only half of the apparent thiophorase activities were observed.

Acetoacetyl-CoA reductase. Acetoacetyl-CoA reductase activity was measured in crude extracts of cells grown for 36 h at two NH_4Cl concentrations. The enzyme activity during 2 min of preincubation without the substrate and NADH, after NADH addition and during incubation with the substrate is shown in Table 3. The enzyme specific activity was higher in the cells grown at the lower NH_4Cl concentration, which were rich in PHB.

Activity of enzymes of the PHB cycle in crude extracts of cells grown at different oxygen concentrations in chemostat culture

A. brasilense cells were grown in chemostat culture with malate as a growth-limiting carbon and energy source, at a moderate dilution rate ($D = 0.14 \text{ h}^{-1}$), under different partial pressures of oxygen, ranging from oxygen limitation (0.024 mM- O_2) to oxygen saturation in water (0.24 mM- O_2). The $p\text{O}_2$ values were changed in the chemostat, and the cells were harvested after 24 h of growth at the particular $p\text{O}_2$. Crude extracts were prepared for assay of BOHB-OH, β -ketothiolase and acetoacetyl-CoA reductase. The enzyme activities were higher in cells grown under oxygen limitation. Under

Fig. 2. BOHB-DH activity during 96 h of starvation in crude extracts of PHB-rich (●) and PHB-poor (▲) cells of *A. brasilense*. (a) Enzyme activity in the hydroxybutyrate oxidation direction; (b) enzyme activity in the acetoacetate reduction direction.

oxygen saturation the enzyme activities were very low, even though the growth rate and the bacterial yield was very high relative to that under oxygen limitation (Table 4).

Activity of PHB cycle enzymes in crude extracts of bacteria grown under starvation conditions

Activity of the enzymes involved in synthesis and degradation of PHB was measured in PHB-rich and -poor cells after 36 h of batch growth at low and high concentrations of NH_4Cl , respectively. These activities are considered to be the starting activities ($t = 0$) in Figs 2–5. These bacteria were kept for an additional 96 h under starvation conditions and samples were taken every 24 h for enzyme activity assays.

BOHB-DH. The enzyme activity in the monomer degradation direction during starvation is shown in Fig. 2(a). The enzyme activity was high in the PHB-rich bacteria and low in the PHB-poor containing bacteria. After more than 24 h of starvation of PHB-rich cells, the specific activity decreased slightly, but it was still higher than that in PHB-poor cells.

The enzyme specific activity in the NADH oxidation direction (Fig. 2b) was lower than that in the reduction direction, in both PHB-rich and -poor cells.

In addition, the activity of BOHB-DH was measured in crude extracts of cells grown in medium containing different concentrations of NH_4Cl and then starved for

Fig. 3. β -Ketothiolase activity during 96 h of starvation in crude extracts of PHB-rich (●) and PHB-poor (▲) cells of *A. brasilense*. (a) Enzyme activity in the condensation direction; (b) enzyme activity in the catalytic cleavage direction.

60 h. The results were similar to those of the above experiment (data not shown).

β -Ketothiolase. β -Ketothiolase activity, in both the condensation (Fig. 3a) and the thiolysis (Fig. 3b) direction was higher in crude extracts of bacteria containing a high level of PHB than in those from PHB-poor cells. The peak of activity in both cases was after 24 h of starvation. In the thiolysis direction, the difference in activity between the two types of cells was much greater.

Thiophorase. Activity of this enzyme was also higher in PHB-rich than in PHB-poor bacteria. There was again a peak of activity after 24 h of starvation (Fig. 4).

Acetoacetyl-CoA reductase. During starvation the activity pattern of this enzyme, which participates in PHB synthesis, was different from those of the enzymes which are involved in PHB degradation (described above). The enzyme activity in the reduction was high after 36 h of growth and decreased to a minimum after 24 h of starvation. In both PHB-rich and -poor cells there was an oscillation of activity during the starvation. During the

Fig. 4. Thiophorase activity during 96 h of starvation in crude extracts of PHB-rich (●) and PHB-poor (▲) cells of *A. brasilense*.

Fig. 5. Acetoacetyl-CoA reductase activity (reduction activity) during 96 h of starvation in crude extracts of PHB-rich (●) and PHB-poor (▲) cells of *A. brasilense*.

first 48 h of starvation there was no significant difference between the two types of cells, but the differences became significant after 72 and 96 h of starvation (Fig. 5).

In the opposite direction of activity the enzyme was active only after 36 h of growth, and there was no activity during the starvation period (data not shown).

Discussion

In this research it was found that PHB metabolism in *Azospirillum brasilense*, as in *Azotobacter vinelandii* (Dawes & Senior, 1973; Dawes, 1986), occurs in an cyclic pathway, in which both synthesis and degradation of the polymer, occur, depending upon the conditions. The

synthesis and degradation of the polymer are under a comparable regulation which is carried out by a chain of reactions. It was shown that the polymer synthesis and degradation cycle in *Azospirillum*, when related to the intermediates and enzymes and to the response to oxygen limitation, is similar to the situation in *Azotobacter* (Senior & Dawes, 1971, 1973; Senior *et al.*, 1972; Jackson & Dawes, 1976; Stockdale *et al.*, 1986; Ward *et al.*, 1977).

It has been shown by various authors that the resistance of micro-organisms to starvation is correlated with the PHB level in the cells and at the beginning of starvation there is a rapid utilization of the polymer (Matin, 1979; Horan & Shanmugan, 1986; Malmcrona-Friberg *et al.*, 1986). This was also shown for *Azospirillum brasilense* (Tal & Okon, 1985). During starvation, coincident with the decline in polymer level in the cells, the elliptic-circular *A. brasilense* cells become rods (Okon, 1985a). In this work the activities of the various enzymes of the PHB cycle were measured in crude extracts of bacteria approaching the end of exponential growth under oxygen- and/or nitrogen-limitation conditions, both of which favour PHB synthesis. The activities of the enzymes were also measured during 96 h of starvation in phosphate buffer. The loss of active synthesis and degradation enzymes, after unbalanced growth and during starvation, in both PHB-rich and -poor cells, indicates that these enzymes are probably constitutive. During PHB synthesis and accumulation the enzyme levels increased. This was shown by the higher enzyme activity achieved in PHB-rich cells, even though these bacteria had grown under unbalanced conditions and had a low growth rate, relative to PHB-poor cells. During starvation, coincident with the decline in polymer level in the cells, the total activity decreased with time. But there was a difference between the synthesis and degradation enzymes: the synthesis enzymes were not active when the degradation enzymes were active, and vice versa. The greatest changes in enzyme activities were observed after 24 h of starvation. The activity of acetoacetyl-CoA reductase, which participates in PHB synthesis, decreased to a minimum level after 24 h of starvation. Activity of the enzymes involved in PHB degradation reactions, BOHB-DH, thiophorase and β -ketothiolase, increased during the first 24 h of starvation. These enzymes are probably always present in the cell, but are more active under conditions in which degradation is favoured. In *Rhizobium japonicum* BOHB-DH and β -ketothiolase activity increase during nitrogenase activity, and thiophorase activity decreases (Karr *et al.*, 1984).

In *A. brasilense* the activity of the enzymes decreased following additional starvation, probably due to the release of inhibitors. Usually the decrease in enzyme activity with time occurred in an oscillatory manner; this

is related to the polymer content in the cell (Tal & Okon, 1985). The increase in activity which follows the decrease was presumably due to the presence of the PHB degradation products, resulting in recovery of the culture for 24 h. After utilization of these products no more endogenous or exogenous carbon and energy sources remain. After 96 h there was still a low level of activity of the degradation enzymes. The synthesis enzymes were probably still present in the cell, and with improvement of the conditions, for example by adding a carbon source, the enzyme activity would be as high as it was before starvation.

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