Secretion of Poly(3-hydroxybutyrate) Depolymerase by *Alcaligenes faecalis* T1

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The relationship between cell growth and the secretion of poly(3-hydroxybutyrate) (PHB) depolymerase by *Alcaligenes faecalis* T1, a gram-negative bacterium, was studied with various carbohydrates added to the medium as sole carbon sources. *A. faecalis* T1 could grow on many kinds of carbon sources, the growth rate depending on the carbon source species. However, among the various carbon sources tested, glucose, PHB and its metabolites, D(−)-3-hydroxybutyrate and acetoacetate, but not succinate or other carboxylic acids, caused the secretion of PHB depolymerase. In the medium containing glucose as a sole carbon source, *A. faecalis* T1 started to grow and to secrete PHB depolymerase after 60 h of cultivation. In contrast, the growth of cells in the medium containing succinate as a sole carbon source started within 2 h and reached a plateau level after 6 h of cultivation, but the cells did not secrete the enzyme into the culture medium. However, succinate-grown cells contained a considerable amount of PHB depolymerase, the level being the same as that in glucose-grown cells. Most of the cellular PHB depolymerase was found to be localized in the membrane fractions prepared from the glucose- and succinate-grown cells. In contrast to the glucose-grown cells, the succinate-grown cells exhibited no ability to incorporate [^{14}C]glucose, although the cells exhibited several glycolytic enzyme activities for glucose oxidation. Therefore, it seems that the glucose availability for this bacterium is dependent on the induction of some protein(s) essential for glucose uptake and that glucose metabolites such as ketone bodies are essential for PHB depolymerase secretion by *A. faecalis* T1.

Keywords poly(3-hydroxybutyrate); polyhydroxybutyrate; depolymerase; poly(3-hydroxybutyrate) depolymerase; polyhydroxybutyrate depolymerase; *Alcaligenes faecalis*; secretion; enzyme secretion

Introduction

Poly(3-hydroxybutyrate) (poly-β-hydroxybutyrate, PHB), the polymeric ester of D(−)-3-hydroxybutyrate, is an intracellular reserve of organic carbon and/or chemical energy in a wide range of microorganisms, and in a cell, PHB is known to be degraded by a specific intracellular depolymerase into free D(−)-3-hydroxybutyrate, which is then oxidized to acetoacetate.[2] On the other hand, some bacteria secrete extracellular PHB depolymerase which degrades environmental PHB and utilize the resulting D(−)-3-hydroxybutyrate as a nutrient.[3]

We previously isolated *Alcaligenes faecalis* T1, a gram-negative bacterium, from activated sludge, which secretes PHB depolymerase into the culture medium. Thereafter, we purified and characterized this enzyme,[4] and determined the nucleotide sequence including a potential signal sequence of deoxyribonucleic acid (DNA) encoding its amino acid sequence.[5]

Compared to the case of gram-positive bacteria, little is known about the way in which proteins are secreted into the growth medium by gram-negative bacteria.[6] Since there are few gram-negative bacteria known to release proteins into the growth medium, *A. faecalis* T1 seems to be useful for studying the secretion of extracellular proteins. We recently found that the secretion of PHB depolymerase by *A. faecalis* T1 was dependent on the carbon sources species in the growth medium. Therefore, in this study, we investigate the relationship between the growth of, and the PHB depolymerase secretion by, this bacterium grown on various carbon sources.

Experimental

Cultivation of the Organism *Alcaligenes faecalis* T1 isolated from activated sludge was maintained as described previously.[4] The bacterium was precultured in a 300-ml Sakaguchi flask containing 50 ml of the basic salt medium [0.1% (w/v) NH_4Cl, 0.05% (w/v) $MgSO_4 \cdot 7H_2O$, 0.01% (w/v) $FeCl_3 \cdot 6H_2O$, 0.0005% (w/v) $CaCl_2 \cdot 2H_2O$ and 66 mM KH_2PO_4/Na_2HPO_4 (pH 6.8)] supplemented with 0.4% (w/v) sodium succinate as a sole carbon source (succinate medium) at 30 °C for 18 h on a reciprocal shaker (100 strokes/min). Aliquots (3 ml) of the culture fluid were transferred to 500-ml Sakaguchi flasks containing 100 ml of the basic salt medium supplemented with glucose (glucose medium) or another carbon source instead of sodium succinate, and cultured under the same conditions. The concentrations of the carbon sources used were 0.4% (w/v) for carboxylic acids, and 0.15% (w/v) for 6-carbon sugars and PHB.

Enzyme Assay PHB depolymerase activity was assayed by measuring the changes in the turbidity of a PHB suspension as described previously.[4] In brief, the reaction mixture (1 ml) comprised 100 μg of purified PHB and 50 μg of potassium phosphate (pH 7.5). The reaction was initiated by the addition of the enzyme, and then the decrease in turbidity due to insoluble PHB was measured at 650 nm and 30 °C with a Shimadzu recording spectrophotometer, model UV-240. Since the PHB depolymerase activity is nonspecifically inhibited by other proteins, the amount of PHB depolymerase in the cells was estimated by means of an enzyme-linked immunosorbent assay[7] involving anti-PHB depolymerase immunoglobulin G raised in a rabbit, the binding of antigen–antibody complex to nitrocellulose being determined.

Glucose-6-phosphate (G-6-P) dehydrogenase,[8] hexokinase,[9] 5′-nucleotidase,[10] malate dehydrogenase,[11] nicotinamide adenine dinucleotide phosphate ($NADP^+$)-linked isocitrate dehydrogenase[12] and D(−)-3-hydroxybutyrate dehydrogenase[13] were assayed as described elsewhere. Protein was determined by the method of Lowry *et al.*[14] with bovine serum albumin as a standard.

Other Assays The glucose[15] and succinate[16] concentrations were determined as described elsewhere.

Localization of PHB Depolymerase in *A. faecalis* T1 *A. faecalis* T1 cells (1 g wet weight) grown in the succinate or glucose medium were subjected to osmotic shock treatment according to the procedure of Neu and Heppel[17] to release periplasmic proteins, followed by centrifugation at 20000 × *g* for 10 min, the supernatant (shock fluid) being saved. The precipitate was suspended in 3 ml of 50 mM Tris–HCl (pH 7.5) and then sonicated for 5 min (20000 Hz, sonifier model W-185; Branson Sonic Power Co., Danbury, CT, U.S.A). After the sonicate had been centrifuged at 100000 × *g* for 60 min, the supernatant (cytosolic fraction) and the precipitate (membrane fraction) were separately collected. The culture medium and shock fluid were concentrated in a dialysis sac using a water adsorbent (Sumikagel N-100; Sumitomo Chemical Co., Osaka, Japan).

Uptake of [^{14}C]Glucose and Release of $^{14}CO_2$ by *A. faecalis* T1 The incorporation of [^{14}C]glucose into *A. faecalis* T1 cells and the release of $^{14}CO_2$ from the cells were measured by the methods of Anraku[18] and Prabhakararao and Jones,[19] respectively. For measurement of the uptake of [^{14}C]glucose, *A. faecalis* T1 cells grown in the succinate or glucose medium were harvested and then 1.6×10^9 cells were suspended in 1 ml of the basic medium supplemented with 40 μg/ml of chloramphenicol, followed by incubation at 30 °C for 3 min in a 10-ml flask. The reac-

tion was initiated by the addition of 0.1 μmol of D-[1-^{14}C]glucose (7.9 mCi/mmol) and incubation was carried out at 30 °C. At time intervals, samples of 0.1 ml were removed and filtered on nitrocellulose membranes (Schleicher and Schull; BA 85, 0.45 μm), followed by washing 3 times with 2 ml of ice-cold 0.01 M Tris–HCl (pH 7.3) containing 0.3 M NaCl and 0.5 mM $MgCl_2$. The filters were dried and then subjected to radioactivity counting with 0.5% 2,5-diphenyloxazole in toluene. For assaying of $^{14}CO_2$ release, 1.6×10^9 cells suspended in 1 ml of the basic medium supplemented with 40 μg/ml of chloramphenicol were preincubated in a Warburg flask, and then a 2.0 × 3.0 cm piece of filter paper, folded to one-eighth of its length and wet with 0.1 ml of 1 N NaOH, was placed in the center well of the flask to absorb $^{14}CO_2$. The reaction was initiated by the addition of 10 μmol of D-[^{14}C]glucose (79 μCi/mmol) and incubation was carried out in time intervals at 30 °C. The reaction was terminated by the addition of 0.1 ml of 1 N HCl. The incubation was continued for another 30 min to complete the absorption of released $^{14}CO_2$ by the NaOH-soaked filter paper. The filter paper was then dried and subjected to radioactivity counting.

Preparations Purified PHB and PHB depolymerase were prepared as described previously.[4]

Materials The following materials were purchased from the sources indicated: nicotinamide adenine dinucleotide (NAD^+), $NADP^+$ and G-6-P dehydrogenase from yeast from Oriental Yeast Co. (Tokyo, Japan); bovine serum albumin from Sigma Chemical Co. (St. Louis, MO, U.S.A.); and D-[1-^{14}C]glucose (5 mCi/mmol) from New England Nuclear Corp. (Boston, MA, U.S.A.). Other chemicals were of reagent grade and obtained commercially.

Results

Effects of Various Carbon Sources on the Growth of and PHB Depolymerase Secretion by *A. faecalis* T1 The effects of various carbon sources on the growth of *A. faecalis* T1 were investigated by measuring the turbidity at 650 nm of the culture medium. As shown in Table I, the growth rate of the bacterium varied according to the carbon source added to the culture medium. However, it only secreted PHB depolymerase on incubation with certain carbon sources, such as glucose, PHB and its metabolites, 3-hydroxybutyrate and acetoacetate. When succinate was used as the sole carbon source, a culture of *A. faecalis* T1 reached the end of the logarithmic growth phase within 6 h, but PHB depolymerase activity was not detected in the culture supernatant. In the glucose medium it took 130 h to reach the end of the logarithmic growth phase, however, high PHB depolymerase activity was found in the culture medium. In order to investigate the characteristics of PHB depolymerase secretion, the succinate and glucose media were used for further studies.

Growth Profiles of *A. faecalis* T1 Cells Figure 1 shows the effect of succinate or glucose on the growth curve of *A. faecalis* T1 cells. In the succinate medium, the bacterium started to grow after 2 h of cultivation and then grew

Table I. PHB Depolymerase Activity in Culture Supernatants of *A. faecalis* T1 Grown on Various Carbon Sources

Carbon source	Growth (h)[a]	Activity (units/ml)[b]
Succinate	6	0
Acetate	14	0
Pyruvate	12	0
Citrate	24	0
Malate	10	0
2-Oxoglutarate	26	0
Fumarate	10	0
3-Hydroxybutyrate	24	0.23
Acetoacetate	19	0.20
PHB	40	0.20
Glucose	130	1.09
Mannose	No growth	—
Galactose	No growth	—

A. faecalis T1 cells were grown as described under Experimental. *a*) The time necessary to reach the end of the logarithmic growth phase. *b*) The maximal enzyme activity found in the culture supernatant.

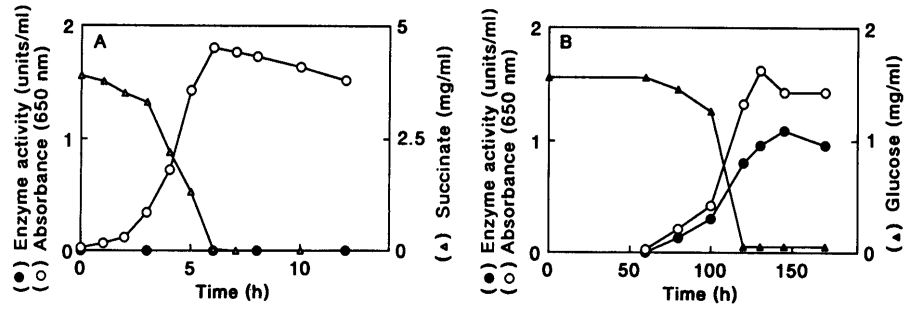

Fig. 1. Time Courses of the Growth of and PHB Depolymerase Secretion by *A. faecalis* T1

A. faecalis T1 cells were grown at 30 °C in a medium containing 0.4% (w/v) sodium succinate (A) or 0.15% (w/v) glucose (B) as the carbon source as described under Experimental. At time intervals, aliquots of the culture were taken and examined as to the absorbance at 650 nm (○), and then centrifuged at 10000 × *g* for 10 min. The PHB depolymerase activity (●), and the amounts of succinate (△, A) and glucose (▲, B) in the supernatant were determined as described under Experimental.

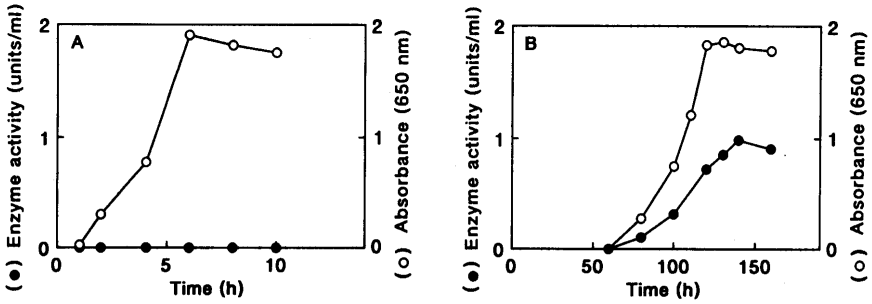

Fig. 2. Effect of the Carbon Source on the Growth Profile of *A. faecalis* T1

3 ml of culture fluid prepared under the conditions in Fig. 1B was transferred to the succinate medium and then grown at 30 °C (A). Cells grown under the conditions in (A) (3 ml of the culture fluid) were then transferred to the glucose medium and grown at 30 °C (B). At time intervals, aliquots of each culture fluid were taken and examined as to the absorbance at 650 nm (○), and then centrifuged at 10000 × *g* for 10 min. Then the PHB depolymerase activity in the supernatant (●) was determined.

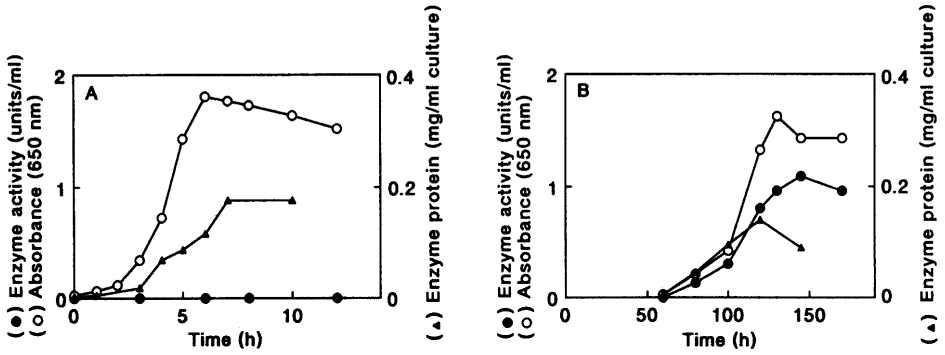

Fig. 3. Time Courses of the Cellular Level of PHB Depolymerase of *A. faecalis* T1 Grown in the Succinate or Glucose Medium

A. faecalis T1 cells were grown at 30 °C in the medium containing 0.4% (w/v) sodium succinate (A) or 0.15% (w/v) glucose (B). At time intervals, aliquots of the culture fluid were taken and examined as to the absorbance at 650 nm (○), and then centrifuged at 10000 × *g* for 10 min. The PHB depolymerase activity in the supernatant (●) and the amount of PHB depolymerase protein in the precipitate (▲) were determined.

TABLE II. Localization of PHB Depolymerase in *A. faecalis* T1

Fractions	Activity (%)		
	G-6-P dehydrogenase	5′-Nucleotidase	PHB depolymerase
Glucose medium			
Culture supernatant	0	0	17
Shock fluid	7	74	1
Cytosol	93	25	2
Membrane	0	1	80
Succinate medium			
Culture supernatant	0	0	0
Shock fluid	3	82	1
Cytosol	97	17	4
Membrane	0	1	95

Each fraction was prepared as described under Experimental and then subjected to the enzyme activity assays. PHB depolymerase was determined by means of an enzyme-linked immunosorbent assay as described under Experimental.

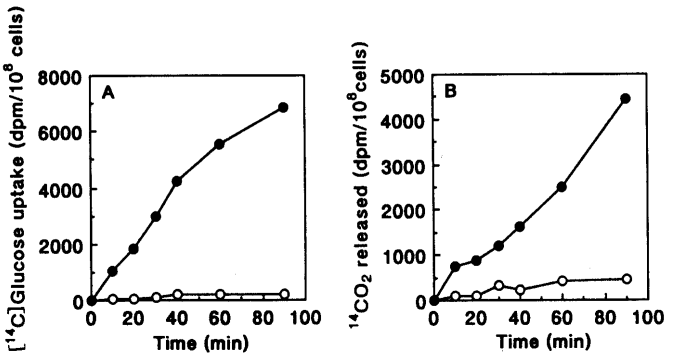

Fig. 4. Uptake of [^{14}C]Glucose and Release of $^{14}CO_2$ by *A. faecalis* T1 Cells

A. faecalis T1 cells grown to the logarithmic phase in the medium containing either 0.4% (w/v) sodium succinate (○) or 0.15% (w/v) glucose (●) were collected by centrifugation at 10000 × *g* for 10 min, and then washed twice with ice-cold 0.01 M Tris–HCl (pH 7.3) containing 0.03 M NaCl. Then [^{14}C]glucose uptake (A) and $^{14}CO_2$-release (B) experiments were carried out as described under Experimental.

exponentially (doubling time, 1 h), but the cells did not secrete PHB depolymerase into the culture medium (Fig. 1A). On the other hand, the cells which were inoculated into the glucose medium did not grow at once, there being a long lag time of about 60 h before the onset of cell multiplication. As the cells grew exponentially (doubling time, 10 h), glucose was utilized and PHB depolymerase was secreted into the culture medium (Fig. 1B). To examine whether or not cell mutation occurred during incubation, aliquots of glucose-grown cells were inoculated and grown in the succinate medium (Fig. 2A), and these succinate-grown cells were then transferred to the glucose medium (Fig. 2B). The results shown in Fig. 2 indicate that the profiles as to growth and PHB depolymerase secretion were almost the same as those in Fig. 1.

Although PHB depolymerase activity was not detected in the culture medium of the succinate-grown cells, the cellular level of this enzyme was found to be almost the same as that in the glucose-grown cells (Fig. 3). Even when the bacterium in the succinate medium was cultured up to 150 h, there was no detectable PHB depolymerase activity in the culture medium (data not shown).

Localization of PHB Depolymerase in *A. faecalis* T1 Next, we compared the enzyme distribution in *A. faecalis* T1 cells grown in the succinate medium with that in cells grown in the glucose medium. As shown in Table II, more than 74% of the activity of 5′-nucleotidase, the marker enzyme of the periplasmic fraction of gram-negative bacteria,[20] was detected in the shock fluid, only 1% of the total PHB depolymerase activity being found in this fraction of the cells. More than 80% of the total PHB depolymerase activity was found to be localized in the membrane fraction of the cells grown in both the succinate and the glucose medium. 17% of the total PHB depolymerase activity was recovered in the culture medium of the glucose-grown cells, however, the secretion of PHB depolymerase does not reflect rupturing of the cells because neither G-6-P dehydrogenase nor 5′-nucleotidase activity was found in the culture supernatant of the glucose medium (Table II).

Metabolism of Glucose by *A. faecalis* T1 Figure 4 shows the uptake and metabolism of [^{14}C]glucose by *A. faecalis* T1 cells. The cells grown in the glucose medium incorporated [^{14}C]glucose (Fig. 4A) and oxidized it, releasing $^{14}CO_2$ (Fig. 4B), while in the case of succinate-grown cells, [^{14}C]glucose was not metabolized during incubation for 90 min.

Then we examined whether or not *A. faecalis* T1 cells grown in the succinate medium have several glycolytic enzyme activities. It was found that specific activity values of G-6-P dehydrogenase and hexokinase were almost the same as those in the glucose-grown cells, respectively (Table III). On the other hand, the glucose-grown cells exhibited more than four-times higher D(−)-3-hydroxybutyrate dehydrogenase activity and nine-times lower isocitrate

TABLE III. Activities Related to Glucose Metabolism in *A. faecalis* T1

	Cells from	
	Glucose medium (units/mg)	Succinate medium (units/mg)
G-6-P dehydrogenase	0.057	0.056
Hexokinase	0.061	0.053
D(−)-3-Hydroxybutyrate dehydrogenase	0.322	0.073
Isocitrate dehydrogenase	0.011	0.096
Malate dehydrogenase	0.322	0.503

A. faecalis T1 cells grown in the glucose medium for 130 h or grown in the succinate medium for 6 h were collected by centrifugation at 10000 × *g* for 10 min and then suspended in 4 volumes of 10 mM Tris–HCl (pH 7.5) containing 1 mM 2-mercaptoethanol. After sonication for 5 min, the homogenates were assayed for the enzyme activities and protein concentration.

dehydrogenase activity than those grown in the succinate medium.

Discussion

In this study, we examined some characteristics of the secretion of PHB depolymerase by *A. faecalis* T1, and showed that whether or not this bacterium secretes PHB depolymerase is dependent on the carbon source species in the culture medium. When a carboxylic acid such as acetate or pyruvate, or an intermediate of the tricarboxylic acid cycle was used as a sole carbon source in the culture medium, the growth rate of the cells was relatively high (especially with succinate), but the enzyme secretion did not occur. Conversely, in the glucose medium, a long lag time was necessary before the onset of the growth, and the growth rate was low, whereas the cells secreted PHB depolymerase into the culture medium (Table I and Fig. 1). Interestingly, the cells grown in the succinate medium also synthesized PHB depolymerase in the cytoplasm, like the glucose-grown cells, suggesting that the succinate-grown cells lack the ability of the translocation of PHB depolymerase across the inner or outer membrane (Fig. 3). It seems unlikely that mutation of the cells had occurred in the glucose medium during the long lag time, since the profiles as to growth and PHB depolymerase secretion of the cells easily changed, depending on the carbon source added to the culture medium (Fig. 2), and the growth profiles of the cells were always reproducible.

Recently, we showed that in transformed *E. coli* cells carrying the PHB depolymerase gene from *A. faecalis* T1, a considerable amount of the expressed enzyme was localized in the periplasmic space of the cells, although a fraction of the enzyme was secreted into the culture medium.[5] According to the results in Table II, however, the majority of PHB depolymerase was found in the membrane fraction of *A. faecalis* T1 cells grown in the glucose or succinate medium, and thus the enzyme does not appear to be localized in the periplasmic space of the cells. Since the PHB depolymerase has a characteristic amino acid composition with very few charged amino acids[5] and a quite hydrophobic nature,[21] it is possible that the enzyme is attached to the membrane of *A. faecalis* T1 cells through a hydrophobic interaction. However, the membrane fraction in which the enzyme is localized has not been determined, since the separation of the outer and inner membrane fractions of *A. faecalis* T1 cells has not been successful yet.

Since succinate-grown *A. faecalis* T1 cells could not incorporate [^{14}C]glucose or oxidize it to $^{14}CO_2$ (Fig. 4), it appears that these cells have no ability to utilize glucose. Considering the apparent lack of alteration in the activities of several glycolytic enzymes (Table III), succinate-grown cells might have no glucose-transporter activity, and it is possible that some protein(s) involved in such activity may be induced in the bacterium on incubation of the succinate-grown cells in the glucose medium.

As shown in Table I and our previous paper,[4] *A. faecalis* T1 was able to grow in a medium containing water-insoluble PHB as a sole carbon source and to secrete PHB depolymerase into the growth medium. In addition, D(−)-3-hydroxybutyric acid and acetoacetic acid, further metabolites of PHB, also caused bacterial growth and PHB depolymerase secretion. These results show that in the basal salt medium supplemented with PHB as a sole carbon source, a trace amount of PHB depolymerase leaked into the medium or the enzyme localized on the surface of the cells would have hydrolyzed PHB, liberating D(−)-3-hydroxybutyrate, which functions as an energy source for cell growth and as a signal for the enzyme secretion.

D(−)-3-Hydroxybutyrate dehydrogenase, which catalyzes the oxidation of D(−)-3-hydroxybutyrate into acetoacetate, was detected in several bacteria which could grow on PHB as an exogenous carbon source.[13] As shown in Table III, D(−)-3-hydroxybutyrate dehydrogenase activity of the glucose-grown cells was much higher than that of the succinate-grown cells. Thus, it is possible that ketone bodies such as D(−)-3-hydroxybutyrate and/or acetoacetate may play the role as an inducer for PHB depolymerase secretion by the cells. Although the ketone body concentration in *A. faecalis* T1 cells has not been determined yet, it is likely that the accumulation of acetoacetate or D(−)-3-hydroxybutyrate occurs in the glucose-grown cells, since the low isocitrate dehydrogenase activity should cause a high level of acetyl-CoA in the cells.

Further investigation is surely needed to determine how the onset of cell growth in the glucose medium occurs after a long lag time and how ketone bodies act as an inducer of PHB depolymerase secretion.

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