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RESEARCH ARTICLE

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Evidence of an association between poly(3-hydroxybutyrate) accumulation and phosphotransbutyrylase expression in *Bacillus megaterium*

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Abstract Molecular analysis of a genomic region of *Bacillus megaterium*, a polyhydroxybutyrate (PHB)-producing microorganism, revealed the presence of a gene coding for the enzyme phosphotransbutyrylase (Ptb). Enzyme activity was measured throughout the different growth phases of *B. megaterium* and was found to correlate with PHB accumulation during the late-exponential growth phase. Ptb expression was repressed by glucose and activated by the branched amino acids isoleucine and valine. Overexpression of Act_{Bm}, a σ^{54} regulator from *B. megaterium* whose gene is located upstream from *ptb*, caused an increase in Ptb activity and PHB accumulation in *B. megaterium*.

Keywords *Bacillus megaterium* · Polyhydroxybutyrate Phosphotransbutyrylase

Introduction

The presence and/or expression of genes involved in the synthesis of ATP at the substrate level, including those coding for the enzymes phosphotransacetylase (Pta), acetate kinase (Ack), phosphotransbutyrylase (Ptb), and butyrate kinase (Buk), have been reported for the model organism of the *Bacillus* genus, *Bacillus subtilis* [6].

In previous experiments, we cloned and molecularly analyzed a 2.53-kb region of the *B. megaterium* chromosome, in which three complete open reading frames (ORFs) were identified: *actBm*, ORF2, and *ptb*. The sequence of ORF2 was found to be homologous to that of sensory transduction histidine kinases [16]. *actBm* encodes an activator homologous to σ^{54} regulators that is able to activate *in trans* the *Escherichia coli* genes for the degradation of short-chain fatty acids [12]. This regulator is highly homologous in structure and function to the BkdR regulator of *B. subtilis*, involved in branched amino acids degradation [3]. The third ORF [16] revealed extensive similarity with *ptb* genes [17]. In *Clostridium acetobutylicum* and other *Clostridium* species, the product of *ptb* is involved in butyric acid synthesis from butyryl-CoA by means of a pathway that results in the production of butyryl phosphate followed by the generation of ATP and butyrate [5]. *B. megaterium*, unlike *B. subtilis*, is a polyhydroxybutyrate (PHB) producer. PHB is a member of the family of the polyhydroxyalkanoates, biodegradable plastics and elastomers, that accumulate in several bacterial species under growth conditions characterized by an excess of carbon source and the lack of one or more essential nutrients. PHB acts as a carbon storage compound and as a sink for reducing equivalents [11]. Previous research has shown that low extracellular pH correlates with the start of PHB accumulation in this species [7]. The enzyme phosphotransbutyrylase is involved in butyrate excretion in *Clostridium,* and as a consequence in the acidification of the medium. In addition, butyrylCoA, a possible intermediate in PHB synthesis, is also a substrate of the Ptb enzyme. Thus, we studied expression of this enzyme in order to analyze its possible relation to PHB production.

This work describes *ptb* expression in *B. megaterium* throughout the different phases of growth and in association with PHB accumulation as well as acidification of the culture medium.

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Materials and methods

Strains and growth conditions

The bacterial strains and plasmids used in this study were: plasmid pS14 [16] containing the act_{Bm} gene in pAT18 [14], *B. megaterium* PV447 [15], and its pS14 transconjugant. *B. megaterium* was grown either in supplemented nutrient broth (SNB)[15], or in minimal medium (MM) [4] supplemented with glucose or gluconate as indicated. SNB contains 5 g glucose/l, unless otherwise indicated. The concentrations used were: glucose and gluconate 5 g/l, valine 1.5 g/l, and isoleucine 3.5 g/l. The medium contained sufficient ammonium so that amino acids were not utilized as a nitrogen source. Ptb specific activity and PHB were measured under each growth condition. Samples were taken at the early stationary growth phase. Cultures of *B. megaterium* PV447 and of the same strain bearing pS14, a recombinant multicopy plasmid containing act_{Bm}, were grown in SNB supplemented with 20 g glucose /l until the early stationary phase of growth.

Analytical procedures

PHB and short-chain fatty acids were determined with a Gow Mac Series 580 gas chromatograph equipped with a flame ionization detector. PHB was esterified as previously described [1], and the resulting methyl esters were run in a 1.80-m glass column packed with Carbowax 20M-TPA on Chromosorb WAW (80/100mesh). Short-chain fatty acids were measured by acidifying the culture supernatants with H_2SO_4 (167 g/l final concentration) followed by extraction with diethyl ether. The corresponding ethyl esters were separated in a 1.80-m glass column packed with 10% Sp 1000-1% H_3PO_4 on Chromosorb WAW (100/120mesh). Determinations were repeated twice in two parallel cultures.

Five ml of bacterial cultures, grown as indicated in each experiment, were used for crude extract preparations following procedures previously described [12]. Ptb activity was determined as described [2], following the increase in A_{412} due to formation of the complex DTNB-CoA ($\epsilon = 1.36\times10^4$/M×cm) after liberation of CoA-SH from butyryl-CoA. Proteins were measured according to the method described in [10], with bovine serum albumin as the standard. Measurements were repeated twice in two parallel cultures.

Results and discussion

Kinetics of Ptb activity in *B. megaterium*

As mentioned above, low pH values of the medium are associated with the start of PHB accumulation in SNB [7]. Thus, in accordance with the functions predicted from the sequence of the *B. megaterium* region analyzed, a decrease in the pH of the medium was expected to correlate with an increase in Ptb activity. These two parameters were measured throughout the exponential and stationary phase of growth. The results are shown in Fig. 1. Enzyme activity and PHB accumulation started when the pH of the medium reached its lowest value, and were highest during stationary phase. Acetic acid was the only short-chain fatty acid detected extracellularly. These results suggest either that short-chain fatty acids other than acetate were not synthesized in detectable amounts or that they were immediately metabolized.

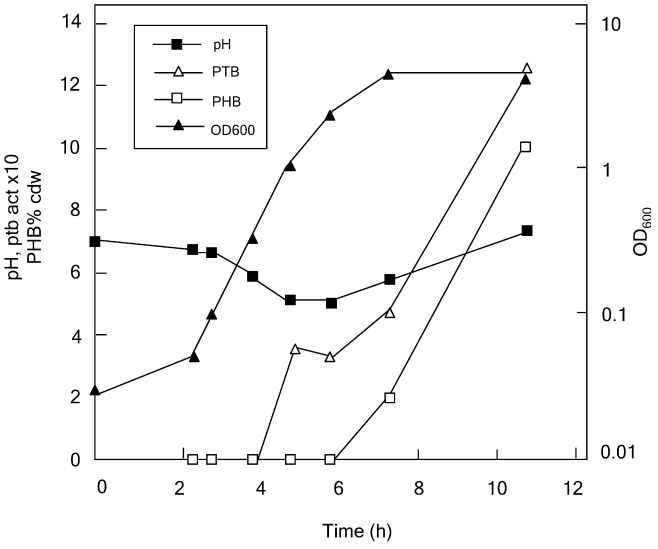

Fig. 1 Ptb expression, PHB accumulation and pH of the medium throughout the growth of *Bacillus megaterium* in supplemented nutrient broth

The addition of 50 mM butyrate to late-exponential phase cultures of *B. megaterium* in SNB caused a 68% reduction of Ptb activity (data not shown). Therefore, the possible metabolism of butyrate might proceed by a pathway not involving this enzyme.

Effect of branched amino acids and carbohydrates on PHB accumulation

Branched amino acids were found to induce the expression of *ptb*, as determined by using *lac* genetic fusions in *B. subtilis* [3]. In order to study the induction of Ptb synthesis and its possible influence on PHB content, *B. megaterium* was grown in MM supplemented as indicated in Materials and methods. This medium contained sufficient ammonium so that amino acids were not used as a nitrogen source. Ptb specific activity and PHB were measured under each growth condition (Table 1).

The results showed that the increase in enzyme activity caused by the addition of branched amino acids correlated with an increase in PHB accumulation. Branched short-chain fatty acids were not detected extracellularly, as in previous experiments.

Effect of Act_{Bm} on PHB accumulation

The results indicated a correlation between PHB accumulation and Ptb activity. In order to exclude the possibility that the increase in PHB content was solely due to the use of the amino acids as a carbon source, Ptb activity was induced without the addition of its natural inducers. It was predicted that overexpression of ActBm, an activator of Ptb synthesis, would as a consequence

Table 1 Effect of branched amino acids on phosphotransbutyrylase expression and polyhydroxybutyrate (PHB) accumulation. *Bacillus megaterium* was grown in minimal medium (MM) supplemented with carbon sources and amino acids

Growth medium	Ptb specific activity (U/mg protein)	PHB (% cell dry weight)
MM + glucose	791	8.83
MM + glucose, valine, and isoleucine	6,616	19.05
MM + gluconate	5,183	11.58
MM + gluconate, valine, and isoleucine	43,555	31.05

Table 2 Effect of ActBm on PHB accumulation in *B. megaterium* grown in SNB supplemented as described

Plasmid	Ptb specific activity (U/mg protein)	PHB (% cell dry weight)
None	17,190	12.4
pS14	23,520	22.3

increase the intracellular concentration of PHB. *B. megaterium* strain PV447 and a transconjugant of this strain containing plasmid pS14 were grown in SNB supplemented with 20 g glucose/l. The results shown in Table 2 indicate that the strain carrying act_{Bm} in a multicopy plasmid had higher Ptb activity and a higher PHB content than the wild-type strain.

These experiments suggest an association between Ptb activity and PHB accumulation in *B. megaterium* for the following reasons: (1) *ptb* expression and polymer accumulation occur simultaneously during growth; (2) branched amino acids, which are inducers of enzyme synthesis, enhance PHB accumulation; (3) multiple copies of act_{Bm} activate *ptb* expression and, at the same time, enhance PHB production, in accordance with the results obtained after induction of the enzyme with branched amino acids. *ptb* from *C. acetobutylicum* has been used for PHB production in *E. coli* recombinants [8].

Enzyme activity and PHB accumulation increased from the mid-exponential to the stationary phase of growth. These findings are in line with what is known about the transition from the exponential to the stationary phase in the sporulating model organism *B. subtilis*. This transient state, previous to sporulation, is characterized by the expression of a variety of genes that are necessary for the scavenging of nutrients [13]. We have previously shown that *B. megaterium* PHB^- mutants commit to sporulation earlier than the wild-type strain [9]. Thus, PHB, as a carbon and energy source, allows this species to remain in the vegetative state longer. Therefore, it is possible that both Ptb expression and PHB accumulation are included in the global regulation system characteristic of the transient state of *Bacillus*.

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