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REVIEW PAPERS

Biosynthesis of Poly(3-Hydroxybutyrate) and Poly(3-Hydroxybutyrate-*co*-3-Hydroxyvalerate) and Its Regulation in Bacteria

Yu. A. Trotsenko and L. L. Belova

Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, pr. Nauki 5, Pushchino, Moscow oblast, 142290 Russia

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Abstract—Recent data on the biosynthesis of poly(3-hydroxybutyrate) (PHB) and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) and its regulation in bacteria are reviewed, with special emphasis on the properties and regulation of the relevant enzymes and their genes. Some conditions promoting the synthesis of PHB and PHBV by natural, mutant, and recombinant producers are considered.

1. Poly(3-Hydroxybutyrate) and Poly(3-Hydroxybutyrate-co-3-Hydroxyvalerate) Producers

After Lemoigne had discovered poly(3-hydroxybutyrate) (PHB) as a storage compound of *Bacillus megaterium* in 1926, 3-hydroxybutyric acid (3HBA) remained the only known constituent of bacterial polyhydroxyalkanoates (PHAs) until other 3HBA constituents (hydroxyvaleric, hydroxyhexanoic, and hydroxyoctanoic acids) were reported in the seventies [1–3]. In this review, we limited ourselves to a discussion of the synthesis of PHB and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) and its regulation in bacteria.

By now, more than 100 bacterial species have been found to synthesize PHB(V) (stands for both PHB and PHBV) as reserve polymers in response to nutritional imbalance caused by a deficiency of the sources of nitrogen, phosphorus, potassium, magnesium, and other elements, and an excess of carbon sources in the medium [1–3].

Most studies on the synthesis of PHB(V) were performed using the *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) mutant that utilizes glucose and accumulates up to 80% PHAs. PHB and PHBV, which are valuable thermoplastic and biodegradable polyesters, are commercially produced by ICI, albeit in limited quantities because of their relatively high production cost (about $15/kg) [1–3].

Recombinant PHB(V) producers, which lack ordinary regulatory mechanisms, appear to have considerable promise in industry. Recently, genes involved in PHB synthesis have been cloned in prokaryotic and eukaryotic organisms, including transgenic plants. The recombinant clones of *Escherichia coli* carrying the genes of *R. eutropha* are able to synthesize PHB when grown in mineral or complex media without sugars. The expression of relevant genes in these clones depends on temperature. The efficiency of PHB production by one of the recombinant *E. coli* clones reaches 3.4 g/(l h), with a relative cellular content of this polymer amounting up to 75% [4–10].

Aerobic methylotrophs, especially those implementing the serine pathway of C_1 metabolism, have also shown promise in producing PHB [12, 13]. Serine methylobacteria cultivated in batch mode are able to synthesize high-chain-length PHB (1300–1800 kDa) with a yield of 0.2 g/g methanol and a cellular content of 25–35% (50–55% at the most) [14]. The maximum efficiency of PHB production by methylotrophs was observed in a continuous culture of *Hyphomicrobium zavarzinii* (0.64 g/(l h)) with a cellular PHB content of 40 to 59% [15]. *Methylobacterium extorquens* K cells cultivated in batch mode with a controlled C/N ratio accumulate up to 60% of PHB, with a yield of 0.2 g/g methanol [16]. Recombinant *Mycoplana rubra* strains synthesize 2.5 times more PHB than the parent strain [17].

In practice, PHB production is commonly improved by using a mixture of two substrates, one of which serves as the carbon source and the other as the energy source. Theoretically, mutant serine methylobacteria can convert up to 80% of the carbon atoms of the glucose-(hyphen) acetate and acetate-(hyphen) methanol substrate pairs to PHB.

Bacteria of the genus *Paracoccus* with a ribulose bisphosphate pathway synthesize PHB in lower amounts than serine methylobacteria, but in greater amounts than methylobacteria with a ribulose monophosphate pathway [12]. The cellular content of PHB in a continuous culture of *P. pantotrophus* with the fractional addition of acetate was 36%. The use of methanol in a mixture with amyl alcohol increased the content of PHBV in *P. denitrificans* cells to 58% and its yield to 0.97 g/g substrate [18].

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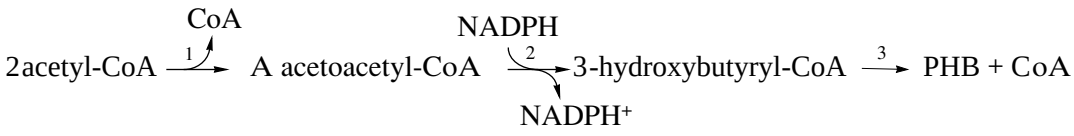
2. Metabolic Pathways Involved in PHB Synthesis and Degradation

PHB is synthesized through the condensation of two acetyl-CoA molecules into acetoacetyl-CoA, its reduction to hydroxybutyryl-CoA, and the polymerization of the latter. Generally, PHB is synthesized in a way similar to that of fatty acids except that: (1) PHB intermediates are CoA derivatives but not acyl-carrier proteins; (2) PHB synthesis is specific for the stereoisomer D(-)-3-hydroxybutyrate and, in most cases, for NADPH; (3) the enzymes involved in PHB synthesis are not assembled in a complex; and (4) the polymerization of hydroxybutyrate occurs at the PHB granule–cytoplasm interface [1, 2].

Most of the prokaryotes studied synthesize PHB from acetyl-CoA under the action of β-ketothiolase (acetyl-CoA C-transferase, EC 2.3.1.9), acetoacetyl-CoA reductase (hydroxybutyryl-CoA dehydrogenase, EC 1.1.1.36), and poly(3-hydroxybutyrate) synthase by the following reaction:



Acetoacetyl-CoA reductase exists in two forms, one of which is specific for NADPH and the other, for NADH. Correspondingly, the reaction product (3-hydroxybutyryl-CoA) has either D(-) or L(+) configuration. It is believed that PHB is synthesized with the involvement of NADPH-dependent D(-)-3-hydroxybutyryl-CoA oxidoreductase. If L(+) reductase is involved, two enoyl-CoA dehydratases catalyze the conversion of L(+)-3-hydroxybutyryl-CoA to D(-)-3-hydroxybutyryl-CoA (via crotonyl-CoA), which is polymerized into PHB. The five-step synthesis of PHB was first studied in *Rhodospirillum rubrum.* Highly active enoyl-CoA dehydratases were revealed in *Methylobacterium rhodesianum* [20].

PHB metabolism is a cyclic process in which the proportion between biosynthetic and degradative reactions is determined by growth conditions. PHB is degraded under the action of the respective depolymerase, 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30), and succinyl-CoA : acetoacetyl transferase (EC 2.8.3.5). The cycle is closed at the level of β-ketothiolase, which converts acetoacetyl-CoA into acetyl-CoA. In *Zoogloea ramigera*, the acetoacetate produced from PHB is esterified by CoA with the involvement of acetoacetyl-CoA synthetase [1].

3. Properties of Enzymes Involved in PHB Synthesis

3.1. β-Ketothiolase. Three β-ketothiolase isoenzymes, which differ in their specificity for substrates with different lengths of carbon chains, catalyze the reversible transfer of activated acetyl group to acyl-CoA. It is believed that the isoenzyme that cleaves β-ketoacyl-CoA molecules containing 4–16 carbon atoms in the backbone chain (for instance, the B form of *R. eutropha* β-ketothiolase or the isoenzyme 2 of *Z. ramigera*) is involved in the β-oxidation of fatty acids [21–26].

The isoenzyme that is specific for shorter substrates (for instance, the A form of *R. eutropha* β-ketothiolase and some other enzymes listed in Table 1) plays a biosynthetic role. In particular, β-ketothiolases specific for C_2 and C_4 substrates catalyze the reactions of condensation and thiolysis, respectively. The third isoenzyme occurs in the eukaryotes that synthesize acetoacetyl-CoA as a precursor to steroids [1].

Various β-ketothiolases possess similar physicochemical properties (Table 1). Typically, they are homotetramers with a molecular mass of 160–190 kDa, whose subunits have a molecular mass of 40–42 kDa. Although the physiological role of β-ketothiolase is to form the acetoacetyl-CoA necessary for PHB synthesis, this enzyme is most active in the direction of thiolysis: its affinity to the substrates of thiolysis is two orders higher than to acetyl-CoA. The cysteine and probably the histidine residues of the active centers of various β-ketothiolases are conservative and, therefore, are presumably necessary for catalysis [26]. Even at low concentrations (<30 μM), CoA inhibits the condensation reaction catalyzed by β-ketothiolases. Reduced pyridine nucleotides are inhibitory to the β-ketothiolases of *Z. ramigera*, *M. rhodesianum*, and *M. extorquens*, while oxidized pyridine nucleotides are inhibitory to the β-ketothiolase of *Azotobacter beijerinckii*. All β-ketothiolases are strongly inhibited by the sulfhydryl alkylating agents *N*-ethylmaleimide and iodoacetamide. The β-ketothiolase genes of various PHB producers are highly homologous, which suggests the significance of β-ketothiolase for PHB synthesis [27].

3.2. Acetoacetyl-CoA reductase. Acetoacetyl-CoA reductase (ACR) is the second enzyme involved in PHB synthesis, which reversibly reduces acetoacetyl-CoA to hydroxybutyryl-CoA with reduced pyridine nucleotides as the donors of hydride ions. The ACRs of *Az. beijerinckii* [28], *Rhodopseudomonas sphaeroides*, *Rhodomicrobium vannielii*, and *Streptomyces coelicolor* do not exist in multiple forms, while the ACRs of *R. eutropha*, *Az. vinelandii* UWD, *Z. ramigera*, *M. rhodesianum* and *M. extorquens* may occur in two isoforms [29–34]. NADH-dependent ACR is active with both L(+)- and D(-)-3-hydroxyacyl-CoA with different

Table 1. Some properties of various bacterial β-ketothiolases

Microorganism and ref.	Molecular mass, kDa	$K_m^{A\text{-}CoA}$, μM	Optimum pH	Inhibitors of	
				condensation (K_i, μM)	thiolysis
A. eutrophus [22]					
A form	170.5	1.1×10^{-3}	5.0	CoA (16)	No
B form	167.5	0.23×10^{-3}	6.4	CoA	NAD(H)
A. latus [1]	170	0.29×10^{-3}	8.3–8.0	CoA (20)	–
Z. ramigera [1]					
Isoenzyme 1	190	0.33×10^{-3}	7.5–8.5	CoA	NAD(P)H
Isoenzyme 2	162		4.4		
Az. beijerinckii [48]	–	–	7.3	CoA	NAD(P)
M. rhodesianum [54]	160	0.5×10^{-3}	7.5	CoA (20)	NAD(P)H

Note: "–" stands for "no data available."

lengths of carbon chain and produces L(+)-hydroxybutyryl-CoA. At the same time, NADPH-dependent ACR is active with C_4–C_6 D(-)-3-hydroxyacyl-CoA, but not with L(+) stereoisomers, and produces D(-)-hydroxybutyryl-CoA. The specificity of NADH-dependent ACR for NADH is less pronounced than the specificity of NADPH-dependent ACR for NADPH.

In vitro experiments with purified β-ketothiolase, ACR, and PHB synthase showed that only NADPH-dependent ACR is involved in PHB synthesis from acetyl-CoA, while NADH-dependent ACR participates in the synthesis and oxidation of fatty acids [22, 29]. In *Rsp. rubrum*, the five-step synthesis of PHB occurs with the involvement of NADH-dependent ACR and two stereospecific dehydratases reversibly converting L(+)-hydroxybutyryl-CoA to D(-)-hydroxybutyryl-CoA [20]. The role of highly active stereospecific crotonases in *M. rhodesianum* cells [32] with the three-step PHB synthesis remains unclear, although it is believed that the implementation of the five-step or three-step PHB synthesis may depend on the cellular level of NADH and NADPH.

The main properties of NADPH-dependent ACRs are presented in Table 2. The molecular masses of the native ACRs of *R. eutropha*, *Az. vinelandii* UWD, and *Z. ramigera* are lower than that of *M. rhodesianum* ACR, although the molecular masses of the subunits are nearly the same. The specificity of the NADPH-dependent ACR of *R. eutropha* for NADH is an order of magnitude lower than for NADPH. All NADPH-dependent ACRs are inhibited by high concentrations of acetoacetyl-CoA and have two to four interacting binding sites. The NADPH-dependent ACRs of *Az. vinelandii* UWD and *M. rhodesianum* are also inhibited by NADPH and $NADP^+$, respectively. Other ACR effectors are still unknown.

3.3. PHB synthases. The substrate specificity of synthases which catalyze the polymerization of D(-)-3-hydroxybutyryl-CoA into PHB determines the composition of the resulting polymer. At present, three types of PHB synthases are known, which differ not only in substrate specificity but also in molecular properties [35]. Synthases of the first type (e.g., the PHB synthases of *R. eutropha*, *Z. ramigera*, and *Synechococcus* sp. MA19) are specific for the CoA-derivatives of short-chain hydroxy acids with 3–5 carbon atoms. Synthases of the second type, which are typical of pseudomonads, are specific for the CoA-derivatives of medium-chain-length hydroxy acids with 6–14 carbon atoms. Both types of PHB synthases are encoded by the same *phaC* gene. PHB synthase of the third type was revealed only in the phototrophic bacterium *Chromatium vinosum*. The substrate specificity of this synthase resembles that of the first type enzymes, but its expression requires two genes, *phaC* and *phaE*.

PHB synthases either occur in soluble form or are bound to PHB granules. It remains unclear whether these two forms of PHB synthases are completely identical. Soluble synthases are usually unstable during purification, except for the soluble PHB synthase of *R. eutropha* expressed in the recombinant clone of *Escherichia coli* [37, 38]. Synthases bound to PHB granules are more stable, but they have not yet been obtained in a homogeneous (soluble) state. If PHB synthesis occurs in vivo. The PHB synthase of *Z. ramigera* is associated with newly synthesized PHB granules. However, under conditions promoting PHB degradation, the activity of PHB synthase is higher in the soluble fraction of cells. Similar results were obtained in the immunocytochemical studies of PHB metabolism in *R. eutropha* and *C. vinosum* cells: the distribution of labeled antibodies between the PHB granules and cytoplasm of cells depended on whether PHB was synthesized or degraded [37–39].

The main characteristics of PHB synthases are summarized in Table 3. The molecular mass of the native *R. eutropha* enzyme in different preparations varies

Table 2. Some properties of various bacterial NADPH-dependent acetoacetyl-CoA reductases

Microorganism and ref.	Molecular mass of enzyme and subunit, kDa	K_m, μM		Inhibitors
		NADPH	Aa-CoA	
Al. europhus [29]	84 (23)	0.05	5	Aa-CoA
Az. vinelandii UWD [53]	77	20	11	Aa-CoA, NADPH
Z. ramigera [1]	92 (25.5)	21	8.3	Aa-CoA
M. rhodesianum [33]	250 (42)	18	15	Aa-CoA, $NADP^+$
M. extorquens [34]	140 (32)	41	12	Aa-CoA, $NADP^+$

Note: Aa-CoA is acetoacetyl-CoA.

Table 3. Some properties of various bacterial PHB synthases

Microorganism and ref.	Enzyme form	Molecular mass, kDa	Optimum pH	K_m, mM	Active group
A. eurtophus [35]	soluble	160	8.0–10.0	0.72	–SH
	granule-bound	–	>10.0	0.68(1.63*)	–SH
E. coli UT5600 (pKAS4) [38]	soluble monomeric	64	–	–	Cys 319, Ser 260
C. vinosum, *phaC*, *phaE* [39]	soluble complex	390 400	7.8	63×10^{-3}	–
Z. ramigera [1]	soluble	–	7.0	53×10^{-3}	–
	granule-bound	–	7.0	0.21	–
Bacillus megaterium [1]	granule-bound	–	7.5	92.5×10^{-3}	–SH
Synechococcus sp.MA19 [1]	membrane	–	–	35×10^{-3}	–

Note: K_m values for C_4 and C_5 substrates.

considerably, probably because of the influence of covalently bound PHB granules. The molecular mass of PHB synthase from the recombinant *E. coli* strain transformed with the *R. eutropha* PHB synthase gene is considerably lower. This enzyme dissociates in solution to catalytically active mono- or dimeric subunits with molecular masses of 64 and 130 kDa, respectively [38].

It is believed that cysteine residues play a decisive role in the reaction of polymerization and that the post-translational modification of *R. eutropha* PHB synthase required for its activity lies in the binding of phosphopantetheine to serine-260. The proposed mechanism of polymerization reaction is similar to the operation mechanism of fatty acid synthase and involves the covalent catalysis of 3-hydroxy acid thioesters and growing polymeric primer. The initiation of primer formation is thought to be the limiting stage of PHB synthesis [38].

The PHB synthase of *C. vinosum* is more complex than that of *R. eutropha*. The former enzyme is composed of ten subunits of two types with molecular masses of 40 and 41 kDa, which are encoded by the *phaC* and *phaE* genes, respectively. The diameter of the enzyme complex is 11.2–12.8 nm. The proportions between different subunits in the enzyme complex is unknown, although it is believed that the *phaC*-encoded protein is a minor component of the enzyme [39]. The attempts to show that PHB synthase is regulated by effectors other than its substrate D(-)-3-hydroxybutyryl-CoA have been unsuccessful for a long time. Recently, however, it has been established that the PHB synthase of the cyanobacterium *Synechococcus* sp. MA19 is activated in vitro by acetylphosphate and that the degree of activation varies within the range of the physiological concentrations of acetylphosphate (up to 3 mM), which are determined by the cellular pool of acetyl-CoA [40]. In actuality, PHB synthase is controlled by phosphotransacetylase.

In vitro experiments with the recombinant PHB synthase of *C. vinosum* ($PhaEC_{Cv}$) allowed for the elucidation of some of the factors influencing the activity of this enzyme. These factors are the incorporation of D(-)-3-hydroxybutyryl-CoA into PHB and the PHB synthesis rate [36]. The presence of some salts ($MgCl_2$, $CaCl_2$, and NaCl), proteins (BSA, lysozyme, and phasin), and the detergent Tween 20, enhanced the incorporation of D(-)-3-hydroxybutyryl-CoA into PHB by 2.5 times, although only some of these factors affected

the activity of $PhaEC_{Cv}$. Increased salt concentrations sometimes inhibited PHB synthase without changing the degree of incorporation of D(-)-3-hydroxybutyryl-CoA into PHB. The oxidized pyridine nucleotides NAD^+ and $NADP^+$, at a concentration of 2 mM, completely inhibited $PhaEC_{Cv}$, while the reduced pyridine nucleotides NADH and NADPH were non-inhibitory. The incubation of $PhaEC_{Cv}$ with D(-)-3-hydroxybutyryl-CoA and $MgCl_2$ gave rise to macroscopic PHB granules. The size and the form of the PHB granules synthesized in vitro depended on the concentration of the $PhaEC_{Cv}$, BSA, and GA24 proteins (the protein of *R. eutropha* associated with PHB granules). The activity of PHB synthase was also found to control the molecular weight and the degree of PHB dispersion in vivo [1].

4. Metabolic Control of PHB Synthesis

4.1. Ralstonia eutropha. All three enzymes of this bacterium involved in PHB synthesis are constitutive and, hence, can be found in actively growing cells. It is believed that the expression of genes involved in PHB synthesis is not regulated at the transcriptional or translational levels but is controlled by the activities of pyruvate dehydrogenase, β-ketothiolase, and citrate synthase, i.e., by the enzymes whose substrate or product is acetyl-CoA [1–3, 22, 29, 41].

It is well known that PHB serves as a cellular reserve of carbon and NAD(P)H. If PHB is not synthesized with the consumption of acetyl-CoA, the oxidation of the latter compound in the Krebs cycle leads to increased production of reducing equivalents and high-energy compounds [1–3, 44]. This will result in a decrease in the activities of some enzymes, and accumulation of cellular metabolites and their excretion into the medium. Pyruvate dehydrogenase complex is subject to stringent feedback regulation by NADH. As a result, batch cultivated *R. eutropha* cells accumulate pyruvate in small amounts (<12 μmol/g cells) [44]. At the same time, mutants with blocked PHB synthesis grown on the gluconate, fructose, lactate, or $CO_2 + H_2 + O_2$ mixture excrete great amounts of pyruvate and, under oxygen deficiency, also excrete β-hydroxybutyrate and some intermediates of the Krebs cycle into the medium. Nongrowing *R. eutropha* cells occurring under conditions of excess glucose or lactate and of nitrogen deficiency synthesize PHB with the concurrent excretion of pyruvate. Some authors believe that PHB synthesis and pyruvate excretion are alternative processes regulated by the cellular level of NADH [42].

In *R. eutropha* mutants with impaired PHB synthesis, the cellular content of acetyl-CoA is high and that of CoA is low. The deficiency of CoA necessary for pyruvate dehydrogenase may be responsible for the accumulation and excretion of pyruvate by these mutants [43].

The role of reduced pyridine nucleotides in the regulation of PHB synthesis has been demonstrated in experiments with *R. eutropha* cells continuously cultivated at $\mu = 0.1\ h^{-1}$. During growth on glucose and formate (the latter compound served as a supplementary source of NADH and/or ATP) under conditions of nitrogen deficiency, the content and the production rate of PHB (q_{PHB}), as well as the consumption rate of the growth substrate (q_s), decreased [42]. Low concentrations (≤ 5 μM) of the effective uncoupler of oxidative phosphorylation carbonyl cyanide m-chrolophenylhydrazone (CCCP) only slightly augmented the cellular content of PHB, q_{PHB}, and q_s. Higher CCCP concentrations considerably diminished the q_{PHB} and the cellular content of PHB, while q_s continued to increase. These data suggest that additional energy in the form of reduced pyridine nucleotides and/or ATP obtained from the oxidation of formate inhibits the consumption of glucose under nitrogen deficiency and thus diminishes q_{PHB} and the cellular content of PHB. CCCP stimulates the consumption of glucose due to uncoupling of the respiratory chain. However, the consumption of glucose in the presence of CCCP is energetically ineffective, and this explains the decrease in q_s and q_{PHB} [42].

The addition of formate to *R. eutropha* cells utilizing glucose and producing PHB led to an increase in q_{PHB} and q_s, obviously due to increased NADH production. The NADH produced diminishes the activity of citrate synthase and the cellular level of CoA, which is inhibitory to β-ketothiolase. Similarly, the intracellular pools of reduced pyridine nucleotides differ under growth and non-growth conditions. This explains the different effects of reduced pyridine nucleotides on the metabolism of *R. eutropha* [41, 42].

The transport of glucose and other substrates can also control PHB synthesis in *R. eutropha*. It was found that the rate of PHB production by nitrogen-deficient *R. eutropha* cells is proportional to the substrate consumption rate. Moreover, cells taken from a culture limited in glucose and lactate rapidly synthesize PHB under conditions of substrate excess and nitrogen deficiency [41].

Analysis of the intracellular concentrations of some metabolites in batch and continuously cultivated *R. eutropha* cells showed that the concentration of CoA in PHB-producing cells is three times higher than in the absence of PHB synthesis, while the concentration of acetyl-CoA is the same in these two cases [45]. The *R. eutropha* mutant defective in isocitrate dehydrogenase synthesized PHB from fructose even when the nitrogen content in the medium was high [46]. These data confirm the hypothesis that CoA plays a key role in the regulation of β-ketothiolase activity and PHB synthesis in *R. eutropha* cells.

The increase in the concentrations of acetoacetyl-CoA and hydroxybutyryl-CoA observed in *R. eutropha* cells immediately after the exhaustion of nitrogen in the medium and their decrease after the subsequent

exhaustion of cellular CoA clearly demonstrate that ACR is a regulatory enzyme, although the effectors of this enzyme in *R. eutropha* have not yet been revealed. These results suggest that NADH and CoA are the main metabolic regulators in *R. eutropha* cells, and control the activities of pyruvate dehydrogenase, citrate synthase, and β-ketothiolase.

4.2. Nitrogen-fixing bacteria. Bacteria of the genus *Azotobacter* synthesize PHB(V) when grown on polycarbon substrates under conditions of oxygen deficiency and, to a lesser degree, under conditions of phosphorus deficienag. Along with the formation of PHB, these bacteria also produce capsules and polysaccharides as storage substances. This diminished their significance as PHB producers and stimulated the search for mutants incapable of encystment. The production of PHB in one of these mutants, UWD, does not require oxygen deficiency and takes place in the exponential growth phase.

The regulation of PHB synthesis was studied in detail in *Az. beijerinckii*. The cells of this species grown in batch mode on 2% glucose accumulate up to 74% PHB by the end of the exponential growth phase [48]. After the exhaustion of glucose in the medium, PHB is rapidly degraded, but the cell biomass does not decrease.

In a chemostat culture of *Az. beijerinckii*, PHB synthesis is stimulated by oxygen deficiency. The mitigation of oxygen deficiency in the presence of excess glucose leads to a partial degradation of PHB [48]. In this case, the rates of O_2 consumption and CO_2 evolution increase rapidly, and the cells accumulate some intermediate products of glucose metabolism. These data were interpreted as indicating that the high $NADH/NAD^+$ ratio in cells grown under conditions unbalanced with respect to oxygen inhibits the enzymes of glucose catabolism and the Krebs cycle, thus suppressing the oxidation of acetyl-CoA in the Krebs cycle and stimulating its conversion into PHB. The mitigation of oxygen deficiency leads to the cessation of PHB synthesis and oxidation of acetyl-CoA in the Krebs cycle [49].

In *Az. beijerinckii*, synthesis of PHB is regulated by its intermediate products at the level of β-ketothiolase and ACR. In this case, acetoacetyl-CoA inhibits both of these enzymes, while acetyl-CoA inhibits only β-ketothiolase. High intracellular concentrations of acetyl-CoA and, correspondingly, low concentrations of CoA due to the limitation of the Krebs cycle, may lead to saturation of β-ketothiolase (K_m of this enzyme with respect to acetyl-CoA is 0.9 mM) and to a decrease in the inhibitory action of CoA with the concurrent initiation of PHB synthesis. Thus, PHB performs a regulatory function in *Az. beijerinckii* cells by maintaining the necessary balance between reduced and oxidized pyridine nucleotides and serving as a reserve of carbon and energy. High intracellular concentrations of NAD(P)H and acetyl-CoA and low concentrations of CoA are prerequisites for PHB synthesis under oxygen limitation. These experimental data made it possible to build a regulatory model for PHB synthesis in *Az. beijerinckii* [48].

The heterotrophic growth of *Az. vinelandii* is accompanied by the formation of cysts and accumulation of intracellular storage polymers which provide the survival of this bacterium in unfavorable conditions. PHB synthesis and encystment occur in parallel. Glucose and butanol enhance these processes. Hydroxybutyrate (the product of PHB degradation) also stimulates encystment, and the synthesis of a mucopeptide that is excreted into the medium in the presence of glucose. Similarly, *Az. chroococcum* cells grown on glucose accumulate PHB and form cysts. Butanol and metabolically related substrates, such as crotonate, hydroxybutyrate, and butyrate, induce encystment but inhibit PHB synthesis [50, 51].

Phosphorus limitation enhances PHB synthesis in *Az. vinelandii* cells and produces some other physiological and morphological effects. One noteworthy effects is the inhibition of oxidative phosphorylation. This stimulates the energy flow for PHB synthesis, decreases the adenylate energy charge and ATP/ADP ratio, and causes some changes in the composition of the cell wall. As a result, the cells fail to form cysts [49].

Unlike the parent strain *Az. vinelandii* UW, which is unable to form capsules, the mutant UWD accumulates up to 65–75% PHB during the exponential growth phase. In this case, PHB production does not depend on oxygen concentration in the medium, although the efficiency of glucose conversion into polyester is inversely proportional to oxygen concentration. The mutant UWD is defective in respiratory NADH oxidase. This mutation is considered to be neutral, since NADH is oxidized during PHB synthesis and is formed in the Entner–Doudoroff pathway (its one cycle gives 1 mole of each ATP, NADPH, and NADH). Electrons from NADH are transferred to oxygen with the involvement of transhydrogenase and NADPH oxidase. Therefore, PHB synthesis in the mutant UWD plays a compensatory role and is not induced by external factors [52].

In *Az. vinelandii*, PHB is synthesized and degraded much as it is in *R. eutropha*, with some minor differences in the regulation of biosynthetic and degradative enzymes. The mutation of NADH oxidase against the functioning background of the non-defective transhydrogenase leads to an increase in the cellular level of reduced pyridine nucleotides, suppression of citrate and isocitrate synthases, and, eventually, to the inhibition of the Krebs cycle and an increase in the acetyl-CoA/CoA ratio [52, 53].

The β-ketothiolase of *Az. vinelandii* UWD is inhibited by CoA, the effect being prevented by high concentrations of acetyl-CoA [53]. Unlike the ACRs of *Az. beijerinckii* and *R. eutropha*, the ACR of *Az. vinelandii* UWD is inhibited by $NADP^+$ (Table 2). Therefore, the transhydrogenase of the latter bacterium can maintain

the low cellular level of $NADP^+$. Like the ACRs of many other PHB producers, the ACR of *Az. vinelandii* UWD is inhibited by high concentrations of acetoacetyl-CoA. However, this inhibition can be prevented by high concentrations of NADPH [53].

4.3. Aerobic methylobacteria. The organization and regulation of PHB synthesis in methylotrophic bacteria with the serine pathway of C_1 metabolism is also of interest. The regulation of PHB synthesis is best studied in *M. rhodesianum* MB126 and *M. extorquens.* In these methylobacteria, PHB synthesis is regulated by mechanisms similar to those described above: namely, by the cellular pools of acetyl-CoA and NAD(P)H. At the same time, there are some peculiarities in the regulation of PHB synthesis during methylotrophic and heterotrophic growth [54, 55].

During methylotrophic growth, the initiating enzymes of PHB synthesis and the Krebs cycle (β-ketothiolase and citrate synthase, respectively) compete for the common substrate, acetyl-CoA. The regulation of these enzymes is due to their different affinities for acetyl-CoA (the K_m of β-ketothiolase and citrate synthase with respect to acetyl-CoA are 500 and 70 μM, respectively) and to changes in the cellular levels of some other effectors [54]. β-Ketothiolase is inhibited by low concentrations of CoA (K_i = 20 μM), while citrate synthase is more sensitive to NADH (K_i = 150 μM). The activity of citrate synthase in cell extracts is lower than that of β-ketothiolase (0.12 and 0.3 U, respectively). The cellular concentration of CoA falls to an undetectable level in response to the induction of PHB synthesis, while the concentration of NADH decreases insignificantly. Under conditions of unbalanced growth on methanol, β-ketothiolase is inhibited by CoA, and acetyl-CoA is metabolized in the Krebs cycle. Under nitrogen limitation, the cellular concentration of NADH is such that the activity of citrate synthase comprises only 25% of its maximum activity. Due to this, the concentration of CoA rapidly diminishes, that of acetyl-CoA increases, and the activity of β-ketothiolase rises considerably.

M. rhodesianum cells grown on fructose in a nutritionally rich medium accumulate PHB in the exponential growth phase. Such a difference in the conditions of PHB synthesis during growth on methanol and fructose is indicative of the different physiological functions of PHB in these two cases. Namely, PHB synthesis in cells grown on methanol under growth-limiting conditions serves as a sink for excess electrons. Conversely, fructose catabolism gives little energy for growth (probably due to the low activity of the Krebs cycle), and this triggers the metabolism of *M. rhodesianum* cells for the synthesis of PHB. This speculation is confirmed by the results of the comparison between carbon flows with the enzyme activities of the Krebs cycle and PHB synthesis, as well as by the effect of formate as a supplementary source of NADH independent of the Krebs cycle activity. Formate added to the medium suppresses PHB synthesis in *M. rhodesianum* cells but not their growth [56].

The concentration of CoA in fructose-grown *M. rhodesianum* cells (0.037 mM) is sufficient to maintain the activity of β-ketothiolase at a level of 20% and to provide for PHB synthesis. The cellular concentrations of NADPH and NADH in fructose-grown cells are such (0.37 and 0.31 mM, respectively) that NADH- and NADPH-dependent ACRs are saturated and cannot control PHB synthesis. The cellular concentrations of all of the aforementioned effectors, except AMP and ADP, decrease when the metabolism of *M. rhodesianum* cells is triggered from methylotrophic growth for PHB synthesis from fructose. In this case, the activities of β-ketothiolase, NADPH- and NADH-dependent ACRs, and citrate synthase do not change activities. These observations are in contradiction with the known properties of these enzymes and suggest that other regulatory mechanisms providing for noninducible PHB synthesis from fructose may exist [56].

Investigation of the relevant metabolic pathways may further contribute to the regulatory model of PHB synthesis in *M. rhodesianum* cells. It is believed that methylotrophic PHB producers, such as *M. extorquens*, implement the three-step pathway of PHB synthesis [34]. However, the presence of stereospecific crotonases in *M. rhodesianum* suggests that PHB synthesis in this bacterium is comprised of five steps (as in *Rsp. rubrum*) and that the mechanism for generating reducing equivalents in this bacterium is peculiar. It is assumed that PHB synthesis in *M. rhodesianum* is specific for NADH, since it is this reduced pyridine nucleotide that is formed by formate dehydrogenase. However, the activities of formate dehydrogenase and transhydrogenase in this bacterium are insignificant [56], and the cellular pool of NAD(P)H can be replenished by the dehydrogenases of the tetrahydrofolate and methanopterine pathways, whose activities are very high in serine methylobacteria [57, 58].

5. Structural Organization of Genes Involved in PHB Synthesis

By now, the PHB synthesis genes (*phaB, phaC*, and *phaA*) have been cloned from more than 20 PHB producers. As a rule, these genes are chromosomal and organized in operons [3].

For instance, in *R. eutropha*, the PHB synthesis genes are clustered and located on the chromosome but not on the megaplasmid pHG1 [60]. The restriction fragment *Sma*I/*Eco*R1, with a minimum size of 5.2 kbp, contains all three genes. This follows from the ability of the recombinant *E. coli* strain transformed with this fragment to synthesize PHB. Analysis of the nucleotide sequence of this locus showed the presence of three open reading frames (ORFs) of the *phaC*, *phaA*, and *phaB* genes that encode proteins with molecular masses of 64, 41, and 26 kDa, respectively [61, 62]. The struc-

tural genes of PHB synthesis form the operon *phaC-A-B*, which encodes PHB synthase, β-ketothiolase, and NADPH-dependent ACR. The promoter of this operon is located 307 bp upstream from the *phaC* gene. The functional role of such a long leader sequence of mRNA remains unclear [62, 63].

In *Z. ramigera*, the respective operon contains only two genes, *phaA* and *phaB*. The structural gene *phaC* was not revealed either in the operon or in the vicinity of it. The site of transcription initiation is located 85 bp upstream from the *phaA* gene, beyond the -35 and -10 regions. The hairpin-like structure located downstream from the *phaB* gene is a potential transcription terminator [3].

The PHB synthase gene of *Acinetobacter* sp. RA3849 has been isolated and cloned in the PHB-defective mutant of *R. eutropha* [64]. The nucleotide sequence of this gene is highly homologous to that of the *phaC* genes of other PHB producers. The PHB synthase gene of this bacterium was found to be both plasmid-borne and chromosome-borne. The *phaC* gene is clustered and codes for a protein with a molecular mass of 67 kDa. This protein belongs to PHB synthases of the first class. Data on the structural organization of other genes involved in PHB synthesis by this bacterium are scarce.

In addition, little is known about the structural organization of the PHB synthesis genes of *M. extorquens* IBT6, although the PHB synthase gene of this bacterium has recently been cloned [65]. This gene has 1815 bp and possesses distinct promoter and terminator sequences located upstream and downstream of its structural part.

As in *Rhodococcus ruber* [66] and *Z. ramigera* [67], the structural PHB synthase gene of *M. extorquens* is separated from the genes of biosynthetic β-ketothiolase and ACR. The predicted molecular mass of PHB synthase (67 kDa) agrees well with the electrophoretically determined molecular mass of granule-associated PHB synthase (65 kDa). It was found that the latter synthase is subject to post-translational modification. The start codons of this gene are at positions 850–919. The putative leader peptide of the PHB synthase discussed presumably contains 24 amino acids.

Recently, the PHB synthesis genes of *P. denitrificans* have also been cloned. The *phaA* and *phaB* genes are organized in an operon and encode proteins with molecular masses of 41 and 26 kDa, respectively. The *phaC* gene is not included into the operon and encodes a protein with a molecular mass of 70 kDa [68, 69].

In the phototrophic bacterium *C. vinosum*, the locus related to PHB synthesis has six ORFs, two of which correspond to the *phaA* and *phaB* genes coding for β-ketothiolase and ACR, respectively. ORF4 and ORF5, located between the aforementioned ORFs, code for proteins with molecular masses of 17 and 14 kDa. The 14-kDa protein, called phasin, is believed to be involved in the formation of PHB granules. The functional role of the 17-kDa protein is unknown, although this protein was found to be homologous to the *E. coli* heat shock proteins, which are also able to bind to granules during the heterologous expression of insoluble proteins. The antiparallel *phaE* and *phaC* genes, which encode the PHB synthase subunits, are located upstream from the *phaA* gene [70, 71].

The PHB synthesis genes of the other phototroph, *Thiocystis violacea*, are structurally similar to the corresponding genes of *C. vinosum*. Upstream of the *phaA* gene, ORF2 and the *phaC* gene are located antiparallel to it. The functional role of ORF4, which is situated between the *phaA* and *phaB* genes, is still unknown [72].

The *phaA* and *phaB* genes of *Rhizobium meliloti* are clustered, like those of *R. eutropha*. ORF1, which is located upstream from the *phaA* gene, is homologous to the ORF4 of *C. vinosum* and *T. violacea*. The *phaC* gene of *Rz. meliloti* is highly homologous to the PHB synthase gene of *M. extorquens* [73]. In general, all the known PHB synthases are characterized by a high degree of homology. This allowed the PHB synthase genes from *Rsp. rubrum*, *Rbd. sphaeroides*, and *Rh. ruber* to be cloned in the PHB synthesis–defective *R. eutropha* mutant with restoration of its ability to synthesize PHB [66, 74].

CONCLUSION

Lemoigne, the discoverer of PHB as a reserve polymer of bacteria, could hardly foresee all the consequences of his discovery. In the last quarter of the twentieth century, the joint efforts of microbiologists, biochemists, geneticists, and biotechnologists has led to establishment of the scientific principles and elaboration of the biotechnology of PHB(V) production on an industrial scale. At present, more than 100 biodegradable plastics of the PHA family are known. Further progress in this field will largely depend on the knowledge gained about the metabolic and genetic organization of PHB synthesis in natural and recombinant prokaryotic and eukaryotic bacterial producers. Using the metabolic design approach and site-directed mutagenesis, researchers search for novel tailor-made biodegradable polyesters with valuable properties [75].

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