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POLY-β-HYDROXYBUTYRATE CONTENT IN CELLS OF VARIOUS *Rhizobium* SPECIES DURING GROWTH WITH DIFFERENT CARBON AND NITROGEN SOURCES

G. A. Bonartseva,[1] V. L. Myshkina,[1] and E. D. Zagreba[1]

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The capacity for poly-β-hydroxybutyrate synthesis was tested in active and less active collection strains of *Rhizobium phaseoli*, *R. meliloti*, and *R. trifolii* during growth on media with different carbon and nitrogen sources. It was found that poly-β-hydroxybutyrate synthesis can be selectively induced either in active or less active *Rhizobium* strains by choosing appropriate sources of carbon and nitrogen. A promising producer of poly-β-hydroxybutyrate, a less active strain *R. phaseoli* 680, was revealed. The poly-β-hydroxybutyrate content in cells of this strain reached 65% of dry cell weight during growth on a medium with sucrose and nitrate.

Poly-β-hydroxybutyrate (PHB) is a widely distributed intracellular reserve substance typical of prokaryotes. It was isolated for the first time 60 years ago from cells of aerobic bacilli [1]. Biopolymers of a similar structure but based on other monomers have been isolated from some microorganisms recently; they were all assigned to the class of poly-β-hydroxyalkanoates [2]. Depending upon the type of the microorganism and the conditions of cultivation, PHB may be not only a store of carbon and energy, but also an electron sink, accumulating excessive reducing equivalents in the form of an osmotically and chemically neutral compound. Some microorganisms may accumulate PHB to 80 – 90% of dry cell weight [3].

In considering PHB synthesis by root nodule bacteria, it should be noted that this property is fully inherent in all *Rhizobium* species. According to Vincent et al. [4] and Tambolini et al. [5], the content of this polymer in rhizobia ranges from 30 to 55% of dry cell weight. PHB constitutes up to 80 – 90% of bacterial and 60 – 65% of bacteroid lipids [1]. Yushkova et al. [6] reported that in *R. lupini* maximal PHB accumulation was observed after growth with mannitol and glutamate.

Storage of energy is probably the main function of PHB in microbial cells. For instance, in *Bacillus megaterium* the polymer serves as an endogenous carbon and energy source providing for sporulation [7]. A direct relation between the amount of the polymer and cyst formation has been revealed in *Azotobacter vinelandii* [8]. The intensity of light emission by luminescent bacteria and the accumulation of PHB in their cells are inversely related [9]. It was shown that the PHB content in bacteroids is negligible precisely during active nitrogen-fixation and respiration, when their requirements for energy and reducing equivalents are particularly high [10]. Our previous work with pure cultures of *R. vigna*, *R. japonicum*, *R. phaseoli*, and *R. meliloti* demonstrated a dependence of the PHB content upon nitrogenase and hydrogenase activity. A strict inverse correlation was revealed between nitrogenase activity and the PHB content during anaerobic growth in the presence of nitrates. This dependence apparently was due to the PHB expenditure on nitrogen fixation under such conditions. A direct correlation between hydrogenase activity and the PHB content was found, which evidently indicates that the presence of hydrogen-consuming hydrogenase gives the studied strains an energy gain as a result of secondary involvement of molecular hydrogen in metabolism; the additional energy is accumulated in the form of PHB [11]. The amount of synthesized PHB may be stabilized by cultivating bacteria under strictly definite conditions, and may be used in this case as a quantitative characteristic of individual *Rhizobium* strains [3, 12]. It was shown that the PHB content may be a criterion for differentiation of active *Rhizobium* strains from strains with a low activity. During cultivation of rhizobia on bean agar with sucrose, a

[1] A. N. Bakh Institute of Biochemistry, Russian Academy of Sciences, Moscow, Russia.

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much greater amount of PHB is accumulated in cells of lowly active strains than in cells of active strains [13, 14]. We have suggested a fluorescence express-test for active *Rhizobium* strains based on staining of PHB with the vital lipophilic stain phosphine 3R in colonies on Petri dishes [15, 16]. The choice of an active strain may be approached in a different manner if the various requirements of the root nodule bacteria for carbon and nitrogen sources are taken into account.

The aim of the present work was to test strains of varying activity, of some species of rapidly growing root nodule bacteria for PHB synthesis during cultivation on different carbon and nitrogen sources.

MATERIALS AND METHODS

The following cultures of root nodule bacteria were employed: *R. phaseoli* strain 673 (active) and strain 680 (less active); *R. meliloti* strain 425a (active) and strain 434a (inactive); *R. trifolii* strain 348a (active) and strain 346a (with low activity). All the strains were obtained from the collection of the Scientific Research Institute of Agricultural Microbiology (Pushkin, Saint Petersburg). They were maintained on a pea medium of the following composition (g/liter): pea, 50; sucrose, 5; K_2HPO_4, 0.5; agar, 15; pH 6.8 – 7.0. In the experiments the microorganisms were cultivated on an agarized synthetic medium of the following composition (mg/liter): $NaH_2PO_4 \cdot H_2O$, 150; $CaCl_2 \cdot 2H_2O$, 150; $MgSO_4 \cdot 7H_2O$, 250; Fe EDTA, 28; $MnSO_4 \cdot 7H_2O$, 10; H_3BO_3, 3; $ZnSO_4 \cdot 7H_2O$, 2; $Na_2MoO_4 \cdot 2H_2O$, 0.25; $CuSO_4 \cdot 5H_2O$, 0.04; $CoCl_2 \cdot 6H_2O$, 0.025; KI, 0.78; biotin, 0.01; pantothenic acid, 0.1; folic acid, 0.01; vitamin B_2, 0.2; B_1, 0.1; B_6, 0.1; B_{12}, 0.02; agar Difco, 1.5%; distilled water, pH 7.0. The following carbon sources were employed (in amounts equalized with respect to the carbon content, g/liter): sucrose, 10; glucose, 10.5; arabinose, 10.5; mannitol, 10.5; Na succinate, 14.35; Na acetate, 14.5; fumaric acid, 10.15. The source of nitrogen in this series of experiments was KNO_3 or $(NH_4)_2SO_4$ (1 and 0.66 g/liter, respectively).

In the experiments with varied nitrogen source the following compounds were used (in amounts equalized with respect to the nitrogen content, g/liter): KNO_3, 1; $(NH_4)_2SO_4$, 0.66; glutamine, 0.73; glycine, 0.75; asparagine, 0.66; $Co(NH_2)_2$, 0.30; the source of carbon was sucrose (10 g/liter).

The bacteria were cultivated in Petri dishes. A 0.1-ml portion of bacterial suspension with an optical density of 0.5 at 550 nm (1 mm cuvette) was applied to the medium and spread with a spatula to obtain continuous uniform growth. Incubation was performed in a thermostat at 28°C for 6 days.

Quantitative analysis of PHB was conducted with the help of IR-spectrophotometry according to the Firordt method [17]. To determine the cell PHB content, bacterial biomass was washed off the agar medium with 15 ml of tap water and the optical density of the suspension was measured on a FEK-56M spectrophotometer at $\lambda = 550$ nm. The cells were centrifuged and washed two times with sterile tap water. The washed biomass was lyophilized and carefully mixed and ground with KBr, after which tablets were pressed for IR-spectroscopy on an IR-20 spectrophotometer (slit program 4, recording speed 160 cm^{-1}/min).

RESULTS AND DISCUSSION

As can be seen from Table 1 and Figures 1 – 6, the nature of the carbon and nitrogen sources utilized during growth of root nodule bacteria determines both their growth yield and PHB synthesis. The maximum PHB content depends upon the type of culture. In *R. trifolii*, maximum PHB content was found in cells grown with sucrose as the carbon source and glutamine as the nitrogen source. PHB content was higher in the active strain than in the strain with low activity, and reached 45% of dry cell weight. The PHB content was much lower when organic acids (succinate, fumarate, or acetate) were used. Testing various combinations of carbon

TABLE 1. Growth of *R. phaseoli*, *R. meliloti*, and *R. trifolii* on Media with Different Carbon and Nitrogen Sources (mg dry biomass/ml)

Strain	Sources of carbon and nitrogen											
	sucrose	glucose	arabinose	mannitol	Na succinate	Na acetate	Na fumarate	$(NH_4)_2SO_4$	glutamine	glycine	asparagine	$CO(NH_2)_2$
R. phaseoli												
673	0.46	0.68	0.44	0.59	0.09	0.09	0.03	0.46	3.01	0.11	1.15	2.49
680	0.60	0.49	0.28	0.67	0.15	0.006	0.14	0.59	2.44	0.12	1.90	2.28
R. meliloti												
425a	0.85	0.58	0.59	0.89	0.28	0.001	0.26	0.05	0.20	0.55	0.52	0.40
434a	0.85	0.84	0.74	0.88	0.17	0.001	0.15	0.09	0.31	0.73	0.77	0.63
R. trifolii												
348a	0.81	0.49	0.99	0.41	0.03	0.001	0.07	0.41	0.91	0.81	2.11	1.46
346a	0.81	1.59	0.65	0.67	0.02	0.01	0.03	0.29	1.75	1.22	2.15	1.59

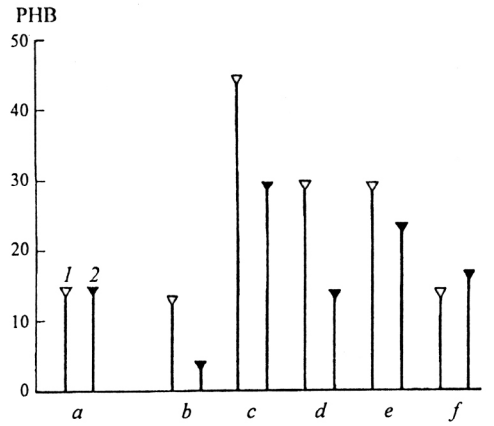

Fig. 1. Content of poly-β-hydroxybutyrate in cells of active (348a) and less active (346a) *R. trifolii* strains grown on sucrose with different nitrogen sources. PHB) Poly-β-hydroxybutyrate — percentage of dry cell weight (the same in Figs. 2 – 6); *a*) KNO_3; *b*) $(NH_4)_2SO_4$; *c*) glutamine; *d*) urea; *e*) asparagine; *f*) glycine; *1*) active strain; *2*) less active strain (the same in Figs. 2 and 3).

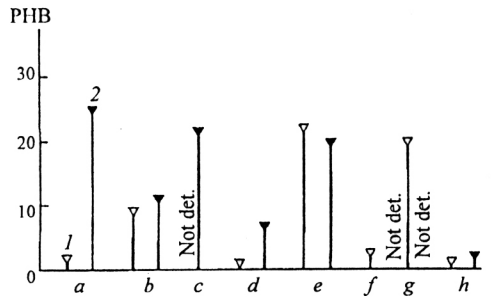

Fig. 2. Content of poly-β-hydroxybutyrate in cells of active (348a) and less active (346a) *R. trifolii* strains grown on different carbon sources in the presence of NO_3^- or NH_4^+. *a*) Glucose; *b*) arabinose; *c*) mannitol *d*) succinate; *e*) succinate + NH_4^+; *f*) fumarate; *g*) fumarate + NH_4^+; *h*) acetate.

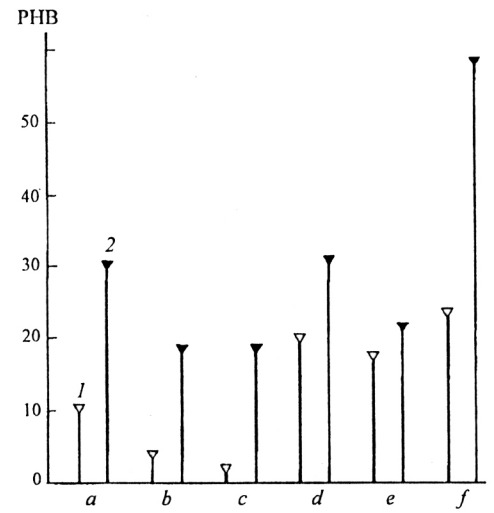

Fig. 3. Content of poly-β-hydroxybutyrate in cells of active (425a) and inactive (434a) *R. meliloti* strains grown on sucrose with different nitrogen sources. *a*) KNO_3; *b*) $(NH_4)_2SO_4$; *c*) glutamine; *d*) urea; *e*) asparagine; *f*) glycine.

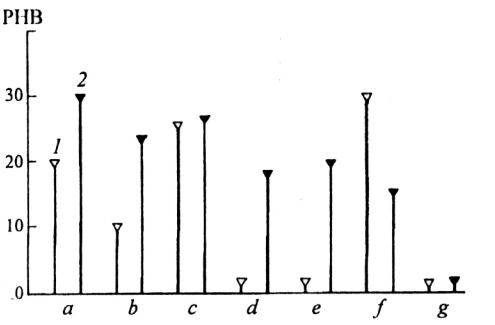

Fig. 4. Content of poly-β-hydroxybutyrate in cells of active (425a) and inactive (434a) *R. meliloti* strains grown on different carbon sources in the presence of NO_3^- or NH_4^+. *1*) Active strain; *2*) inactive strain; *a*) glucose; *b*) arabinose; *c*) mannitol; *d*) succinate; *e*) fumarate; *f*) fumarate + NH_4^+; *g*) acetate.

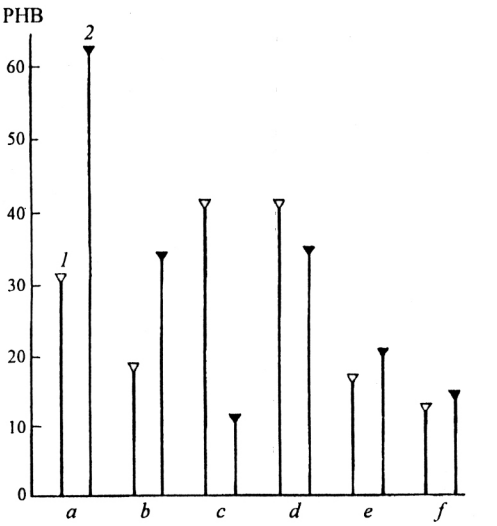

Fig. 5. Content of poly-β-hydroxybutyrate in cells of active (673) and less active (680) *R. phaseoli* strains grown on sucrose with different nitrogen sources. *1*) Active strain; *2*) less active strain; *a*) KNO_3; *b*) $(NH_4)_2SO_4$; *c*) glutamine; *d*) urea; *e*) asparagine; *f*) glycine.

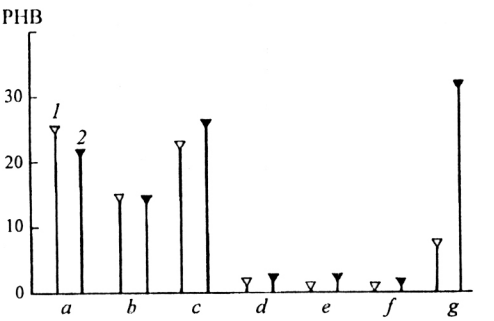

Fig. 6. Content of poly-β-hydroxybutyrate in cells of active (673) and less active (680) *R. phaseoli* strains grown on different carbon sources in the presence of NO_3^- or NH_4^+. *1*) Active strain; *2*) less active strain; *a*) glucose; *b*) arabinose; *c*) mannitol; *d*) succinate; *e*) fumarate; *f*) acetate; *g*) fumarate + NH_4^+.

and nitrogen sources for cultivation of *R. trifolii* showed that with combined use of glucose and nitrate, the PHB content was many times higher in the less active strain than in the active strain.

In the group of lucerne root nodule bacteria, the PHB content was highest in the less active strain *R. meliloti* 434a grown on sucrose and glycine (up to 59% of dry cell weight). On the whole, for *R. meliloti* the PHB synthesis was higher in the lowly active strain than in the active strain on most carbon and nitrogen sources tested. However, when fumarate was the carbon source and ammonium sulfate was the nitrogen source, the active strain accumulated more PHB than the strain with low activity. Just like *R. trifolii*, *R. meliloti* showed low PHB accumulation during growth on media containing organic acids.

Among all *Rhizobium* strains cultivated on different sources of carbon and nitrogen, the highest level of PHB accumulation (up to 65% of dry cell weight) was observed in the less active *R. phaseoli* strain grown on sucrose and nitrate.

The fact that among all sugars and organic acids tested it was sucrose that induced maximum PHB accumulation in all the studied species of root nodule bacteria is of interest in light of data published by V. Romanov [10]. He showed that it is precisely sucrose that makes up the largest proportion of photoassimilates entering bacteroids from the plant part of the root nodule. In other words, the substrate which is most habitual for rhizobia in the natural environment also proves to be the best one for energy storage.

Thus, we have shown that, depending upon the utilized sources of carbon and nitrogen, PHB synthesis may be selectively induced both in inactive (which is traditional) and active *Rhizobium* strains. Therefore, active *Rhizobium* strains may be directly selected by our previously suggested fluorometry method (i.e., according to the accumulation of PHB by active strains) instead of indirect selection through discarding lowly active strains [15, 16]. Species of root nodule bacteria differ in the maximum PHB content. On the basis of data obtained in the present work, *R. phaseoli* strains capable of PHB accumulation up to 65% of dry cell weight were selected, which may be employed as industrial producers after optimization of the conditions of PHB synthesis.

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