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EXPERIMENTAL ARTICLES

Physiological and Biochemical Characteristics and Capacity for Polyhydroxyalkanoates Synthesis in a Glucose-Utilizing Strain of Hydrogen-Oxidizing Bacteria, *Ralstonia eutropha* B8562

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Abstract—The physiological, biochemical, genetic, and cultural characteristics of the glucose-utilizing mutant strain *Ralstonia eutropha* B8562 were investigated in comparison with the parent strain *R. eutropha* B5786. The morphological, cultural, and biochemical characteristics of strain *R. eutropha* B8562 were similar to those of strain *R. eutropha* B5786. Genetic analysis revealed differences between the 16S rRNA gene sequences of these strains. The growth characteristics of the mutant using glucose as the sole carbon and energy source were comparable with those of the parent strain grown on fructose. Strain B8562 was characterized by high yields of polyhydroxyalkanoate (PHA) from different carbon sources (CO_2, fructose, and glucose). In batch culture with glucose under nitrogen limitation, PHA accumulation reached 90% of dry weight. In PHA, β-hydroxybutyrate was predominant (over 99 mol %); β-hydroxyvalerate (0.25–0.72 mol %) and β-hydroxyhexanoate (0.008–1.5 mol %) were present as minor components. The strain has prospects as a PHA producer on glucose-containing media.

Key words: hydrogen-oxidizing bacteria, glucose-utilizing strain, polyhydroxyalkanoates, synthesis.

Due to the broad prospects for their application, polyhydroxyalkanoates (PHAs), the biodegradable natural polyesters produced by prokaryotic microorganisms as storage compounds, are an object of intense study. Various substrates can be used for PHA production that differ in degree of reduction, in energy content, and in price. Both individual chemical compounds (various sugars, alcohols, acids, hydrocarbons, and carbon dioxide) and complex substrates, including industrial and agricultural wastes, are known to be utilized [1]. Sugars are considered potential substrates for polyhydroxyalkanoates synthesis by various microorganisms, including the hydrogen-oxidizing bacteria *Ralstonia eutropha* and *Alcaligenes latus*. Of the spectrum of sugars, the natural strains of these taxa are known to utilize only fructose. Mutants, however, can be easily obtained that utilize glucose and sucrose via the Entner–Doudoroff pathway; the theoretical estimate of sugar consumption for PHA synthesis is 2.5 g/g [2]. A limited number of *R. eutropha* strains capable of glucose utilization have been characterized in available publications. For instance, *Alcaligenes eutrophus* strain H16 (subsequently renamed *Ralstonia eutropha* H16) was used by the company ICI for the production of the polyhydroxybutyrate Biopol®, the first of the polyhydroxyalkanoate polymers. After 40 h of cultivation, the biomass yield of this strain was up to 20 g/l with a polymer content of 70% of the dry weight; 3.0–3.3 g of sugars were consumed per gram of polyhydroxybutyrate [3]; glucose concentrations of over 40 g/l inhibited the strain. To develop the regime of PHA biosynthesis with glucose, various strategies are presently available, including nitrogen or phosphorus limitation [4, 5], obtaining strains resistant to high (up to 100 g/l and higher) glucose levels in the medium [6] and increasing oxygen tension. For example, with the recently obtained glucose-consuming strain *R. eutropha* NCIMB 11599, which has an optimal glucose concentration in the medium of about 9 g/l, in a fermentor with high mass exchange characteristics and 20% oxygen in the gas phase, a polyhydroxybutyrate yield of 53–62% can be achieved in dense cultures (up to 153–208 g/l) [7]. Glucose-utilizing strains are important for the use of sugar-containing industrial waste and hydrolyzed wood pulp in PHA production.

The goal of the present work was to investigate the physiological, biochemical, genetic, and cultural characteristics of the glucose-utilizing *R. eutropha* strain B8562 and assess the capability of synthesizing polyhydroxyalkanoates.

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MATERIALS AND METHODS

The glucose-utilizing mutant strain of a hydrogen-oxidizing bacterium *Ralstonia eutropha* B8562 was studied in comparison with the parent strain *Ralstonia eutropha* B5786 [8]. The strains are deposited in the All-Russia Collection of Industrial Microorganisms (VKPM). The morphological, physiological, genetic, and cultural characteristics of the strain were investigated. Colony morphology was determined after inoculation of nutrient agar and incubation for 3 days at 33°C. The physiological and biochemical characteristics (utilization of sugars, organic acids, and amino acids; assimilation of different nitrogen species; and proteolytic and amylolytic activities) were determined using the standard techniques.

Since the fatty acid composition is useful for identification, the fatty acid composition of strain B8652 was analyzed in comparison with strain B5786. Fatty acids were isolated from individual colonies by saponification; fatty acids were then extracted from acidified solutions and methylated according to the standard procedure [9]. Methyl ethers of fatty acids were analyzed using a GCD plus (Hewlett Packard, United States) chromatograph–mass spectrometer. The conditions of the analysis and the principal approaches to fatty acid identification are described in [10]. The net content of polyhydroxyalkanoates and their structure were analyzed as described previously [11]. To determine glucose-6-phosphate dehydrogenase activity, the sample (1 g of wet biomass in 10 ml of 0.01 M Tris HCl buffer, pH 8.0) was sonicated (4×1 min at 4°C) and centrifuged (10000 *g*, 10 min); the enzymatic activity was then determined spectrophotometrically using a UVICON 943 registering spectrophotometer (Italy). The reaction mixture contained 1 ml of 60 mM Tris HCl buffer, pH 9.0; 0.1 ml of 45 mM NAD; and 0.1 ml of 45 mM glucose-6-phosphate (G6P) [12]. The value accepted for extinction coefficients for NADPH and NADP was 6.22×10^{-3} μM^{-1} cm^{-1}. The amount of the enzyme required to catalyze conversion of 1 μmol G6P per minute per 1 mg protein was accepted as an activity unit (U). Protein content in the extract was determined by the Lowry method.

The genetic test performed in order to determine the relations between the mutant and the parent strain included determination of G+C content in genomic DNA and of the nucleotide sequence of 16S rRNA genes. DNA extraction was performed according to the standard procedure by Marmur and Doty [13]. The content of G+G base pairs was determined using Millikhrom-1a chromatograph (Russia) with a KKhA-2 column filled with Silosorb C18, in the gradient of methanol concentrations 0–3% in 0.02 M ammonium acetate at pH 5.0 (elution rate, 50 μl/min), with detection at $\lambda = 260$ nm. Amplification of 16S rRNA genes was performed using a polymerase chain reaction (PCR) with universal bacterial primers: direct 8F (5'-AGA GTT TGA TCC TGG CTC AG-3') and reverse 1492R (5'-GRT TAC CTT GTT ACG T-3') [14]. The PCR mixture (20 μl) contained single-strength reaction buffer (75 mM Tris HCl, pH 8.8 at 25°C; ammonium sulfate, 20 mM; Tween, 0.1%); deoxynucleotide triphosphate mixture, 0.05 mM; magnesium chloride, 1.5 mM; primers, 10 pmol each; *Taq* DNA polymerase (Fermentas, Lithuania), 2 U; and genomic DNA, 100 ng. The temperature regime included initial denaturation at 95°C (3 min), 30 cycles at 94°C (1 min), annealing at 55°C (1 min), and elongation at 72°C (1.5 min). The final polymerization was performed at 72°C for 10 min. The sample with an equal volume of sterile deionized water served as negative control. The size, amount, and purity of PCR products were assessed by electrophoresis in agarose gel. The PCR products were cloned in *Escherichia coli* by AT cloning using pBluescript vector (Stratagene, United States). The clones containing insertions were selected for sequencing and for phylogenetic analysis by "white-blue" screening. To analyze the size of insertions, PCR was performed using the cell suspension as a template and the primers complementary to the plasmid segments in the insertion region (M13 direct and reverse). The nucleotide sequences of the insertions of the necessary size were determined using fluorescence-labeled dideoxynucleotide triphosphates with an ALFexpress II automatic sequencer (Amersham Pharmacia Biotech, United States) belonging to the Center for Collective Equipment Usage Yenisei (Krasnoyarsk State University). Sequencing was performed with 750 ng of amplified DNA and 10 pmol of primers: M13 and internal universal bacterial primers 514F (5'-CG TGC CAG CAG CCG CGG TAA-3') and 988R (5'-CCT GGT AAG GTT CTT CGC GTT GC-3'), using a Thermo Sequenase Cy5 Dye Terminator Kit (Amersham Pharmacia Biotech, United States) according to the protocol recommended by the manufacturer. The nucleotide sequences of 16S rRNA genes were compared with the sequences from the GenBank and EMBL online databases using the FASTA software package (http://www.ebi.ac.uk/fasta33/nucleotide.html). For comparative analysis, the amplified sequences were aligned with the sequences from the databases using the ClustalW software package (http:/www.ebi.ac.uk/clustalw/index.html). Phylogenetic trees based on the comparison of 16S rRNA genes were built by the neighbor-joining method using the TREECON software package [15]. The statistical reliability of branching was estimated by bootstrap analysis of 100 alternative trees.

Bacteria *R. eutropha* B8562 were grown in batch culture in a Schlegel mineral medium [16] at pH 7.0 and 30°C, with glucose as a source of carbon and energy. The medium with 10 g/l of fructose was used for comparison of the growth characteristics of the mutant strain and of strain *R. eutropha* B5786. Bacteria were grown in 250 and 500 ml flasks filled with the medium to 25–30%. Stable growth of *R. eutropha* B8562 was scaled up in a 10-l laboratory fermentor. The previously developed growth regime with nitrogen

Table 1. Physiological and biochemical characteristics of strains *Ralstonia eutropha* B8562 and B5786

Substrate	Strain		Substrate	Strain	
	B8562	B5786		B8562	B5786
$CO_2 + H_2$	+	+	Succinate	–	–
Arabinose	–	–	Benzoate	–	–
Glucose	+	–	Oxalate	–	–
Xylose	–	–	Urea	+	+
Mannose	–	–	Glutamic acid	+	+
Lactose	–	–	DL-Valine	+	+
Rhamnose	–	–	DL-Leucine	+	+
Fructose	+	+	L-Asparagine	+	+
Maltose	–	–	DL-Methionine	+	+
Sucrose	–	–	DL-Tyrosine	+	+
Galactose	–	–	X, g/l (CO_2)	3.5 ± 1.2	4.1 ± 0.8
Mannitol	–	–	X, g/l (fructose)	4.9 ± 1.0	5.4 ± 1.8
Indole	–	–	PHA synthesis (from fructose)	+	+
Acetate	+	+	PHA synthesis (from CO_2)	+	+
Lactate	+	+	pH	6.7–7.2	7.0 ± 0.1
Pyruvate	+	+	*T*, °C	31 ± 1.0	31 ± 0.5
Citrate	+	+	G+C	66.0%	66.1%

limitation [1] was used to assess the ability of the strain for polyhydroxyalkanoate synthesis in batch culture. In the course of cultivation, the optical density of the culture (FEK-M, blue filter), cell concentration (drying of washed biomass at 105°C for 24 h), polymer content in the biomass, and residual carbon source concentration were determined. Glucose concentration was determined spectrophotometrically at 470–540 nm by the glucose oxidase method using a Fotoglucoza kit (Impact Ltd., Moscow, Russia); fructose was determined by the resorcinol method [17].

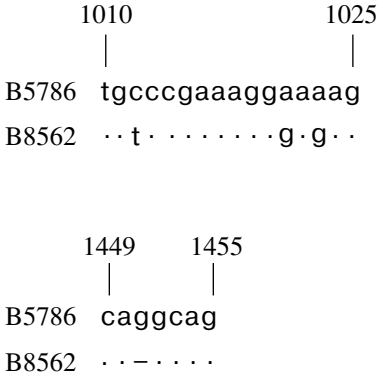

Fig. 1. Alignment of 5' terminal variable regions of the 16S rRNA genes of *Ralstonia eutropha* B5786 and B8562.

RESULTS AND DISCUSSION

The morphological and cultural characteristics of the strains *R. eutropha* B8562 and *R. eutropha* B5786 were found to be similar. The cells of both strains are motile rods of 0.3–0.5 × 1.2–2.0 μm. Both strains formed lightly cream-colored, shiny, opaque colonies 2–6 mm in diameter with a slightly elevated, slightly curving edge and a prominent center with radial grooves. Older colonies acquire light brown pigmentation and grow into the agar. In liquid medium (Schlegel mineral medium with glucose), strain B8562 formed a homogeneous suspension.

Biochemically, strain B8562 is an obligate aerobe and a facultative chemolithoorganotroph; it retains the ability to grow with CO_2 and H_2 as the sole sources of carbon and energy, respectively. Nitrates, ammonium salts, urea, and amino acids can be used as nitrogen sources. It is oxidase positive. There are no hydrolytic enzymes; gelatin is not liquefied and starch is not hydrolyzed. Of all the sugars analyzed, apart from fructose, only glucose was utilized. The strain can grow on media with organic acids and amino acids. Growth factors are not required. The optimal growth temperature is 30–32°C, and pH is 7.0 ± 0.2 (Table 1).

The fatty acid composition of strain B8562 in comparison with strain B5786, which is a systematic feature for microbial identification, is presented in Table 2. The fatty acid spectra of these strains are characteristic for gram-negative bacteria [18]. Palmitic (35–40% of

the total fatty acid), myristic (6.4–6.7%), and stearic acid (1.7–1.8%) are the predominant saturated acids. Palmitoleic (17.8–19.3%) and cis-vaccenic acids (10.8–11.3%) are the main monounsaturated acids. Traces of cyclopropanoic acid were found. In the fatty acid spectra of the biomass of both strains, β-hydroxy-acids were identified: components of the lipopolysaccharides of gram-negative bacterial cell walls [19]. Among these acids, β-OH-myristic acid was dominant in both strains; its content was between 10 and 13%. No statistically reliable differences between the strains in fatty acid composition were found.

The content of G+C nucleotide pairs for strain B8562 was 66 mol %; this value is comparable with that of strain B5786 (Table 1). In strain B8562, the nucleotide sequence of 15S rRNA gene was 1490 bp long; in strain B5786, 1491 bp. The degree of homology between 16S rRNA gene sequences was 99.73%. The differences were: three single-nucleotide replacements in strain B8562 in the variable region V3 [20] and a deleted sequence in the variable region V5 (Fig. 1). The nucleotide sequences of 16S rRNA genes of *R. eutropha* strains VKPM B5786 and VKPM B8562 were deposited in the EMBL database with accession numbers AJ633674 and AJ633675. Analysis of the sequences of strains B8562 and B5786 using the GenBank and EMBL databases revealed that 16S rRNA gene sequence of *A. eutrophus* ATCC 17697 was the closest one, 98.66% identical to the sequence of B8562 and 98.73% identical to that of B5786. The phylogenetic analysis of the sequences obtained and of 11 sequences of *Ralstonia* collection strains deposited in the GenBank and EMBL databases is presented in Fig. 2.

When grown in batch culture following Schlegel, biomass accumulation by strains B8562 and B5786 was comparable both under autotrophic and heterotrophic (with fructose) conditions (Table 1). The strain synthesized polyhydroxyalkanoates, and, in the stationary phase with 100% nitrogen supply, their yield both in autotrophic and in heterotrophic (with fructose) culture was also comparable with PHA yields for strain B5786 under the same conditions (Table 1). Strain B8562 exhibited glucose-6-phosphate dehydrogenase activity of 0.002–0.004U with all the carbon sources tested.

Investigation of the growth characteristic of strain B8562 grown in shaken flasks under different glucose supplies revealed that the highest biomass yield (6.5 ± 0.8 g/l) was achieved at 10–15 g/l of glucose; glucose concentration of 5 g/l limited growth; and initial glucose concentration of 20 g/l inhibited growth, causing also a prolonged (up to 15- to 20-h) lag phase (Fig. 3). The same result was obtained with fructose as substrate. These values are comparable with biomass yields of strain *R. eutropha* B5786 under similar growth conditions. Feed-batch culture resulted in biomass yields up to 11–12 g/l. Cultivation of this strain with glucose as the sole carbon source did not result in increased glucose-6-phosphate dehydrogenase activity.

Table 2. Fatty acid composition of the biomass of *R. eutropha* B8562 and B5786

Fatty acid	Strain		
	B8562 M ± *m***	B5786 M ± *m*	$t_{B8562–B5786}$
12 : 0*	0.1 ± 0.08	0.2 ± 0.10	0.81
14 : 0	6.4 ± 1.70	6.7 ± 0.82	0.87
14 : 1ω5	0.4 ± 0.39	0.1 ± 0.04	0.87
14 : 1	0.3 ± 0.04	0.3 ± 0.01	0.97
15 : 0	0.4 ± 0.08	0.5 ± 0.12	0.10
16 : 0	35.4 ± 3.05	38.5 ± 0.58	0.67
16 : 1ω7	19.3 ± 3.71	17.8 ± 0.07	0.39
16 : 1ω9	1.9 ± 1.54	2.7 ± 0.21	0.46
17 : 0	0.5 ± 0.05	0.7 ± 0.06	1.79
C-17	traces	traces	
17 : 1ω9	traces	traces	
β-OH-12 : 0	0.1 ± 0.01	0.1 ± 0.01	0.49
2-OH-12 : 0	1.8 ± 0.09	1.7 ± 0.26	0.49
18	1.9 ± 0.47	2.4 ± 0.29	0.94
18 : 1ω7	11.3 ± 2.24	10.8 ± 0.64	0.23
18 : 1ω9	1.8 ± 0.29	1.7 ± 0.02	0.29
β-OH-14 : 0	18.0 ± 1.88	15.8 ± 2.61	0.68
β-OH-16 : 0	0.1 ± 0.01	0.1 ± 0.01	0.45
β-OH-18 : 0	0.3 ± 0.05	0.2 ± 0.03	0.57

* The first number indicates the number of carbon atoms; the second, the number of double bonds; and the third, the double bond position from the methyl end.

** M are average values; *m*, standard errors; *t*, Student's criterion for the differences between the strains B8562 (n = 3) and B5786 (n = 3).

In order to assess the efficiency of polyhydroxyalkanoate production by strain B8562, bacteria were grown on salt medium with glucose under nitrogen lim-

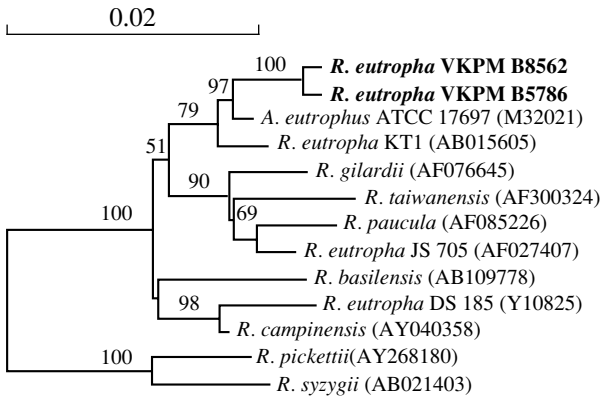

Fig. 2. Dendrogram based on phylogenetic analysis of 13 16S rRNA gene sequences of bacteria of the genus *Ralstonia*, including strains *R. eutropha* VKPM B5786 and VKPM B8562 (in bold). Scale bar shows the evolutionary distance corresponding to 2 nucleotide replacements per 100 nucleotides. Figures show reliability of the branching order, as determined by bootstrap analysis (values above 50 were considered reliable).

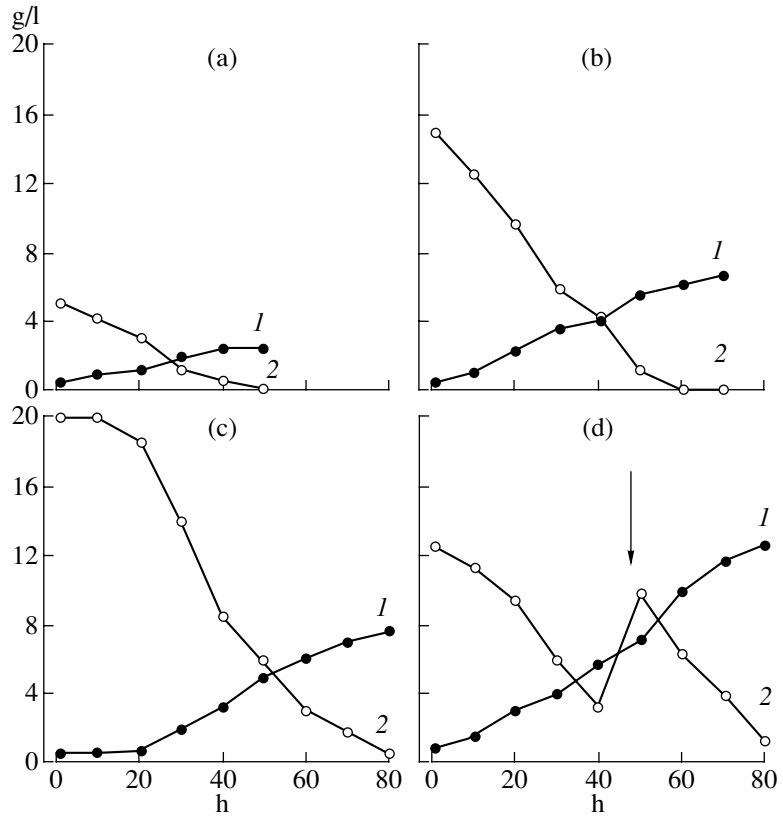

Fig. 3. Dynamics of (*1*) biomass yield and (*2*) glucose concentration in the medium for *R. eutropha* B8562 cultures on media with (a) 5, (b) 15, and (c) 20 g/l of glucose and (d) in feed-batch culture.

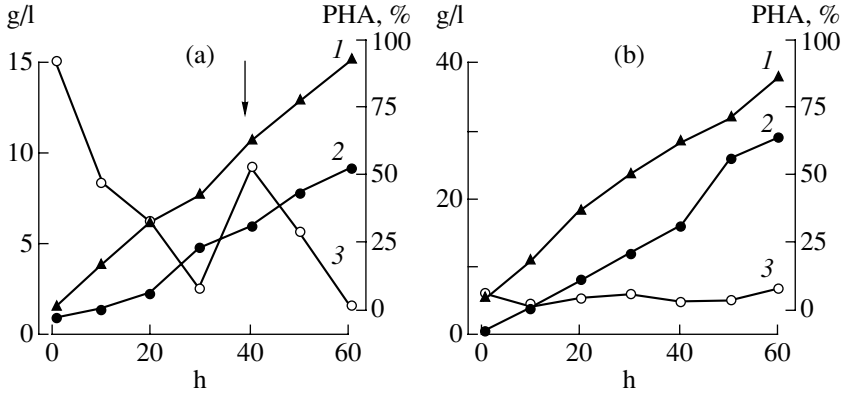

Fig. 4. (*1*) Polyhydroxyalkanoate accumulation, (*2*) biomass yield, and (*3*) glucose concentration in cultures of *R. eutropha* B8562 grown (a) in flasks and (b) in a fermentor.

itation (Fig. 4a). The yield of PHA was 9.2 g/l after 70 h, and the polymer concentration in the cells was 92%. This value is comparable with polyhydroxyalkanoate yield for strain B5786 on fructose under similar growth conditions. The glucose expense for PHA synthesis was 2.7 ± 0.3 g/g.

Scaling up of the process in a 10-l laboratory fermentor in fed-batch mode with the current glucose concentration of about 5 g/l for 60 h resulted in a biomass yield of 29.2 g/l (as dry cell biomass) and a polymer yield of 8.46 g/l with a concentration in the cells of 92% (Fig. 4b). The polymer synthesized by the glucose-utilizing strain of hydrogen-oxidizing bacteria *R. eutropha* B8562 on glucose-containing medium consisted mostly of poly-β-hydroxybutyric acid (over 99 mol %). The monomers of hydroxyvaleric (0.25–0.72 mol %) and hydroxyhexanoic (0.08–1.5 mol %) acids were identified as minor components. Mass spectrometry data con-

firmed the presence of hydroxyvalerate and hydroxyhexanoate in PHA.

The investigated glucose-consuming strain of hydrogen-oxidizing bacteria *R. eutropha* B8562 can be considered as a polyhydroxyalkanoate producer on sugar-containing substrates with glucose.

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