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Cloning and Molecular Organization of the Polyhydroxyalkanoic Acid Synthase Gene (*phaC*) of *Ralstonia eutropha* Strain B5786

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Abstract—Class I polyhydroxyalkanoic acid (PHA) synthase gene (*phaC*) of *Ralstonia eutropha* strain B5786 was cloned and characterized. *R. eutropha* B5786 features the ability to synthesize multicomponent PHAs with short- and medium-chain-length monomers from simple carbohydrate substrate. A correlation was made between the molecular structure of PHA synthase and substrate specificity and the ability of strain-producers to accumulate PHAs of this or that structure. A strong similarity of PHA synthase of *R. eutropha* strain B5786 with PHA synthase of *R. eutropha* strain H16, which, as opposed to strain B5786, enables to incorporate medium chain length PHAs if hexanoate is used as carbon source, exhibited 99%. A correlation between the structure of PHA synthase of B5786 strain with synthases of microorganisms which synthesize short and medium chain length PHAs similarly to B5786 strain, showed an identity level from 26 to 41% (homology with synthase of *Rhodospirillum rubrum* makes 41%, *Ectothiorhodospira shaposhnikovii* makes 26%, *Aeromonas punctata* makes 40%, *Thiococcus pfennigii* makes 28%, *Rhodococcus ruber* makes 38%, and with PhaCl and PhaC2 synthases of *Pseudomonas* sp. 61-3 makes 34 and 37%, respectively). This allows for speaking about the absence of a direct connection between the molecular organization of PHA synthases and their functional abilities, namely, the ability to synthesize PHAs of a particular composition.

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INTRODUCTION

Natural polyhydroxyalkanoate polyesters (PHAs) are synthesized by prokaryotes during the complex multi-level biosynthesis process with catalyzing of each step by a specific enzyme. PHA-synthase, one of the key enzymes of polymer synthesis, catalyzes the formation of ether connections during the polymerization of some oxidation derivative fatty acids [1]. Considering the narrow substrate specificity of synthases all known PHAs were subdivided into three groups: short chain length (PHA_{SCL}), medium chain length (PHA_{MCL}) and long chain length (PHA_{LCL}) [2]. PHA_{SCL} consisted of monomers with carbon chain length from three to five atoms (C_3–C_5), PHA_{MCL} (from C_6 to C_{14}) and PHA_{LCL} (more than C_{18}). Currently, there are four classes of synthases divided by size, structure and substrate specificity [1, 3]. Synthases of classes I and II are enzymes consisting of one type of subunits (PhaC) with molecular weight between 61 and 73 kDa. Prototype of class I synthases is an enzyme of *Ralstonia eutropha*, phototrophic purple nonsulphur bacteria, and enzymes of most of heterotrophic bacteria. These types of synthases interact as substrates mainly with short chain length β-oxyacids. Class II synthases are of 60–65 kDa molecular weight and interact with medium chain length oxyacids. These enzymes consist of two subunits and are coding by two genes (*phaC1* and *phaC2*). This type synthases are common for *Pseudomonas oleovorans*. Synthases of purple sulphur bacteria, e.g., *Allochromatium vinosum*, cyanobacteria [4] and sulphate-reducing bacteria [5] belong to class III. This type of enzyme comprises two subunits of different structure: PhaC with molecular weight of approximately 40 kDa (homology with synthases of types I and II is 21–28%) and PhaE with similar molecular weight 40 kDa (homology with PhaC is absent). PHA-synthases of class IV (e.g., *Bacillus megaterium*) have similarity with class III enzymes except for the second subunit presented by PhaR with molecular weight of approximately 22 kDa. This type synthases interact only with short chain length fatty acids.

Molecular organization of synthases is of great interest nowadays. In 1999, primary structures of 30 synthases were known, in 2002, 42 were known, and, by the end of 2003, 59 synthases structures were described from 45 bacteria strains [3]. Until recently it was considered that synthases are highly specific to substrates, and, therefore, microorganisms were able to accumulate only PHA_{SCL} or PHA_{MCL}. The ability of *R. eutropha* bacteria to synthesize two- and three-component copolymers consisting of short and medium chain length monomers first was revealed in natural strain B5786 [6]. It was determined that strain

B5786 featured the ability to synthesize three-component copolymers consisting of hydroxybutyrate, hydroxyvalerate, and hydroxyhexanoate (C_4, C_5, C_6) even while growth on monocarbon substrate (CO_2) or acetate, or fructose and were able to include oxyhexanoate in polymer. On mixed carbon source with supplemental even hydrocarbon acids (hexanoate and octanoate), this strain is able to include in PHA until 6–8 mol % of hydroxyhexanoate. This was confirmed by few research works [7, 8], in which authors showed that some *R. eutropha* natural strains are capable to synthesize medium chain length PHAs (hydroxyhexanoate and hydroxyoctanoate) besides short chain length PHAs (copolymers of hydroxybutyrate and hydroxyvalerate) during the growth on mixed carbon source containing octanoate as substrate-inducer for PHA_{MCL} synthesis. Currently, in addition to *R. eutropha*, there were described few more organisms that featured the ability to generate PHA containing PHA_{SCL} and PHA_{MCL} at the same time. This ability is typical to *Rhodospirillum rubrum* [9], *Rhodococcus ruber* [10], *Thiococcus pfennigii* [11], *Aeromonas punctata* FA440 [12], *Pseudomonas* sp. 61-3 [13], and *Ectothiorhodospira shaposhnikovii* [14]. Molecular organization of synthases of these organisms, at present, is fully described [14–19] and, according to primary structure, they were divided into three different classes (I, II, or III).

These results fundamentally change the idea about the narrow substrate specificity of PHA-synthases. But there were no studies described dedicated to revealing the connection between the structure of PHA-synthases, substrate specificity, and ability to synthesize one or other type of polymers. Study of microorganisms' ability to synthesize different multicomponent PHAs discovers possibilities for the directed production of tailor-made polymers and also widens the basic knowledge on metabolism of PHAs and functional qualities of PHA-synthases.

The aim of the present work was to study the molecular organization of PHA-synthase of the *R. eutropha* B5786 strain capable to synthesize PHA_{SCL} and PHA_{MCL} simultaneously.

METHODS

Bacterial strains, plasmids, and cultivation conditions. *R. eutropha* B5786 bacteria, obtained from the Russian National Collection of Industrial Microorganisms, were cultivated on Shlegel mineral medium with fructose (0.5%) as carbon and energy source under 30°C. *Escherichia coli* XL-10 Gold (Stratagene, USA) was grown under 37°C in Luria-Bertani broth with the addition of suitable antibiotics. For cloning, pBluescript SK^- vector (Stratagene, USA) was used.

DNA manipulations. DNA extraction, molecular cloning, and analysis of recombinant *E. coli* strains were performed by the standard methods [20] with the use of commercial enzymes (New England Biolabs, Gibco-BRL, Stratagene, MBI Fermentas) and NucleoTrap kits (MachereNagel GmbH, Germany) for DNA extraction from agarose gel.

Cloning of *phaC* gene from *R. eutropha* B5786. The *phaC* gene was amplified from genomic DNA of *R. eutropha* B5786 using polymerase chain reaction (PCR) with Platinum®*Pfx* polymerase (Invitrogen, USA). For PCR, two pair of primers were designed and synthesized in accordance with the nucleotide sequence of the *phbC* gene of *R. eutropha* H16 (GenBank accession number J05003) [21]: 5'-GCAATGCCCGGAGCCGGTTC-3' (792-811) and 5'-GGAG**GGATCC**TGCAGGCCTGC-3' (2671-2651). The second primer included additional restriction site *Bam*HI (indicated in bold). PCR mix (50 μl) included: *Pfx* reaction buffer, 0.05 mM dNTP mix, 1 mM $MgCl_2$, 10 pmol of each primer, 2 units of *Pfx* polymerase and 100 ng of genomic DNA. Amplification conditions were as follows: primary denaturation step at 95°C for 3 min; 25 cycles: DNA denaturation at 94°C for 20 s, primers annealing at 57°C for 30 s, and elongation at 68°C for 2 min 20 s. Length, quantity, and purity of PCR-products were assessed by electrophoresis in 0.8% agarose gel. Extracted and purified from gel PCR-product of needed length was ligated by *Eco*RV restriction site in pBluescript SK^- vector. *E. coli* XL-10 Gold cells were transformed by purified ligase mix. Obtained clones were examined by restriction analysis to include the insertion of appropriate length. Selected plasmids with cloned fragments were used for sequencing.

Sequencing and analysis of nucleotide sequences. Sequencing was performed both ways with the use of universal bacterial primers on automatic DNA sequencer LI-COR 4000L (MWGBiotech, Germany). Analysis of primary structure of cloned *R. eutropha* B5785 PHA-synthase and alignment of the sequence with known synthases was performed in accordance with NCBI GenBank BLAST data base (http://www.ncbi.nlm.nih.gov/BLAST/) and with the Genamics Expression program. Amino acids sequence was aligned with the ClustalW program. Phylogeny tree was constructed by the use of methods realized in the TREECON program.

Sequence accession. The 1877 bp DNA fragment that included nucleotide sequence of *phaC* gene coding the PHA-synthase from *R. eutropha* B5786 was accessed in the GeneBank database under the number AY836680.

RESULTS AND DISCUSSION

The 1877 bp fragment, which included the PHA-synthase gene (*phaC*), was amplified from genomic DNA of *R. eutropha* B5786 and cloned in pBluescript SK^- plasmid. PHA-synthase gene of the cloned fragment is coding the protein of 588 amino acids. The amino acid sequence of synthase from the B5786 strain was compared to known microorganisms PHA-

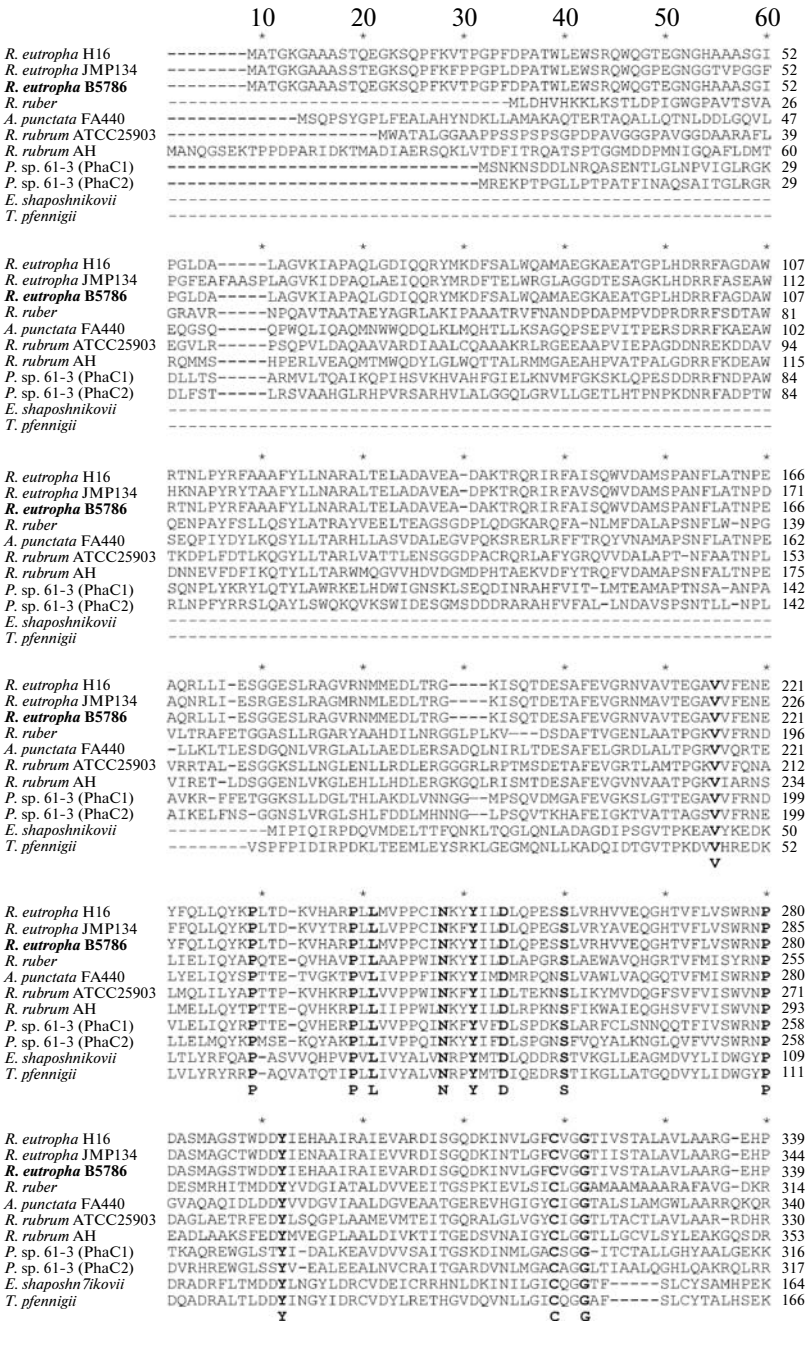

Fig. 1. Alignment of amino acid sequences of 11 PHA synthases of microorganisms capable to synthesize PHA_{SCL} and PHA_{MCL}. Identical amino acids in all PHA-synthases are indicated in bold. In the primary structure of *R. eutropha* B5786, nonhomologous to the sequence of *R. eutropha* H16 amino acids are underlined. Amino acids are indicated in standard one-letter code.

synthases capable to synthesize short and medium chain length PHAs simultaneously (Fig. 1, Table 1).

In spite of the fact that there had been a lot of *R. eutropha* strains described, the primary structure of PHA-synthase, until recently, was defined only for the strain most studied abroad—*R. eutropha* H16 [21]. Current analysis of nucleotide and amino acid sequences of *R. eutropha* B5786 capable to synthesize PHAs of different structures (Table 2) revealed high similarity (99%) with synthases of *R. eutropha* H16. Differences in amino acids structure of PHA-synthases between two strains localized in short nonidentical fragment of C-terminal region, between 561 and 572 amino acids in sequence (in accordance with the position for *R. eutropha* H16). It was also determined that homology of PHA-synthase of B5786 strain with synthase of other strain *R. eutropha* JMP134 was very high as well and equaled 98%. Strain JMP134 synthase

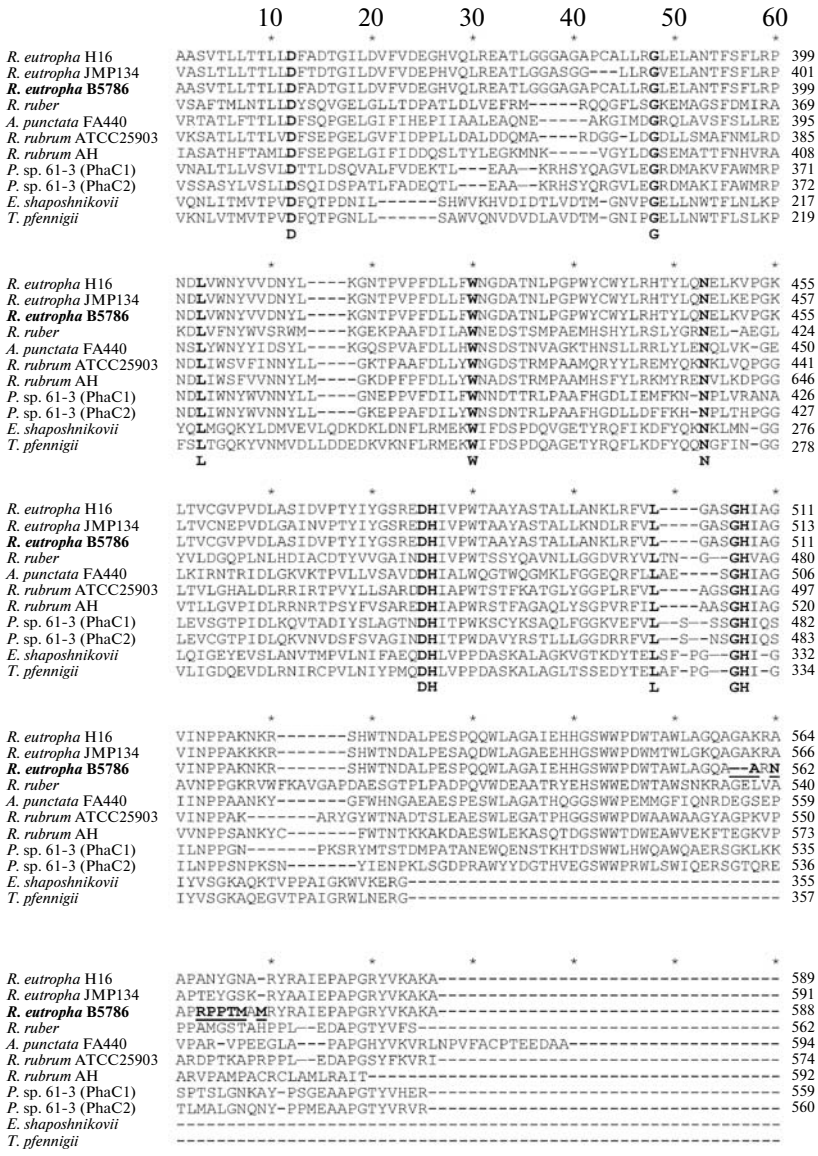

Fig. 1. Contd.

was sequenced under the NCBI genome project (October, 2004) but there was no information of its substrate specificity mentioned.

Figure 2 demonstrates the phylogeny tree based on the analysis of molecular organization of 60 PHA-synthases described to present day. Clusterization supports classification of synthases into four classes [3]. Comparison of amino acid sequences revealed the presence of a different homology degree between characterized enzymes (from 8 to 96%). *Pseudomonas* synthases cluster belonging to class II differed genetically from all others (homology with other enzymes did not exceed 34–35%). At the same time, synthase PhaC1 of pseudomonas notably differed from that of PhaC2 (similarity between these groups was 52–58%). *Gordonia rubripertinctus* and *Rhodococcus ruber* synthases, in spite of belonging to class I according to substrate specificity, genetically more related to class II synthases typical for pseudomonas (homology to this group 36–43%) while homology to class I synthases was 27–39%. The next cluster (class III) was presented by synthases of phototrophic sulphur bacteria and cyanobacteria *Synechocystis* (homology in this group of synthases reached 87%). This is interesting as the analysis of small rRNA subunit homology did not reveal any phylogeny connections between these groups of bacteria. Higher homology was established for *Rickettsia prowazekii* (PhbC1) synthases to class III (homology 36%) than to class I synthases (21%). The fourth cluster presented by two species earlier was related to class III. Only recently its structure was defined. Other synthases from organisms of different

Table 1. PHA-synthases of microorganisms capable to synthesize short and medium chain length PHAs simultaneously

Species, Strain	Synthase class	GeneBank accession	Reference
Aeromonas punctata FA440	I	D88825	[12, 13]
Ectothiorhodospira shaposhnikovii N1	III	AF307334	[14]
Pseudomonas sp. 61-3	II	AB014758	[13, 17]
Ralstonia eutropha B5786	I	AY836680	[6]
Ralstonia eutropha H16	I	J05003	[7, 21]
Rhodospirillum rubrum ATCC25903	I	AF178117	[9, 18]
Rhodospirillum rubrum HA	I	AJ245888	[9, 18]
Rodococcus ruber	I	X66407	[10, 14]
Thiococcus pfennigii 9111	III	A49465	[11, 19]

taxons (*Rhizobium, Rhodospirillum, Ralstonia, Aeromonas, Alcaligenes, Rhodobacter*) were clustered into two more groups (class I).

Based on the performed analysis of molecular organization of *R. eutropha* B5786 PHA-synthase for which the reaction substrates were short and medium chain length hydrooxyacids we attempted to reveal relation degree of this synthase with PHA-synthases of other microorganisms capable of the simultaneous synthesis of short and medium chain length PHAs.

Only few synthases were revealed which featured the ability to interact with short and medium chain length acids and to form PHA_{SCL} and PHA_{MCL} (Table 1). This ability is typical for *Thiococcus pfennigii* and *Aeromonas punctata* synthases, accumulating copolymers consisting of short and medium chain length acids respectively, copolymers (C_4, C_6, C_8) and (C_4, C_6) [11, 12]. By structure, they belonged to PHA-synthases of different types: *T. pfennigii* enzyme to synthases of class III and *A. punctata* belong to class I [16, 19]. Homology between synthases of these organisms that are characterized by the same ability to synthesize copolymers with addition, besides short chain length oxybutirate and oxyvalerate the medium chain length oxyhexanoate, makes 25.5%. It was established that PHA-synthases from *R. eutropha* bacterial strain B5786 were similar to synthases from *T. pfennigii* and *A. punctata* on 40 and 28%, respectively.

Table 2. Structure of PHAs synthesized by *Ralstonia eutropha* B5786 during the growth different carbon sources

Source	PHA structure, mol %		
	butyrate C_4	valerate C_5	hexanoate C_6
Fructose	93.6	5.7	0.7
CO_2	94.4	4.9	0.7
$CO_2 + C_5$	75.0	24.2	0.8
$CO_2 + C_6$	83.7	10.0	6.3
$CO_2 + C_7$	80.8	16.9	2.3
$CO_2 + C_8$	91.9	0.1	8.0
$CO_2 + C_9$	97.6	1.0	0.7
$CO_2 + C_{10}$	98.0	0.7	1.3

The ability to include in PHA oxyhexanoate as copolymer during anaerobe growth on oxyhexanoate or oxyheptanoate was demonstrated for *Rhodospirillum rubrum* as well [9]. Molecular organization of synthases of two strains of this species was published [18]. Synthases of these strains belonged to class I and homology between them was 51.9%. *Rhodococcus ruber* bacteria was also capable to form copolymers of short and medium chain length PHAs during the growth on hexanoate [10]. Synthase of this bacterium was identified as PHA-synthase of class I [15]. Similarity with *R. eutropha* B5786 synthase was 38%.

One more recently discovered organism capable to synthesize PHAs consisted of short (oxybutirate) and medium chain length (oxyhexanoate) copolymers. This was *Ectothiorhodospira shaposhnikovii* [14]. In accordance with its structure and substrate specificity synthase of this organism belonged to class III. Similarity with synthase of B5786 strain was 26%. Among class II, PHA-synthases enzymes from PhaC1 and PhaC2 of *Pseudomonas* sp. 61-3 featured the ability to synthesize PHA_{SCL} and PHA_{MCL} [13]. These bacteria were able to produce copolymers of 3-hydroxybutirate with hydroxalkanoates of long chain with 4–12 carbon atoms. Based on the expression analysis of cloned *phb* and *pha* genes from *Pseudomonas* sp. 61-3 in PHA-negative mutant *P. putida* GPp104 and *R. eutropha* $PHB^{-}4$, we demonstrated that only synthases from the *pha* locus coded by *phaC1* and *phaC2* genes showed more substrate affinity and ability to synthesize PHA_{SCL} and PHA_{MCL} [17]. Homology of amino acids sequences of synthases PhaC1 and PhaC2 with that of *R. eutropha* B5786 synthase was 34 and 36%, respectively.

Alignment of amino acids sequences of 11 synthases capable to synthesize short and medium chain length PHAs (Fig. 1) demonstrated the significant variation of the N-terminal area (approximately 100 amino acids), which proved its minor role in

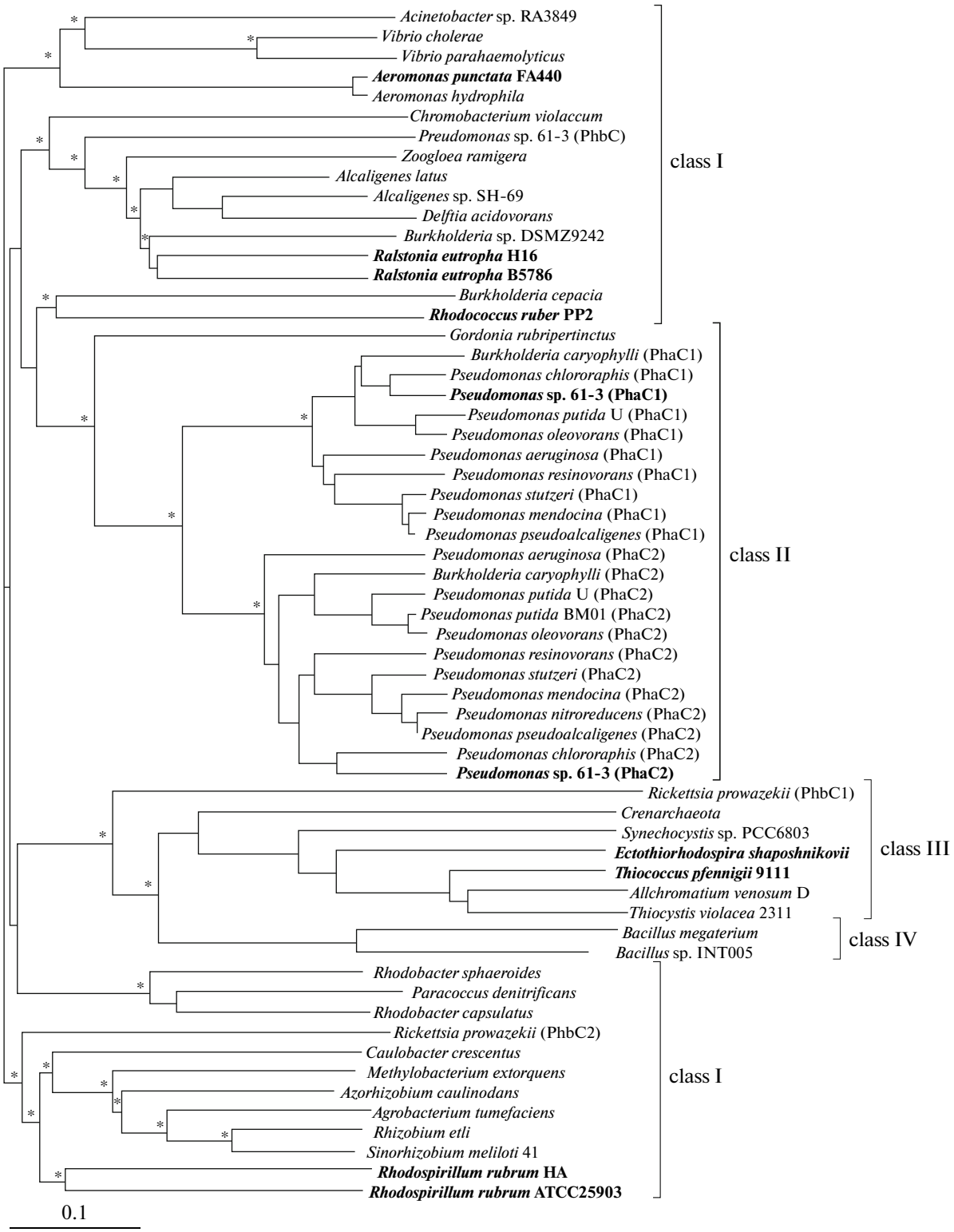

Fig. 2. Phylogeny tree of 60 PHA-synthases. Synthases of bacteria capable to form PHA consisted of PHA_{SCL} and PHA_{MCL} simultaneously were distinguished. The scale corresponds to one amino acid change for each ten amino acid sequences. The value of "bootstrap" index more than 50% is indicated by asterisk.

enzyme functioning. It was shown earlier [22] that *R. eutropha* PHA-synthase saved functional activity after the removal of fragment from amino acids 36 to 100. Comparative analysis of primary structure also demonstrated that C-terminal site (approximately 40 amino acids) plays a significant role in the enzyme activity for the synthases of class I and II but is absent in *E. shaposhikovii* and *T. pfennigii* synthases of class III. Thus C- and N- terminal sites do not influence the substrate specificity of synthases. In all analyzed PHA-synthases, we revealed 22 conservative amino acid residues: Val-216, Pro-230, Pro-239, Leu-241, Asn-248, Tir-251, Asp-254, Ser-260, Pro-280, Tir-292, Cys-319, Gly-322, Asp-351, Gly-387, Leu-402, Trp-425, Asn-448, Asp-480, His-481, Leu-503, Gly-507, and Gis-508 (in accordance with the position for *R. eutropha* H16). This observation testifies to the significance of these amino acids for enzyme functioning. It was established [23] that three of the amino acids (Cys-19, Asp-480 and His-508) formed a catalytic triad. Catalytic polymerization mechanism occurs by the formation of an enzyme-substrate complex PhaC with hydroxyacyl CoA with amino acid Cis-319 as the active site; at the same time His-508 plays a role of nucleophilic activator, and activation of 3-hydroxyl in 3-hydroxybutyril-CoA molecule caused by Asp-480. Authors suggested that cysteine plays a key role in polymerization reaction during addition of 4-phosphopantetheine to serine.

In our work, we demonstrated that the structure of synthase of strain B5786, which features the ability to include oxyhexanoate in polymer even during the growth on monocarbon substrate (CO_2 or acetate, or fructose), on mixed carbon substrate was able to include hydrocarbon acids with an even number of carbon atoms as additional carbon source, capable to accumulate oxyhexanoate in PHA until 6–8 mol %, was similar (homology 99%) to synthase of H16 strain which (opposite to the B5786 strain) is capable to include oxyhexanoate in polymer only at the presence in media carbon substrate (hexanoate or octanoate). The comparison of strain B5786 synthase structure to synthases of microorganisms, also capable to synthesize copolymers of short and medium chain length PHAs, demonstrated the following: homology with synthase from *Rhodospirillum rubrum* was 41%, *Ectothiorhodospira shaposhnikovii* was 26%, *Aeromonas punctata* was 40%, *Thiococcus pfennigii* was 28%, *Rhodococcus ruber* was 38%, and with synthases PhaC1 and PhaC2 from *Pseudomonas* sp. 61-3 was 34 and 37%, respectively. At the same time, *R. rubrum*, *A. punctata*, and *R. ruber* synthases according to their substrate specificity and structure were placed to class I similarly as *R. eutropha*, *Pseudomonas* sp. 61-3 synthases to class II, and synthases from *E. shaposhnikovii* and *T. pfennigii* to class III.

Thus, we revealed that primary structure of *R. eutropha* B5786 synthase to a lesser degree (26–41%) coincides with molecular organization of bacterial synthases that formed short and medium chain length polymers on monocarbon substrate and to a greater extend (99%) with synthases that formed only short chain length polymers. This observation allowed to exclude the presence of a direct connection between molecular organization of PHA-synthases and its' functional properties, namely, the ability to synthesize short and medium chain length PHAs.

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