# **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<sub>SCL</sub>), medium chain length ( $PHA_{MCL}$ ) and long chain length ( $PHA_{LCL}$ ) [2]. PHA<sub>SCL</sub> consisted of monomers with carbon chain length from three to five atoms ( $C_3-C_5$ ), PHA<sub>MCL</sub> (from  $C_6$  to  $C_{14}$ ) and PHA<sub>LCL</sub> (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  $\beta$ -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 threecomponent 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<sub>MCL</sub> synthesis. Currently, in addition to *R. eutro*pha, there were described few more organisms that featured the ability to generate PHA containing PHA<sub>SCL</sub> and PHA<sub>MCL</sub> 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<sup>-</sup> 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 (Gen-Bank accession number J05003) [21]: 5'-GCAATGC-CCGGAGCCGGTTC-3' (792-811) and 5'-GGAGG-GATCCTGCAGGCCTGC-3' (2671-2651). The second primer included additional restriction site BamHI (indicated in bold). PCR mix (50 µl) included: Pfx reaction buffer, 0.05 mM dNTP mix, 1 mM MgCl<sub>2</sub>, 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<sup>-</sup> 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 PHAsynthase gene (*phaC*), was amplified from genomic DNA of *R. eutropha* B5786 and cloned in pBluescript SK<sup>-</sup> 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-

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	10	20	30	40	50	60
R. eutropha H16 R. eutropha JMP134 R. eutropha B5786 R. ruber A. punctata FA440 R. rubrum ATCC25903 R. rubrum AH P. sp. 61-3 (PhaC1)	MATGKGA MATGKGA MATGKGA MATGKGA MANQGSEKTPPDPAR	AASTQEGKS AASTEGKS AASTQEGKS QPSYGPLFE OPSYGPLFE	QPFKVTPGPFD QPFKFPPGPLD QPFKVTPGPFD ALAHYNDKLLA TALGGAAPPSS ERSQKLVTDFI MSNK	DPATWLEWSRQ DPATWLEWSRQ DPATWLEWSRQ ILDHVHKKLKS IMAKAQTERTA SPSPSGPDPAV TRQATSPTGG INSDDLNRQAS	WQGTEGNGH# WQGPEGNGGI TLDPIGWGP# QALLQTNLDI GGGPAVGGD# MDDPMNIGQ# ENTLGLNPVI	AASGI 52 VPGGF 52 AASGI 52 VTSVA 26 DLGQVL 47 ARAFL 39 IFLDMT 60 GLRGK 29
P. sp. 61-3 (PhaC2) E. shaposhnikovii T. pfennigii	*	*	MREK	*	TFINAQSAI1	* CGLRGR 29
R eutropha H16 R. eutropha JMP134 R. eutropha B5786 R. ruber A. punctata FA440 R. rubrum ATCC25903 R. rubrum AH P. sp. 61-3 (PhaC1) P. sp. 61-3 (PhaC2) E. shaqoshnikovii T. pfennigii	PGLDALAGVW PGFEAFAASPLAGVA FGLDALAGVW GRAVRPWQDU EGVLRPWQDU EGVLRPSQPV RQMMSHPERI DLLTSARWU DLLTSTLRSVA	IIAPAQLGDI IIAPAQLGDI IIAPAQLGDI IQAQMNWWQ ILDAQAAVAR VEAQMIMWQ ITQAIKQPIH: AHGLRHPVR	2QRYMKDFSAI 2QRYMRDFTEI 2QRYMKDFSAI RLAKI PAAATF DQLKLMQHTLI DIAALCQAAAK DYLGLWQTTAI SVKHVAHFGIE SARHVLALGGC	WQAMAEGKAE WRGLAGGDTE WQAMAEGKAE KVFNANDPDAP KRLRGEEAAPV RMMGAEAHPV LKNVMFGKSK LLGRVLLGETL	ATGPLHDRRF SAGKLHDRRI ATGPLHDRRI ITPERSDRRF ITPERSDRRF IEPAGDDNRF ATPALGDRRF LQPESDDRRF HTPNPKDNRF	AGDAW 107 ASEAW 112 AGDAW 107 SDTAW 81 KAEAW 102 KDDAV 94 KDEAW 115 NDPAW 84 ADPTW 84
	*					*
R. eutropha H16 R. eutropha JMP134 R. eutropha B5786 R. ruber A. punctata FA440 R. ruberum ATCC25903 R. ruberum AH P. sp. 61-3 (PhaC1) P. sp. 61-3 (PhaC2) E. shaposhnikovii T. nfennieii	RTNL PYRFAAAFYLI HKNAPYRYTAAFYLI RTNL PYRFAAFYLI OENPAYFSLLOSYLA SEQPIYDYLKOSYLI TKDPLEDTIKQGYLI DNNEVFDFIKQTYLI SQNPLYKRYLQYTLS RLNPFYRRSLQAYLS	NARALTELA NARALTELA NRALTELA TRAVVEELT TARHLLASV TARLVATTL TARWMQGVV WRKELHDWI WQKQVKSWI	DAVEA-DAKTF DAVEA-DPKTF DAVEA-DAKTF EAGSGDFLQDG DALEGVPQKSF ENSGGDFACRQ HDVDGMDPHTA GNSKLSEQDIN DESGMSDDDRA	QRIRFAISQW QRIRFAISQW QRIRFAISQW KKARQFA-NLM RERLRFFTRQY RLAFYGRQVV LEKVDFYTRQF RRAHFVIT-LM ARAHFVFAL-L	VDAMSPANFI VDAMSPANFI VDALAPSNFI VDALAPSNFI VNAMAPSNFI DALAPT-NFA VDAMAPSNFA TEAMAPTNSA NDAVSPSNTI	ATNPE 166 ATNPD 171 ATNPE 166 W-NPG 139 ATNPE 162 ATNPE 153 LTNPE 175 -ANPA 142 L-NPL 142
1. pjenngu						
R. eutropha H16 R. eutropha JMP134 R. eutropha B5786 R. ruber A. punctata FA440 R. ruberum ATCC25903 R. ruberum AH P. sp. 61-3 (PhaC1) P. sp. 61-3 (PhaC1) E. shaposhnikovii T. pfennigii	AQRLLI-ESGESLF AQRLLI-ESGESLF VLTRAFETGGASLLF -LLKLTLESDGQNLV VRRTAL-ESGGKSLI VIRET-LDSGEGNLJ AVKR-FFETGGKSLI AIKELFNS-GGNSLV VSFFPIC	* AGVRNMMED AGVRNMMED GARVAAHDI GGARVAAHDI 'RGLALLAED 'KGLEHLLHD 'RGLSHLFDD 'IRPDKLTEEI	LTRGKIS LTRGKIS LNRGGLPLKV- LERSADQLNIF LERGGGRLRPT LURNGGMPS LMHNNGLPS LTFQNKLTQC MLEYSRKLGEC	* QTDESAFEVG QTDESAFEVG QTDESAFEVG LITDESAFELG MSDETAFEVG QVDMGAFEVG QVTKHAFEIG SLQNLADAGDI MQNLLKADQI	* RNVAVTEGAV RNMAVTEGAV ENLAATPGAV RDLALTPGAV RTLAMTPGKV KSLGTTEGAV KTVATTAGSV PSGVTPKEAV DTGVTPKDVV	* VFENE 221 VFENE 226 VFENE 221 VFRND 196 VQRTE 221 VFQNA 212 TIARNS 234 VFRND 199 VFRNE 199 VFRNE 199 YKEDK 50 HREDK 52
R. eutropha H16 R. eutropha JMP134 R. eutropha B5786 R. ruber A. punctata FA440 R. rubrum ATCC25903 R. rubrum AH P. sp. 61-3 (PhaC1) P. sp. 61-3 (PhaC2) E. shaposhnikovii T. pfennigii	* YFQLLQYKPLTD-KV FFQLLQYKPLTD-KV YFQLLQYKPLTD-KV LYELIQYAPTTE-V LMQLLLYAPTTP-KV LMELLQYTPTTE-QV ULELLQYRPTTE-QV LLLYRPQAP-ASVVQ LVLYRYRRP-AQVAT P	* YTRPLLVP HARPLLVP HARPLLVP HAVPLAAP GKTPVLIVP HKRPLLVVP HKRPLLVVP HERPLLVVP HERPLLVVP QTIPLLVY PL	* PCINKYYILDI PCINKYYILDI PCINKYYILDI PWINKYYILDI PWINKYYILDI PWINKYYILDI PQINKYYILDI PQINKYYILDI PQINKYYILDI ALVNRPYMTDI ALVNRPYMTDI N Y D	* QPESSLVRHV QPESSLVRHA QPESSLVRHA RPQNSLVAWL TEKNSLIKYM RPKNSLIKYM SPDKSLARFC SPGNSFVQYA QDDRSTVKGL S	* VEQGHTVFLV VEQGHTVFLV VQHGRTVFLV VQHGRTVFMI VAQGQVFWI IEQGHSVFVI LENNQQTFIV LENNGLQVFVV LEAGMDVYLI LATGQDVYLI	* 280 SWRNP 285 SWRNP 285 SWRNP 280 SYRNP 280 SYRNP 280 SWNP 271 SWVNP 271 SWVNP 273 SWRNP 278 SWRNP 278 SWRNP 258 DWGYP 109 DWGYP 111 P
R. eutropha H16 R. eutropha JMP134 R. eutropha B5786 R. ruber A. puncitat FA440 R. rubrum ATCC25903 R. rubrum AH P. sp. 61-3 (PhaC1) P. sp. 61-3 (PhaC2) E. shaqosha Tikovii T. pfennigii	DASMAGSTWDDYIEH DASMAGCTWDDYIEN DASMAGSTWDDYIEN GVAQAQIDLDDYVVU DAGLAETREDIXLSC EADLAAKSFEDYMVE TKAQREWGLSTYI-D DVRHREWGLSSTYI-D DRADREITMDDYLNG DQADRALTLDDYING	* AAIRAIEVA JAAIRAIEVVA GIATALDVVI GVIAALDGVI GPLAALDGVI GPLAALDIVI JALKEAVDVVI ALKEAVDVVI YLDRCVDEI YYIDRCVDYL	* RDISGQDKINV RDISGQDKINV EEITGSPKIEV EAATGEREVHO TEITGGRALGI KTITGEDSVNA SAITGSKDINM RAITGARDVNI CRRHNLDKINI RETHGVDQVNI	* LGFCVGGTIV PLGFCVGGTIV PLGFCVGGTIV PLGFCVGGTIV PLGFCIGGTAL VGYCIGGTLL AIGYCLGGTLL MGACAGGLTI LGGCQGGTF- LLGICQGGF-	* STALAVLAAF STALAVLAAF AMAAARAFAV SLAMGWLAAF ACTLAVLAAF GCVLSYLEAF CTALLGHYAA AALQGHLQAF SLCYSA	* GG-EHP 339 GG-EHP 344 (G-EHP 339 G-DKR 314 RQKQR 340 -RDKR 310 GQSDR 353 LGEKK 316 RQLRR 317 MHPEK 164 LHSEK 166

**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

	10	20	30	40	50	60
R. eutropha H16 R. eutropha JMP134 R. eutropha JMP134 R. ruber A. punctata FA440 R. rubrum ATC25903 R. rubrum AH P. sp. 61-3 (PhaC1) P. sp. 61-3 (PhaC2) E. shaposhnikovii T. pfennigii	AASVTLLTTLIDFAD VASUTLLTTLIDFAD VSAFTMLNTLLDFAG VSAFTMLNTLLDFAG VKSATLLTTLDFSG VKSATLTTLUDFSG VNALTLUVSVLDTTL VSSASVLVSLLDSGI VQNLITMVTPVDFGI VKNLVTMVTPVDFGI D	* TGILDVFVD TGILDVFVD TGILDVFVD PGELGLTTC PGELGIFIC DSQVALFVD DSQVALFVD DSPATLFAD "PDNIL	* DEGHVQLREATI DEGHVQLREATI DEGHVQLREATI DEGHVQLREATI DEGHVQLREATI DEVILDALDDQ DQSLTYLEGKU DEKTLEAA- DEQTLEAA- SHWVKHVDI SAWVQNVDV	* LGGGAGAPCAJ LGGASGG LGGGAGAPCAJ (RCQGI (AKG) (AKG) (	LIRGUELANTF LIRGVELANTF FLSGKEMAGSF INDGRQLAVSF LDCDLLSMAF LEGRDMAKVF VLEGRDMAKVF VVPGELLNWTF G	* SFLRP 39 SFLRP 39 DMIRA 30 SLLRE 39 NMLRD 38 NHVRA 40 AWMRP 37 'LNLKP 21 'LSLKP 21
R. eutropha H16 R. eutropha JMP134 R. eutropha JMP134 R. ruber A. punctata FA440 R. rubrum ATCC25903 R. rubrum AH P. sp. 61-3 (PhaC1) P. sp. 61-3 (PhaC2) E. shaposhnikovii T. gfennigii	* NDLVWNYVDNYL NDLVNNYVVDNYL KDLVENYWVSNWA- NSLYWNYYIDSYL NDLIMSYFINNYLL- NDLIMSYFINNYLL- NDLIMYWVNNYLL- YQLMGQKYLDMVEVI FSLTGQKYVNMVDLI L	KGNTPVP KGNTPVP KGKPAA KGQSPVA GKTPAA GKDPFP GKEPPA GKEPPA .QKDKLDNF .DEDKVKNF	* PFDLLFWNGDAT PFDLLFWNGDAT FDLLFWNGDAT FDLLAWNEDST FDLLYWNSDST FDLLYWNSDST FDLLYWNSDST FDLLYWNSDST FDLLYWNSDST FDLLYWNSDST FDLLYWNSDST FDLLYWNSDST FDLLYWNSDST W	* TNLPGPWYCW TNLPGPWYCW TSMPAEMHSH TNVAGKTHNSI TRMPAAMQRY TRMPAAMGRY TRLPAAFHGDI PDQVGETYRQI PDQAGETYRQI	* VLRHTYLQNEL VLRHTYLQNEL VLRSLYGRNEL LLRRLYLENQI VLREMYQKNKL VLREMYQKNKL LIEMFKN-NPI LLDFFKH-NPI FIKDFYQKNKI FLKDFYQKNKI N	+ KVPGK 4: KEPGK 4: KVPGK 4: -AEGL 4: VQPGG 44 KDPGG 64 VRANA 4: THPGG 4: MN-GG 2: 'IN-GG 2:
R. eutropha H16 R. eutropha JMP134 R. eutropha B5786 R. ruber A. punciata FA440 R. rubrum AH P. sp. 61-3 (PhaC1) P. sp. 61-3 (PhaC2) E. shaposhnikovii T. pfennigii	* LTVCGVPVDLASIDV LTVCGVPVDLASIDV ITVCGVPVDLASIDV YVLDGQPLNLHDIAC LKIRNTRIDLGKVKT ITVLGHALDLRRIKT UTLGVPIDLRNKT LEVSGTPIDLQKVW LQIGEYEVSLANVTM VLIGDQEVDLRNIKC	* VPTYIYGSRE VPTYIYGSRE VPTYIYGSRE VPTYIYGSRE VPTYIYGSRE VPYLLSARC VPYLLSARC VDSFSVAGIN VDSFSVAGIN VDSFSVAGIN VPVLNIFAEC VPVLNIYPMC	* DHIVPWTAAY/ DHIVPWTAAY/ DHIVPWTAAY/ DHIVPWTSYC DHIALWQGTW( DHIALWQGTW( DHIAPWRSTFF DHITPWKAYYF DHITPWDAYYF DHIVPPDASK/ DHIVPPDASK/ DH	* ASTALLANKLI ASTALLANKLI STALLANKLI JAVNLLGGDVI DGMKLFGGEQI (ATGLYGSPU (SAQLFGGVI SSTLLGGDRI ALAGKYGSKD) ALAGLTSSED	* RFVLGAS RFVLGAS VPULGAS VPUL	* GHIAG 51 GHIAG 55 GHIAG 55 GHIAG 52 GHIAG 52 GHIAG 52 GHIQS 48 GHIQS 48 GHIQS 48 GHIQS 48 GHIG 33 GHI-G 33 GHI-G 33
R. eutropha H16 R. eutropha JMP134 R. eutropha B5786 R. ruber A. punctata FA440 R. rubrum ATCC25903 R. rubrum ATC P. sp. 61-3 (PhaC1) P. sp. 61-3 (PhaC2) E. shaposhnikovii T. pfennigii	VINPPAKNKR VINPPAKNKR AVNPPGKWKKAVG IINPPGNKVY VINPPAKKYA VINPPSNKYC ILNPPGNF ILNPPSNPKSN IYVSGKAQKTVPPAI IYVSGKAQEGVTPAI	* 	* LPESPQQWLAG LPESPQQWLAG LPESPQQWLAG LPADPQVWDBJ LEAESPESWLAG TSLEAESWLEG (KAKDAESWLEG (LSGDPRAWYYI	* SAIEHHGSWWI SAIEHHGSWWI SAIEHHGSWWI SATRYEHSWWI SATPHGGSWWI SATPHGSWWI STKHTDSWWI SGTHVEGSWWI	* PDWTAWLAGOP PDWTAWLAGOP SDWTAWSNKRA PDMAGFIQNRE PDWARWAGYP PDWARWAGYP TDWEAWVEKFT LHWQAWQAERS PRWLSWIQERS	AGAKRA 50 AGAKRA 50 AGAKRA 50 AGELVA 54 AGELVA 54 AGPKVP 55 AGPKVP
R. eutropha H16 R. eutropha JMP134 R. eutropha B5786 R. ruber A. punctata FA440 R. rubrum ATCC25903 R. rubrum AH P. sp. 61-3 (PhaC1) P. sp. 61-3 (PhaC2) E. shaposhnikovii T. ofenniövii	APANYGNA-YRAIE APTEYGSK-RYAAIE AP <b>RPTMAM</b> RYRAIE PPAMGSTARPPI- VPAR-VPEEGLA ARDPTKAPRPPI-E ARVPAMPACRCLAMI SPTSLONKAY-PSGE TLMALGNQNY-PPME	* PAPGRYVKA PAPGRYVKA DAPGRYVKS PAPGHYVKV DAPGSYFKV RAIT	* KA KA KA RLNPVFACPTH 'RI 'R	+ EEDAA		* 58 59 50 50 50 50 50 50 50 50 50 50 50 50 50



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

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

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

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<sub>SCL</sub> and PHA<sub>MCL</sub> (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-syn-

**Table 2.** Structure of PHAs synthesized by *Ralstonia eutropha* 

 B5786 during the growth different carbon sources

Source	PHA structure, mol %				
Source	butyrate C <sub>4</sub>	valerate C <sub>5</sub>	hexanoate C <sub>6</sub>		
Fructose	93.6	5.7	0.7		
CO <sub>2</sub>	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		

thases from *R. eutropha* bacterial strain B5786 were similar to synthases from *T. pfennigii* and *A. punctata* on 40 and 28%, respectively.

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<sub>SCL</sub> and PHA<sub>MCL</sub> [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 PHAnegative mutant P. putida GPp104 and R. eutropha PHB<sup>-4</sup>, we demonstrated that only synthases from the pha locus coded by phaC1 and phaC2 genes showed more substrate affinity and ability to synthesize PHA<sub>SCL</sub> and PHA<sub>MCL</sub> [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  $6-8 \mod \%$ , 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 bacte-

rial 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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