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ORIGINAL ARTICLE

Amplification and sequence analysis of *phaC* gene of polyhydroxybutyrate producing *Vibrio azureus* BTKB33 isolated from marine sediments

Raghul Subin Sasidharan[1] · Sarita Ganapathy Bhat[1] · Muthusamy Chandrasekaran[1,2]

Abstract Vibrios have shown their potential as polyhydroxyalkanoates (PHAs) producers, but until recently little information was available about their PHA-related genes. The present study attempts to characterize the *phaC* genes from a potent PHA-accumulating bacterium, *Vibrio azureus* strain BTKB33, isolated from marine sediments. The molecular detection of class I PHA synthase gene in the *V. azureus* strain BTKB33 gave the required amplicon and was confirmed by subsequent seminested PCR; however, a class II PHA synthase gene was not detected. The in silico characterization of the PCR product helped to deduce the presence of class I PHA synthase, particularly a polyhydroxybutyrate polymerase. Sequence alignment of the nucleotide sequence of class I PHA synthase of strain BTKB33 and other related *Vibrio* sp. showed intra-generic variation within genus *Vibrio* and this is revealed in the dendrogram. The multiple sequence alignment of the in silico-translated *phaC* gene of BTKB33 with the protein sequence of PHA synthase of related organisms showed the conserved regions of protein sequences within the genus *Vibrio* and the dendrogram constructed showed the relatedness based on the deduced amino acid sequences.

Introduction

Bacteria accumulate polyhydroxyalkanoates (PHAs) under unbalanced growth conditions of a carbon substrate in excess of other nutrients like nitrogen, sulfur, phosphorus or oxygen (Madison and Huisman 1999; Kim and Lenz 2001). PHAs are strong candidates for biodegradable plastics due to their properties either as thermoplastics or as elastomers, and therefore find applications in domestic (Glazer and Nikaido 1994), agriculture (Holmes 1985; Hocking and Marchessault 1994; Dobbelaere et al. 2001), marine (Asrar and Gruys 2002), medicine (Williams and Martin 2002) and industrial (Chen et al. 2000; Bucci and Tavares 2005) fields. Approximately 140 different hydroxyalkanoic acids are known to be incorporated into PHAs (Steinbuchel 2005), with microbial species from over 90 genera being reported to accumulate these polyesters (Zinn et al. 2001). The variation in PHA composition in bacteria influence its physical and chemical properties and can be modified by several factors, including the type of microorganism in which PHA synthase, a crucial enzyme in PHA synthesis, plays a key role, medium composition and production conditions (Keshavarz and Roy 2010; Singh et al. 2014).

PHAs are mainly of two types, short-chain-length PHAs (scl-PHAs) with 3–5 carbon atoms and medium-chain-length PHAs (mcl-PHAs) with 6–14 carbon atoms. The number of carbon atoms in their side chains modifies their mechanical properties. The scl-PHAs are generally thermoplastics, whereas mcl-PHAs are elastomers. The composition of the monomeric units depends on the substrate used and a class of highly versatile enzymes, the PHA synthase, which are not specific to

✉ Sarita Ganapathy Bhat
saritagbhat@gmail.com

1   Department of Biotechnology, Cochin University of Science and Technology, Kalamassery, Cochin-22, Kerala, India

2   Department of Botany & Microbiology, College of Science, King Saud University, PB No. 2455, Riyadh 11451, Kingdom of Saudi Arabia

any one type of hydroxyalkanoic acid (Steinbuchel et al. 1992), but nevertheless crucial in all PHA synthesis (Anderson and Dawes 1990; Madison and Huisman 1999; Rehm and Steinbuchel 1999).

PHA synthases can be categorized into three types based on their primary amino acid sequences and in vivo substrate specificities (Rehm and Steinbuchel 1999). Class I PHA synthases are preferentially active towards coenzyme A thioesters of various scl 3-hydroxyalkanoates comprising three to five carbon atoms, exemplified by polyhydroxybutyrates (PHB), the smallest known PHA having myriad applications in medicine and industry. Class II PHA synthases are active towards coenzyme A thioesters of various mcl-3 hydroxyalkanoates comprising at least five carbon atoms; both types being encoded by the same *phaC* gene (Trotsenko and Belova 2000). Class III PHA synthases detected in *Chromatium vinosum* comprise two different subunits, the *phaC* and the *phaE* (Trotsenko and Belova 2000), and they prefer coenzyme A thioesters of scl 3-HA. There are reports that bacteria like *Rhodococcus ruber* (Haywood et al. 1991) and *Aeromonas caviae* (Fukui et al. 1998) have PHA synthases that exhibit specificity for both scl and mcl-PHAs. McCool and Cannon (2001) reported a fourth class of PHA synthases in *Bacillus megaterium,* consisting of two subunits, *phaC* (Bm) and *phaR* (Bm).

Based on the organization of the gene locus and the structure–function properties of PHA synthase enzymes, the PHA biosynthesis genetic system (*pha* gene locus) is divided into four (Solaiman and Ashby 2005). The class I locus consists of *phaC* (coding for PHA synthase), *phaA* (β-ketothiolase), and *phaB* (acetoacetyl-CoA reductase) genes, as observed in *Wautersia eutropha*. The class II locus consists of an operon with two PHA synthase genes (*phaC1* and *phaC2*) flanking a PHA depolymerase gene (*phaZ*), as seen in *Pseudomonas* sp. (Solaiman and Ashby 2005). The class III pha operon with class III PHA synthases and the *phaA* and *phaB* genes, transcribed in the opposite direction, are found adjacent to these two synthase genes. The class IV pha loci comprising *phaR* and *phaC* coding for the two hetero-subunits of the active PHA synthase and a *phaB* between the two has been reported in *Bacillus megaterium* (McCool and Cannon 2001).

Phenotypic detection methods such as Sudan black B (Schlegel et al. 1970), Nile blue A (Ostle and Holt 1982) and Nile red (Spiekermann et al. 1999) staining methods have been widely used for screening PHAs accumulation in microbes. These methods are laborious and do not involve differentiation of PHA types. Hence, alternative methods like Fourier transform infrared spectroscopy (Hong et al. 1999) and the polymerase chain reaction (PCR)-based technique (Sheu et al. 2000; Solaiman et al. 2000; Romo et al. 2007) can be used. Vibrios from marine sediments were among the first bacteria to be reported as PHA producers (Baumann et al. 1971; Oliver and Colwell 1973). PHA production in vibrios

isolated from marine environments have been reported by several workers (Sun et al. 1994; Rawte and Mavinkurve 2004; Chien et al. 2007; Arun et al. 2009; Wei et al. 2011; Sasidharan et al. 2014). These ubiquitous microbes from the aquatic environment have been implicated as significant PHA producers. The search for novel microbial PHA producers has yielded a potential PHA producing marine *Vibrio azureus* strain BTKB33 from marine sediments. A PHB content of 426.88 mg/g of cell dry weight was obtained and major bioprocess variables influencing its PHB production were optimized (Sasidharan et al. 2014). Information about the presence of PHA synthase genes in vibrios has been reported only by complete genome sequence analysis of vibrios and is available in NCBI (Heidelberg et al. 2000; Makino et al. 2003; Goudenège et al. 2013). Until recently, little information was available about the *phaC* genes in vibrios. This study discusses the detection and differentiation of the PHA synthase gene by PCR-based analysis and the in silico analysis of *phaC* genes in a potent PHB accumulating marine bacterium, *V. azureus* strain BTKB33.

Materials and methods

Bacterial strains and growth conditions

PHA-producing bacterial strain *V. azureus* strain BTKB33 (Accession No. HM346671), deposited in Microbial Culture Collection, Microbial Genetic Lab, Dept. of Biotechnology, Cochin University of Science and Technology, Cochin, India, was used. Nutrient agar medium (HiMedia, Mumbai, India) was used for maintenance of the culture. The strain BTKB33 was grown in nutrient medium with 1 % NaCl at 35 °C on a rotary shaker at 120 rpm for 12 h.

Molecular characterization of PHA synthase gene

Genomic DNA extraction was according to Sambrook et al. (2000). The purity of extracted DNA was checked by reading the absorbance ratio A_{260}/A_{280} using a UV–VIS spectrophotometer (Shimadzu, Japan), followed by the agarose gel electrophoresis (GeNei, Bengaluru, India) and the visualization with G: BOX Fluorescence Gel Documentation system (Syngene, USA).

PCR and semi-nested PCR detection for class I PHA synthase gene

The class I PHA synthase gene represents the gene for scl-PHAs. The PCR reaction for the class I PHA synthase gene (*phaC*) was performed according to Sheu et al. (2000) and the primer details are as in Table 1. The PCR-based detection involved two steps. In the first PCR reaction, the forward

Table 1 Details of primers used for PCR analysis of *phaC* gene

Primer	Sequence 5'-3'	Reference
CF1	ATCAACAARTWCTACRTCYTSGACCT	Sheu et al. (2000)
CF2	GTSTTCRTSRTSWSCTGGCGCAACCC	Sheu et al. (2000)
CR4	AGGTAGTTGTYGACSMMRTAGKTCCA	Sheu et al. (2000)
I-179 L	ACAGATCAACAAGTTCTACATCTTCGAC	Solaiman et al. (2000)
I-179R	GGTGTTGTCGTTGTTCCAGTAGAGGATGTC	Solaiman et al. (2000)

primer phaCF1 and the reverse primer phaCR4 were used. The PCR mixture consisted of 0.5 μL of 100 ng/mL template DNA (100 ng/mL), 2 μL of 2.5 mM of dNTP, 1U units of Taq DNA polymerase (Sigma-Aldrich, India), 2 μL of 10× Taq buffer, 1 M of betain, 0.03 % of DMSO, 1 μL of acetylated BSA, 5 μL of each primers and added sterile distilled water to make a final volume of 20 μL. PCR mixture was pre-incubated at 94 °C for 5 min, 51 °C for 2 min, and 72 °C for 2 min. The PCR cycle consisted of 20 s of denaturation at 94 °C, 45 s of annealing at 61 °C (decreased by 1 s/cycle), and 1 min of extension at 72 °C. This cycle was repeated 35 times and then incubated at 72 °C for 10 min for the final extension. PCR was performed in a thermal cycler (Bio Rad, USA) and the predicted PCR product was ~496 bp in length.

A semi-nested PCR was performed using the above PCR product for the confirmation of the scl-PHA synthase gene in strain BTKB33 using primers phaCF2 and phaCR4. For positive products, 1 μL of 100-fold diluted colony PCR products was used for semi-nested PCR. For negative products, 1 μL of undiluted colony PCR products was directly supplied as DNA templates for semi-nested PCR. PCR reactions were done as detailed above and a predicted product of ~406 bp indicates the presence of positive amplification, visualized after agarose gel electrophoresis (GeNei) with G: BOX Fluorescence Gel Documentation system (Syngene).

PCR for class II PHA synthase gene

The class II PHA synthase gene codes for mcl-PHAs. PCR for class II PHA synthase genes was performed in a thermal cycler (Bio Rad) with the primers I-179 L and I-179R (Table 1) according to Solaiman et al. (2000).

The PCR reaction mixture consisted of 0.5 μL of template DNA (100 ng/mL), 2 μL of 2.5 mM of dNTP, 1 U of Taq DNA polymerase (Sigma-Aldrich, India), 2 μL of 10× Taq buffer, 5 μL of each primers and added sterile distilled water to a final volume of 20 μL. The PCR mixture was pre-incubated at 94 °C for 5 min, 51 °C for 2 min, and 72 °C for 2 min. The PCR cycle was repeated 35 times and consisted of 20 s of denaturation at 94 °C, 45 s of annealing at 57 °C and 1 min of extension at 72 °C, followed by incubation at 72 °C for 5 min and a final incubation at 4 °C (Sujatha et al. 2005). The expected positive PCR product was ~540 bp.

In silico analysis of the sequence

Products after PCR amplification were purified by a gene cleaning kit (GeNei) and were sequenced by Sanger's Dideoxy method using an ABI 3730 DNA Analyzer (Applied Biosystems, USA). The sequenced PCR products were compared with those available from GenBank using online BLAST tools: nucleotide blast (http://www.ncbi.nlm.nih.gov/blast). Nucleic acid sequences of other PHA synthase genes were obtained from NCBI database. Multiple sequence alignment for the selected nucleotide sequences was done by using the Clustal X program (Thompson et al. 1997) in BIOEDIT software (Hall 1999). The phylogenetic tree was constructed using the Neighbor-Joining method (Saitou and Nei 1987) using the nucleotide-based TN84 evolutionary model in MEGA5: Molecular Evolutionary Genetics Analysis (MEGA) software ve.5.0 (Tamura et al. 2011). The evolutionary distances were computed using the Tajima-Nei method (Tajima and Nei 1984) and are in the units of the number of base substitutions per site. Statistical support for branching was estimated using 1000 bootstrap steps.

The nucleic acid sequence of the PHA synthase from BTKB33 was translated in silico into its corresponding protein sequence by Expasy (http://web.expasy.org/translate) and the deduced amino acid sequence were compared with other protein sequences using Conserved Domain Database services available in NCBI (Marchler-Bauer et al. 2011) using online BLAST tool, blastx (http://www.ncbi.nlm.nih.gov/blast). Multiple sequence alignment for the assembled amino acid sequences was carried out by using the Clustal W program in BIOEDIT software (Hall 1999). The phylogenetic tree was constructed as detailed above with 1000 bootstrap steps. The evolutionary distances were computed using the method outlined by Nei and Kumar (2000) and are in the units of the number of amino acid differences per sequence.

Results and discussion

In this study, two separate PCR-based protocols were adopted for the detection and differentiation of the PHA synthase gene in strain BTKB33. The method outlined by Sheu et al. (2000)

for the detection of the class I PHA synthase gene (scl-PHA synthase gene) gave the desired amplicon of ~496 bp for the strain BTKB33 and a ~406-bp amplicon for the seminested PCR of the previously obtained PCR products and is represented in Fig. 1. Preliminary PCR analysis showed that strain BTKB33 harbored the class I PHA synthase gene type representing single-chain-length (scl) PHAs. The class II PHA synthase gene (mcl-PHA synthase gene) was not detected by this method (result not shown). Class I PHA synthases among others are important as they are preferentially active towards coenzyme A thioesters of scl 3-HA comprising three to five carbon atoms, including polyhydroxybutyrate (PHB), polyhydroxyvalerate (PHV), polyhydroxybutyrate-valerate (PHBV), etc. There are previous reports on the presence of PHB in vibrios (Chien et al. 2007; Arun et al. 2009; Wei et al. 2011). PHB is the smallest molecule among scl-PHAs coded by class I PHA synthase gene (Anderson and Dawes 1990; Madison and Huisman 1999); the results of the present study concur with the previous one. Chien et al. (2007) studied PHA accumulation in marine vibrios from sediments and identified it as only PHB. It was reported that vibrios produce only PHB despite alteration in carbon sources and these findings conclude that the variety of PHAs produced in marine environments may not be as diverse as in other environments. PHB production in *Vibrio* sp. was evidenced by the structural characterization by FTIR and NMR analysis (Chien et al. 2007; Arun et al. 2009; Sasidharan et al. 2014).

In silico analysis of the partial sequence of *phaC* gene

The semi-nested PCR product of the class I *phaC* gene from BTKB33 was sequenced and showed 98 % similarity with the PHA synthase gene of *V. parahaemolyticus* RIMD 2210633 (Accession no. BA000032) following BLAST analysis. This outcome indicated that the partial gene sequence obtained was
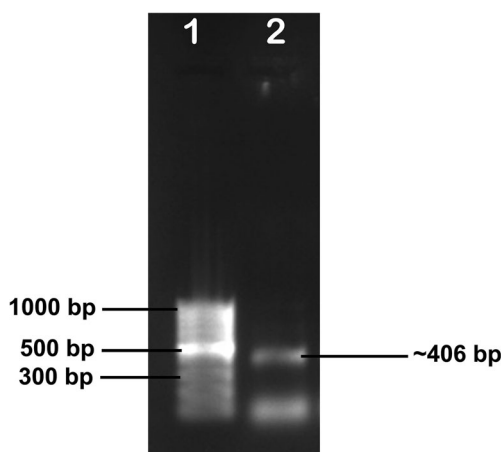

Fig. 1 Semi-nested colony PCR for the identification of single-chain-length (scl) PHA producers; Estimated PCR product length ~406 bp. Lane *1* 100-bp ladder; lane *2* PCR product of *Vibrio azureus* BTKB33

indeed that encoding class I PHA synthase and was submitted to GenBank with accession number JQ781052. The class I *phaC* gene has been observed in the complete genome analysis of vibrios such as *V. cholerae, V. parahaemolyticus, V. alginolyticus, V. splendidus, V. harveyi, V. rotiferianus, V. nigripulchritudo, V. owensii, V. campbellii* and *V. vulnificus* and is available in the NCBI database (http://www.ncbi.nlm.nih.gov/). This preliminary study helped to conclude that a PHA synthase class I, specifically a PHB polymerase is present in *V. azureus* BTKB33. To date, no other information has been reported as to the presence of class II PHA synthase in vibrios. The result of this study is highly relevant considering that this sequence (JQ781052) of the *phaC* gene is the first deposit in GenBank from the *V. azureus* species of genus *Vibrio*, as the review of the description of the *V. azureus* (Yoshizawa et al. 2009) has not included PHA production by *V. azureus*. This report adds to the description of this organism and hence can be used as a variable in taxonomic evaluation of the species.

Multiple sequence alignment of the nucleotide sequences of class I PHA synthase from *V. azureus* strain BTKB33 with other related *Vibrio* sp. is represented in Fig. 2. Nucleotide sequence from *V. azureus* strain BTKB33 in comparison showed maximum similarity with that of PHA synthase from *V. parahaemolyticus* strain RIMD 2210633 (Accession no. BA000032) and *V. parahaemolyticus* strain BB22OP (Accession no. CP003973), forming a clade. The alignment showed intra-generic variation within genus *Vibrio*. A dendrogram was constructed with the nucleotide sequence of the class I *phaC* gene of *V. azureus* strain BTKB33 with other related *Vibrio* sp. (Fig. 3). From the tree, it was observed that all the *Vibrio* strains claded together to form a single clade and separated from the out group *Paracoccus denitrificans* (Accession no. CP000489). The partial nucleic acid sequence of the *phaC* gene obtained was translated into its amino acid sequence and aligned using PSI-BLAST. An ORF encoding 74 amino acid residues was obtained, with maximum E value of 6e-43 and 93 % identity with the amino acid sequence of the *phaC* gene of *V. alginolyticus* strain 40B (Accession no. NZ ACZB01000148). The deduced amino acid sequence was deposited in GenBank under accession number AFK87747. The multiple sequence alignment of this deduced 74 aminoacid sequence from strain BTKB33 (Accession no. AFK87747), with related bacteria from NCBI (Fig. 4), showed maximum similarity with PHA synthase of other *Vibrio* sp. like *V. alginolyticus* 40B (Accession no. ZP 06182878), *V. harveyi* 1DA3 (Accession no. ZP 06174179), *V. parahaemolyticus* AQ4037 (Accession no. ZP 05908852) and *Vibrio* sp. Ex25 (Accession no. ZP04921203) included in this study. In addition, the alignment showed some intra-generic variation within genus *Vibrio*; the divergence of amino acid sequences is indicated in Fig. 4. On the analysis of the multiple sequence alignment, it was noted that the translated
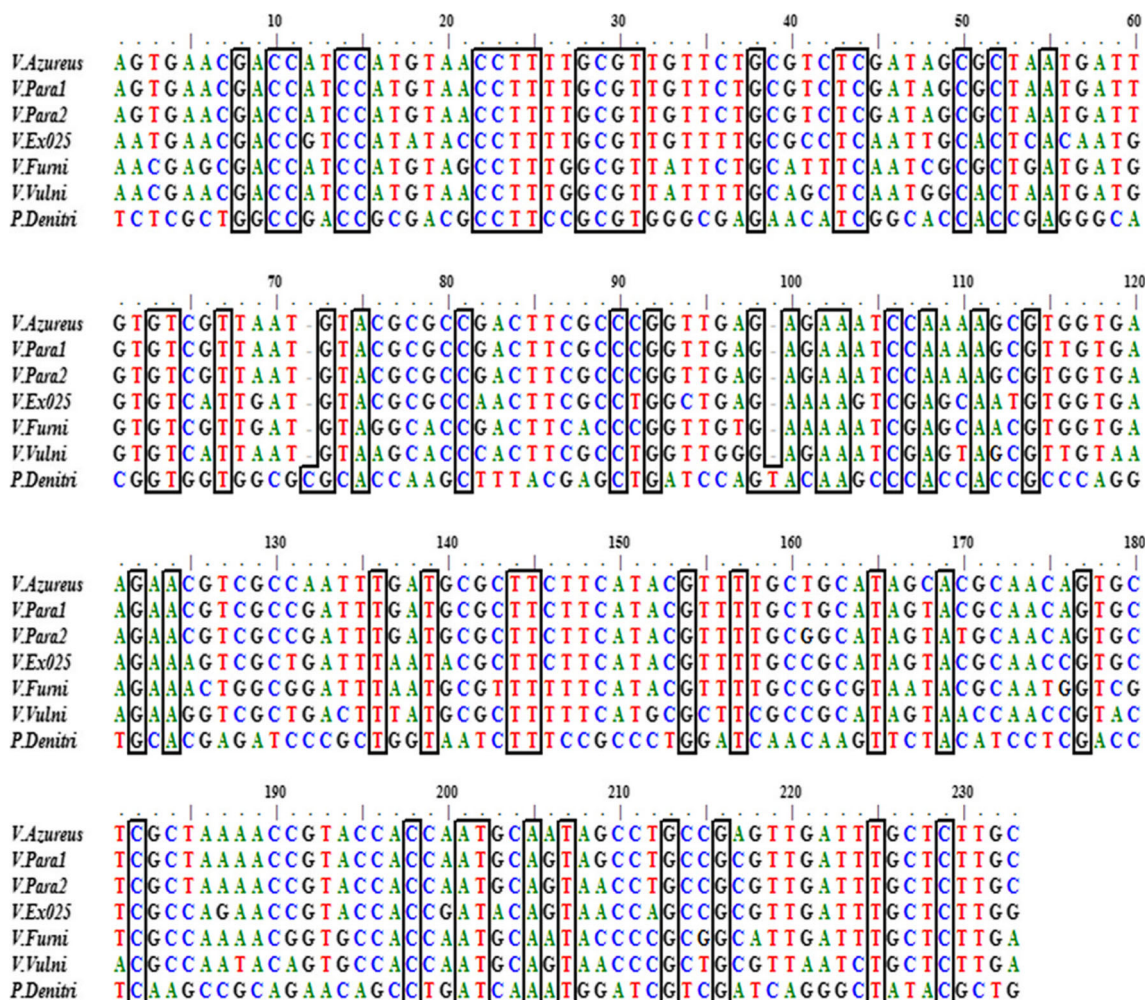
Fig. 2 Multiple sequence alignment of the nucleotide sequences of class I PHA synthase from *V. azureus* strain BTKB33 and other related *Vibrio* sp. The nucleotide sequences were aligned using Clustal W programme using BIOEDIT software. The identical sequences are marked inside the outline. *V.Azureus*, *V. azureus* strain BTKB33 (JQ781052); *V.Para1*, *V. parahaemolyticus* strain RIMD 2210633 (BA000032); *V.Para2*, *V. parahaemolyticus* strain BB22OP (CP003973); *V.Ex025*, *Vibrio* sp. Ex25 (CP001806); *V.Furni*, *V. furnissii* (CP002378); *V.Vulni*, *V. vulnificus* (AE016796); *P.Denitri*, *Paracoccus denitrificans* (CP000489). GenBank accession numbers are presented in *brackets*

phaC gene sequence of BTKB33 had serine instead of alanine in the 6th position. Likewise, there was cysteine instead of tyrosine in the 22nd and leucine instead of alanine in the 34th positions. Apart from these differences, the multiple
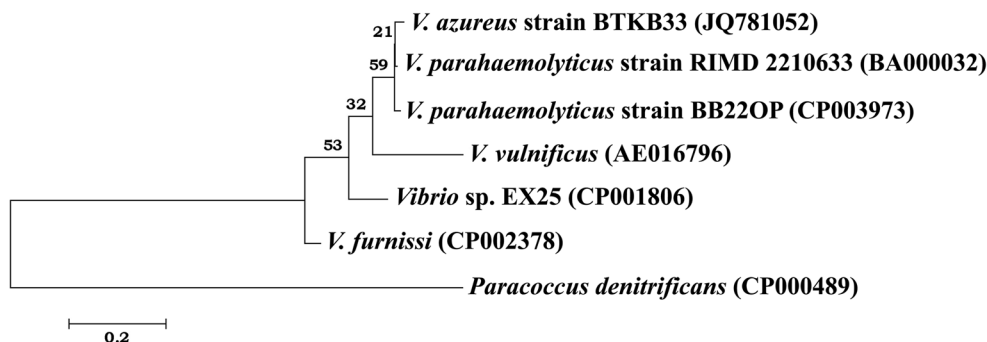

Fig. 3 Phylogenetic relationship based on nucleotide sequences of class I PHA synthase gene from *V. azureus* strain BTKB33 with related taxa. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The *numbers at the nodes* indicate the levels of bootstrap support based on 1000 replicates. The evolutionary distances were computed using the Tajima–Nei method (Tajima and Nei 1984) and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA 5.0 (Tamura et al. 2011). *Paracoccus denitrificans* (Accession no. CP000489) was used as outgroup
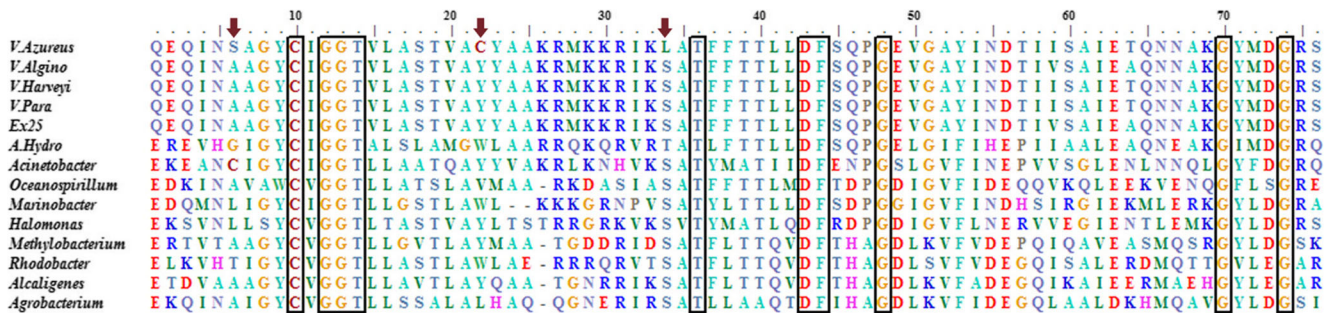
Fig. 4 Multiple sequence alignment of the amino acid sequence of class I PHA synthase from *V. azureus* strain BTKB33 and other related bacteria. The protein sequences were aligned using Clustal W programme using BIOEDIT software. The identical sequences are marked inside the outline. *V.Azureus*, *V. azureus* strain BTKB33 (AFK87747); *V.Algino*, *V. alginolyticus* 40B (ZP 06182878); *V.Harveyi*, *V. harveyi* DA3 (ZP 06174179); *V.Para*, *V. parahaemolyticus* AQ4037 (ZP 05908852); *Ex25*, *Vibrio* sp. Ex25 (ZP04921203); *A.Hydro*, *Aeromonas hydrophila* (AAL77053); *Acinetobacter*, *Acinetobacter* sp. (AAA52191); *Oceanospirillum*, *Oceanospirillum* sp. MED 92 (ZP 01166141); *Marinobacter*, *Marinobacter manganoxydans* (ZP 09161036); *Halomonas*, *Halomonas* sp. TD01 (ZP 08636397); *Methylobacterium*, *Methylobacterium nodulans* ORS 2060 (YP 002501157); *Rhodobacter*, *Rhodopseudomonas palustris* HaA2 (YP 486582); *Alcaligenes*, *Alcaligenes eutrophus* H16 (AAB24910); *Agrobacterium*, *Agrobacterium tumefacians* str. C98 (NP 354598). GenBank accession numbers are presented in *brackets*

sequence alignment clearly revealed the conserved regions of the protein sequences of PHA synthase within the genus *Vibrio*, if only in this short stretch.

A dendrogram was constructed with deduced amino acid sequence of class I *phaC* gene of *V. azureus* strain BTKB33 with other related bacteria and is presented in Fig. 5, which showed that all the *Vibrio* strains claded separately from the rest of the bacteria included in the study. Within the clade, *V. azureus* strain BTKB33 showed some divergence from other *Vibrio* sp. due to

the amino acid variations of the class I PHA synthase among them, as already revealed in the sequence alignment analysis.

A comparison using the putative conserved domains search service (RPS-BLAST) revealed that the translated protein sequence of *phaC* of *V. azureus* strain BTKB33 showed maximum resemblance to the conserved domain model TIGR01838, with an E value of 2.18e-37, which represented the class I subfamily of poly(R)-hydroxyalkanoate synthases, which polymerizes hydroxyacyl-CoAs with three to five

Fig. 5 Phylogenetic tree of deduced amino acid sequence of class I PHA synthase from *V. azureus* strain BTKB33, related *Vibrio* sp. and other bacteria. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the method (Nei and Kumar 2000) and are in the units of the number of amino acid differences per sequence. The analysis involved 15 amino acid sequences. Evolutionary analyses were conducted in MEGA 5.0 (Tamura et al. 2011)
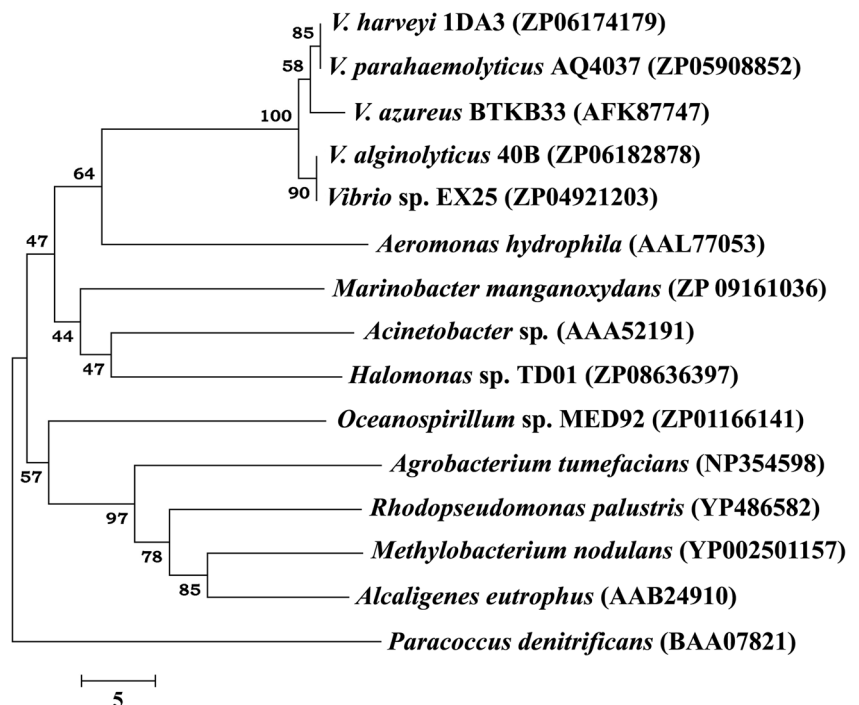
carbons in the hydroxyacyl backbone into aliphatic esters, termed poly(R)-hydroxyalkanoic acids.

Conclusion

The outcome of this study allowed us to conclude that the partial *phaC* gene sequence of *Vibrio azureus* strain BTKB33 is of class I PHA synthase, especially a poly-β-hydroxybutyrate polymerase. The PHAs extracted from vibrios were characterized as PHB and the molecular characterization of the PHA synthase gene in this study supports these previous findings. PHB are of considerable interest to the polymer industries due to the similarity of their physical and chemical characteristics to synthetic plastics. Their biodegradable nature makes them most interesting potential natural product. This study shows the relatedness of the PHA synthase in strain BTKB33 to that in other vibrio species and their differences from distant organisms based on in silico characterization of the PHA synthase gene. To conclude, marine vibrios which are fast growing and easy to manipulate organisms can serve as an important source for PHB production.

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