Computational Molecular Biology

2013, Vol.3, No.3, 16-23 http://cmb.sophiapublisher.com

BioPublisher

Research Report Open Access

Identification of a Novel *phaC1* Gene from Native *Pseudomonas putida* KT2442 as a Key Gene for PHA Biosynthesis

Jamshid Raheb, Saeed Saadat, Fatemeh Nazari

National Institute of Genetic Engineering and Biotechnology, Tehran, Iran

Corresponding Author email: jamshid@nigeb.ac.ir; Author

Computational Molecular Biology, 2013, Vol.3, No.3 doi: 10.5376/cmb.2013.03.0003

Copyright © 2013 Raheb et al. This is an open access article published under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Abstract The global dependence on petroleum-derived plastics has increased dramatically over the years. They have harmful effects on nature that leading scientists to attention to the biodegradable polymers. Polyhydroxyalkanoates (PHAs) are a family of polyhydroxy esters of 3, 4, 5 and 6 hydroxyalkanoic acids produced by a wide range of bacteria such as *pseudomonas* bacteria by PHA synthetase gene, a key enzyme for PHA biosynthesis, as granules in the cytoplasm of the cells under growth limiting conditions with carbon excess. In this study, PHA produced by native *Pseudomonas putida* strain was investigated by Methanolysis and FT-IR methods. After proving the ability of bacteria to produce PHA, by using appropriate primers and the polymerase chain reaction, PHA synthetase gene of the organism, was amplified, sequenced and compared to NCBI registered sequences. The results showed that the bacteria is capable of producing PHA. The similarity of the sequenced gene with the other registered sequences of the NCBI site can be 80 to 90 percent that showed new ORF, in this bacteria. Therefore, this gene was named *phaC1*. Increasing research on biopolymers has been ongoing in both Europe and Japan and to some extent in the US. Private sector is the main funder of biopolymer research in the EU. In conclusion, with the currently increased interest level and the resulted extensive research being carried out in this area, PHAs are potentially emerging as the next generation of environmentally friendly materials with a wide range of applications.

Keywords Biodegradable polymers; Polyhydroxyalkanoates; *Pseudomonas putida*

Introduction

Hydrocarbon wastes from petroleum-derived plastics produce harmful toxic compounds during their lengthened decomposition periods. Depleting natural resources resulted from contemporary lifestyle and overpopulation of the world demands rational, efficient and sustainable use of environment potentials. Short production/degradation cycle of environmentally friendly plastic materials such as polyhydroxyalkanoic acids (PHAs) reduces unfavorable wastes and emissions (Koller et al., 2010; Levis and Barlaz, 2013).

Under nutrient-limited conditions, various bacteria produce these carbon and energy storage polyesters (Liu et al., 2013). These biopolymers are accumulated as granules in bacterial cytoplasm when a carbon source is provided in excess and one essential growth nutrient is limited (Tanamool et al., 2013). PHAs are optically active biopolyoxoesters composed of (R) 3-hydroxy fatty acids, representing a complex class of biopolymers (Martínez et al., 2012). Hydroxyalkanoic acids (HAs), monomers of polyhydroxyalkanoates, are R-enantiomerically pure chemicals (Adkins et al., 2012). Since PHAs are similar to petrochemical polymers in physical properties, these bacterial biodegradable thermoplastics are considered promising alternatives for petro-chemically derived plastics (Poli et al., 2011). There are two classes of PHAs depending upon the carbon chain length of the monomer units. PHAs containing repeat-unit monomers of 3 to 5 carbon atoms are classified as short-chain-length (SCL-) PHAs. On the other hand,

Preferred citation for this article:

Raheb et al., 2013, Identification of a Novel *phaC1* Gene from Native *Pseudomonas putida* KT2442 as a Key Gene for PHA Biosynthesis, Computational Molecular Biology, Vol.3, No.3 16-23 (doi: 10.5376/cmb.2013.03.0003)

Received: 06 Nov., 2013 | Accepted: 05 Dec., 2013 | Published: 26 Dec., 2013

medium-chain-length (MCL-) PHAs are comprised of 6 to 14 carbon atoms (Gangoiti et al., 2012). Chiral building blocks of polyhydroxyalkanoates monomers may be utilized for synthesis of fine chemicals like antibiotics, vitamins, aromatics and pheromones (Guo-Qiang, 2009). It has been reported that some of polyhydroxyalkanoic acids have antimicrobial activity (Shuping, 2013; Ruth, 2007). PHA biosynthesis genes vary in different PHA-producing organisms. PHA biosynthesis loci have been categorized into three major classes. In the type I system, PHA synthase-encoding gene (*phbC*) is adjacent to *phbA* and *phbB*, as exemplified by the pha locus of *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*). These two genes, respectively code for *b-ketothiolase* and *acetoacetyl-CoA* reductase, two enzymes closely related to the biosynthesis of scl-PHA. A locus in which two synthase genes (*phaC1* and *phaC2*) are separated by a PHA-depolymerization gene (*phaZ*) is of type II PHA genetic organization system. The mcl-PHA-producing Pseudomonads bears the type II system. In type III PHA biosynthesis genetic organization, the synthase enzyme is composed of two polypeptide subunits encoded by *phbE* and *phbC* genes. The *phbA* and *phbB* genes are also located in this locus, but are usually transcribed divergently from the *phbE* and *phbC* genes. This type has been observed in *Chromatium vinosum*, *Synechocystis* spp, and *Thiocystis violacea*. Later, the pha locus of *Bacillus megaterium* was characterized as type IV PHA biosynthesis genetic system (Hernandez-Eligio et al., 2011; Poirier et al., 1995). In this paper, we report a rapid and sensitive PCR procedure for isolation and amplification of PHA synthase gene, *phaC1*, in a native *Pseudomonas* strain. The main aim of our work was the cloning of *phaC1* gene from native *Pseudomonad* strains to be expressed later in an appropriate host for the intention of production of biodegradable plastics, hence substitute the use of petroleum-derived polymers as a non-degradable plastic, Because of PHA synthase is a key enzyme essential for bacterial synthesis of polyhydroxyalkanoic acid biodegradable polyesters.

1 Results

1.1 FT-IR analysis and confirmation of PHA production

Purified PHB, mcl-PHA and P(HB + mcl-HA) showed their strongest band at 1728 /cm, 1740 /cm and 1732 /cm respectively in FT-IR spectra (Shamala et al., 2009). The strongest band in spectrum of mcl-PHA was methylene C-H vibration near 2928 /cm. Other characteristic band for mcl-PHA was visible near 1165 /cm in spectrum (Figure 1). It is demonstrated that the band between 1728 /cm and 1744 /cm is characteristic of PHA (Shamala et al., 2009).

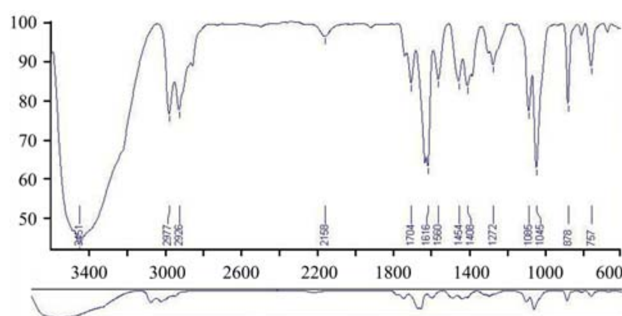

Figure 1 FT-IR analysis of PHA produced by native *Pseudomonads*

1.2 PCR amplification of *phaC1* gene

The PCR protocol was developed using genomic DNAs purified from *P. putida*. This organism has been reported to produce mcl-PHA. In this study, a distinct 540-bp PCR product was obtained (Figure 2). The size of the PCR product agrees with the length of the *phaC1* and *phaC2* genes flanked by the I-179L/I-179R primer-pair. When ELONGASE, formulated by its manufacturer for long PCR amplification was used, an additional ca. 3.4-kb amplicon was observed which represents the DNA sequence flanked by the I-179L and I-179R annealing sites in the *phaC1* and *phaC2* genes, respectively, and included the entire *pha Z* gene. The PCR protocol developed using the purified genomic-DNAs was then tested for the detection of *phaC* gene sequences in lysates prepared by heating the suspension of bacterial colonies. The results showed that the characteristic 540-bp PCR product was produced even from the crude cell-lysates of microorganisms shown to contain *phaC1* and *phaC2* genes (Steinbuchel and Schlegel, 1991).

1.3 Sequence analysis of the amplicon from *phaC1* gene

The 540-bp PCR product from native *P. putida* sample was purified and sequenced. The purification product is showed in Figure 3.

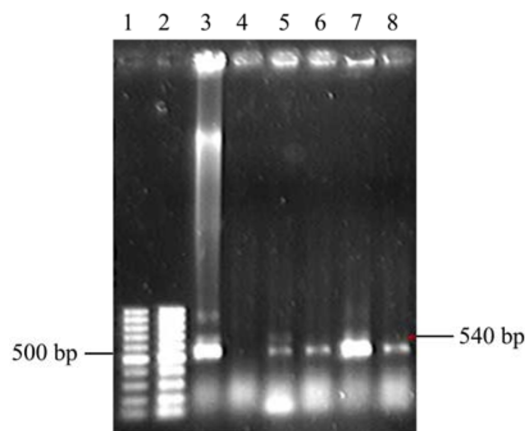

Figure 2 Gel electerophoresis of PCR production of 540 fragment from high conservative area in *phaC1* Gene

Note: Lane 1 and 2, 50 bp molecular weigh, Lane3-8, 540 fragment PCR product from high conservative sequences in *phaC1* gene in *pseudomonas putida* KT2442, (different reaction)

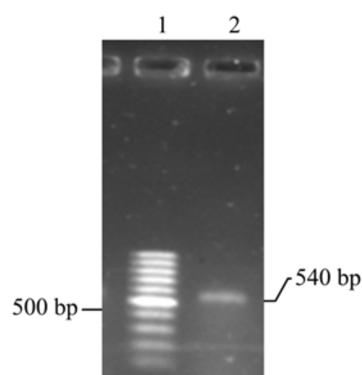

Figure 3 The purification of 540 bp PCR fragment from *P. putida* KT2442

Note: Line 1, 50bp molecular weigh and line 2, 540 bp PCR product

The 540 bp PCR fragment were purified and sequenced using the Forward and Revers primer as demonstrated in Figure 4 and Figure 5 respectively.

A BLASTX search using the selected 190-bp *P. putida phaC1* gene sequences as query yielded an amino acid sequence matches with more than 20 PHA synthases (data not shown). The alignments with the most homology were those with the PHA synthase 1&2 from *Pseudomonas aeruginosa* PHA synthase 1&2 from *P. putida* etc. Seemingly, the particular bacterial clone chosen for the sequence analysis contained an amplified segment of the *Pseudomonas putida phaC1* gene. The sequences in FASTA format were used in Mega5 application to built the phylogenic tree.

1.4 Phylogenetic tree

The ClustalW method in Mega5 application was used to make a phylogenic tree. The “Phylip Tree” section gives the tree in newick format. Figure demonstrated the phylogenetic tree in newick format (Figure 6).

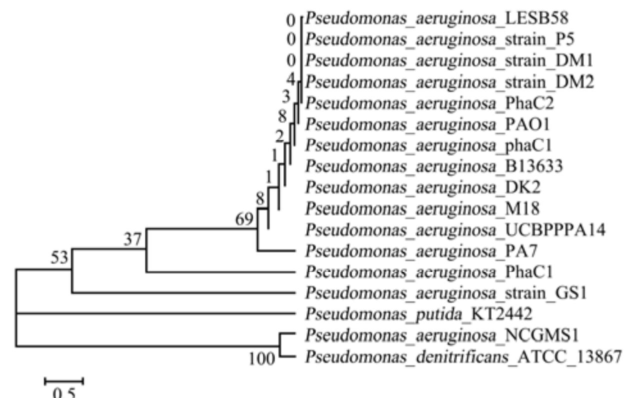

Figure 6 The phylogenic tree of *phaC1* gene from *pseudomonas putida* KT2442

2 Conclusion

Because of The PHA numerous applications in industry, medicine and agriculture, the need for extensive research on genes involved in the production, it is very much needed. Multiple use of plastics in the world is increasing day by day, so that the global market for biodegradable polymers to 114 million pounds, up until 2010, was rape, For this reason, the production of PHAs are highly regarded scientists and the significant research on biopolymers is flow in Europe and Japan and to some extent in America (European Bioplastics, 2007).

What has attracted great industrial interest is the idea that application-specific, novel polymers with physical and chemical properties can be synthesized merely by controlling the composition of the carbon source. It is important to note that several economic and technical barriers to the production of PHAs and

Interdisciplinary nature of the biopolymer science, are created its challenges for large-scale studies in this area that leads scientists towards affordable ways to produce biodegradable polymers, Because of the high cost of PHA production, would threaten the rapid advance of the commercial exploitation of these (Pandey et al., 2005). Finally, the PHAs have vast and varied applications that Among these, medical applications are the most viable as economically. Now, with increasing interest and extensive research in this field is ongoing, PHA's potential as the next generation of environmentally friendly materials with a wide range of applications are emerging. Due to the small number of studies on genes involved in the synthesis of PHA in the world and the potential of *phaC1* gene, in production of PHA and that gene is new, transfer this gene into suitable expression vector and transformation of that in *E. coli* bacteria in order to expression of that, it is very important. Also, the transfer of this gene with *phaD* and *phaG* genes to bacteria and also transfer of this simultaneously to several different bacterial species, can be a very good approach to increase gene expression as more efficiency and studies. The PHA synthase identification of other indigenous bacteria and effort to produce this substance on large scale and commercial is also very helpful.

3 Materials and Methods

3.1 Bacterial strains and cultivation conditions

The taxonomic characteristics of *Pseudomonas putida* KT2442 have been reported (Hume et al., 2009). Cells were pre-cultured in 100-mL Erlenmeyer flasks containing 25 mL Luria broth [(5 g/L yeast extract, 10 g/L tryptone, 5 g/L NaCl) Merck Darmstadt, Germany] for 24 h at 30℃ in a rotary incubator at 150 rpm (Akhavan Sepahy et al., 2011). For production and extraction of PHA, cells were cultivated in a shaker incubator (180 rpm) at 30℃ for 72 h in 500-mL flasks containing 100 mL of a nitrogen-limited mineral salt (MS) medium. The standard mineral salts medium is composed of (per liter of distilled water): 1.269 g CH_3CH_2COOH, 600 mg $MgSO_4 \cdot 7H_2O$, 160 mg NH_4Cl, 100 mg ethylenediamine tetraacetic acid (EDTA), 92 mg K_2HPO_4, 45 mg KH_2PO_4, 70 mg $CaCl_2 \cdot 2H_2O$ and 2 mL of trace elements solution. The trace solution is consisted of (per liter of distilled water): 1 500 mg $FeCl_3 \cdot 6H_2O$, 150 mg H_3BO_3, 150 mg $CoCl_2$ $6H_2O$, 120 mg $MnCl_2 \cdot 4H_2O$, 120 mg $ZnSO_4 \cdot 7H_2O$, 60 mg $Na_2MoO_4 \cdot H_2O$, 30 mg $CuSO_4 \cdot 5H_2O$ and 30 mg of KI. Filter-sterilized carbon sources were also added to the medium (Watcharakul et al., 2012).

3.2 Sample preparation for FT-IR confirmation of PHA

For qualitative and quantitative analysis of PHA, 7~10 mg of lyophilized cells or of isolated polyesters was subjected to methanolysis in the presence of 15% (v/v) sulfuric acid suspended in methanol. PHA was extracted from lyophilized cells by chloroform in a Soxhlet apparatus, and subsequently precipitated in 10 vol. methanol. The precipitate was dissolved in acetone and was again precipitated in methanol in order to obtain highly purified PHA. Purified PHA was dissolved in chloroform and layered on the KRS-5 window. After evaporation of the chloroform, the PHA polymer film was subjected to FT-IR analysis (Mai et al., 2004; Hong et al., 1999).

3.3 PCR Amplification Method

Total genomic DNA was isolated by standard procedures using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI) (Sambrook et al., 1989). PCR was performed in a Techne PCR Thermocycler (Techne, England) and sequencing primers were synthesized by PCR premix was purchased from fermentas and was used according to the manufacturer's protocols. To amplify the PHA synthase gene, *phaC1*, from genomic DNA of *Pseudomonas putida*, PCR was performed with two specific primers, Forward primer: ACAGATCAACAAGTTCTACATCTTCGAC, Reverse primer GGTGTTGTCGTTGTTCCAGTAGAGGATGTC. PCR conditions were as follows: 30 cycles of denaturation at 94℃ for 30 sec, annealing at 60℃ for 30 second, elongation at 72℃ for 1 min. The PCR products were analyzed by agarose gel electrophoresis in 1 × TBE (Tris-Borate-EDTA) buffer and gel was stained with 0.5 ug/mL ethidium bromide.

3.4 Nucleotide sequence analysis

PCR-amplified *phaC1* gene with forward and reverse primers was sequenced by the modified dideoxy chain

termination method as described (Tomris et al., 2007) with a 310 Genetic Analyzer (Perkin Elmer). The resulting nucleotide sequence was analyzed with genetic information processing software Bioedit. The bioinformatics of the amplified sequences were extracted by using NCBI BLAST program (Wiese et al., 2011).

3.5 Bioinformatic Method for Phylogenetic Tree

A set of homologous DNA sequences made to build a tree. The sequence from the PCR-amplified phaC1 gene and feed it into the blastn web interfaces at the NCBI by pasting it into the main search box. The following sequence were applied in blastn:

*Pseudomonas_putida*_KT2442
aagagcttggtccgcttgtgctggagaacagctggcagactttcatcatcca
ttgggggcaccccgacaactaccacgccaatggggcctgaccacctatgt
cgaggccctcaacgaggccatcgaggtcatcctgtagatcaccggcagcc
acgacctgaacctggtcggcgcctgctccggcg

A list of hits were obtained for which the seventeen sequences were selected to built a phylogenetic tree for (partially *phaC1* genes:)

*Pseudomonas_putida*_KT2442,
*Pseudomonas_aeruginosa*_LESB58,
*Pseudomonas_aeruginosa*_PAO1,
*Pseudomonas_aeruginosa*_phaC1,
*Pseudomonas_aeruginosa*_B136-33,
*Pseudomonas_aeruginosa*_DK2,
*Pseudomonas_aeruginosa*_M18,
*Pseudomonas_aeruginosa*_UCBPP-PA14,
*Pseudomonas_aeruginosa*_strain_GS1,
*Pseudomonas_aeruginosa*_NCGM2.S1,
*Pseudomonas_denitrificans*_ATCC_13867,
*Pseudomonas_aeruginosa*_strain_DM2,
*Pseudomonas_aeruginosa*_strain_DM1,
*Pseudomonas_aeruginosa*_PA7,
*Pseudomonas_aeruginosa*_PhaC2,
*Pseudomonas_aeruginosa*_PhaC1,
*Pseudomonas_aeruginosa*_strain_P5

Acknowledgements

This work partially received financial support from National Institute of Genetic Engineering and Biotechnology, NIGEB.

References

Adkins J., Pugh S.h., McKenna R., and Nielsen D.R., 2012, Engineering microbial chemical factories to produce renewable “biomonomers”, Front Microbiol, 3: 313-318 http://dx.doi.org/10.3389/fmicb.2012.00313 PMid:22969753 PMCid:PMC3430982

Akhavan Sepahy A., Ghazi Sh., and Akhavan Sepahy M., 2011, Cost-Effective Production and Optimization of Alkaline Xylanase by Indigenous Bacillus mojavensis AG137 Fermented on Agricultural Waste, Enzyme Res., 20: 1-11 http://dx.doi.org/10.4061/2011/593624 PMid:21904670 PMCid:PMC3166571

Gangoiti J., Santos M., Prieto M.A., Mata I.D., Serra J.L., and Llama M.J., 2012, Characterization of a Novel Subgroup of Extracellular Medium-Chain-Length Polyhydroxyalkanoate Depolymerases from Actinobacteria, Appl Environ Microbiol, 78: 20-28 http://dx.doi.org/10.1128/AEM.01707-12 PMid:22865072 PMCid:PMC3457088

Guo-Qiang C.H., 2009, A microbial polyhydroxyalkanoates (PHA) based bio and materials industry, Chem Soc Rev., 38: 2434-2446 http://dx.doi.org/10.1039/b812677c PMid:19623359

Hernandez-Eligio A., Castellanos M., Moreno S., and Espín G., 2011, Transcriptional activation of the Azotobacter vinelandii polyhydroxybutyrate biosynthetic genes *phbBAC* by *PhbR* and *RpoS*, Microbiology, 157(Pt 11): 3014-3023 http://dx.doi.org/10.1099/mic.0.051649-0 PMid:21778206

Hume A.R., Nikodinovic-Runic J., and O'Connor K.E., 2009, FadD from *Pseudomonas putida* CA-3 Is a True Long-Chain Fatty Acyl Coenzyme A Synthetase That Activates Phenylalkanoic and Alkanoic Acids, J Bacteriol., 191(24): 7554-7565 http://dx.doi.org/10.1128/JB.01016-09 PMid:19820085 PMCid:PMC2786608

Koller M., Salerno A., Dias M., Reiterer A., and Braunegg G., 2010, Modern biotechnological polymer synthesis: A review, Food Technol Biotechnol, 48(3): 255-269

Levis J.W, and Barlaz M.A., 2011, Is Biodegradability a Desirable Attribute for Discarded Solid Waste? Perspectives from a National Landfill Greenhouse Gas Inventory Model, Environ Sci Technol., 45: 5470-5476 http://dx.doi.org/10.1021/es200721s PMid:21615182

Liu H., Luo Y., Han J., Wu J., Wu Z., and Feng D., 2013, Proteome reference map of Haloarcula hispanica and

comparative proteomic and transcriptomic analysis of polyhydroxyalkanoate biosynthesis under genetic and environmental perturbations, J Proteome Res, 12(3): 1300-1315
http://dx.doi.org/10.1021/pr300969m
PMid:23301558

Mai M., Hoyer J.D., McClure R.F., 2004, Use of multiple displacement amplification to amplify genomic DNA before sequencing of the α and β haemoglobin genes, J Clin Pathol, 57(6): 637-640
http://dx.doi.org/10.1136/jcp.2003.014704
PMid:15166271 PMCid:PMC1770312

Martínez V., Peña F., García-Hidalgo J., Mata I., Garcíaa J., and Prieto M.R., 2012, Identification and Biochemical Evidence of a Medium-Chain-Length Polyhydroxyalkanoate Depolymerase in the Bdellovibrio bacteriovorus Predatory Hydrolytic Arsenal, Appl. Environ Microbiol., 78(17): 6017-6026
http://dx.doi.org/10.1128/AEM.01099-12
PMid:22706067 PMCid:PMC3416617

Poirier Y., Nawrath C., and Somerville C., 1995, Production of polyhydroxyalkanoates, a family of biodegradable plastics and elastomers, in bacteria and plants, BioTechnology, 13: 142-150
http://dx.doi.org/10.1038/nbt0295-142
PMid:9634754

Poli A., Donato P.D., Abbamondi G.R., and Nicolaus B., 2011, Synthesis, Production, and Biotechnological Applications of Exopolysaccharides and Polyhydroxyalkanoates by Archaea, Archaea, Article ID 693253
PMid:22007151 PMCid:PMC3191746

Ruth K., Grubelnik A., Hartmann R., Egli T., Zinn M., and Ren Q., 2007, Efficient production of (R)-3-hydroxycarboxylic acids by biotechnological conversion of polyhydroxyalkanoates and their purification, Biomacromolecules, 8: 279-286
http://dx.doi.org/10.1021/bm060585a
PMid:17206818

Shuping Wu, Yumin Du, Yunzi Hu, Xiaowen Sh., and Lina Z., 2013, Antioxidant and antimicrobial activity of xylan–chitooligomer–zinc complex, Food Chemistry, 138(2-3): 1312-1319
http://dx.doi.org/10.1016/j.foodchem.2012.10.118
PMid:23411248

Tanamool V., Imai T., Danvirutai P., and Kaewkannetra P., 2013, Biopolymer generation from sweet sorghum juice: screening, isolation, identification, and fermentative polyhydroxyalkanoate production by Bacillus aryabhattai, Turk J Biol, 37: 259-264

Watcharakul S., Umsakul K., Hodgson B., Chumeka W., Tanrattanakul V., 2012, Biodegradation of a blended starch/natural rubber foam biopolymer and rubber gloves by Streptomyces coelicolor CH13, Electron J Biotechnol., 15: 1-8

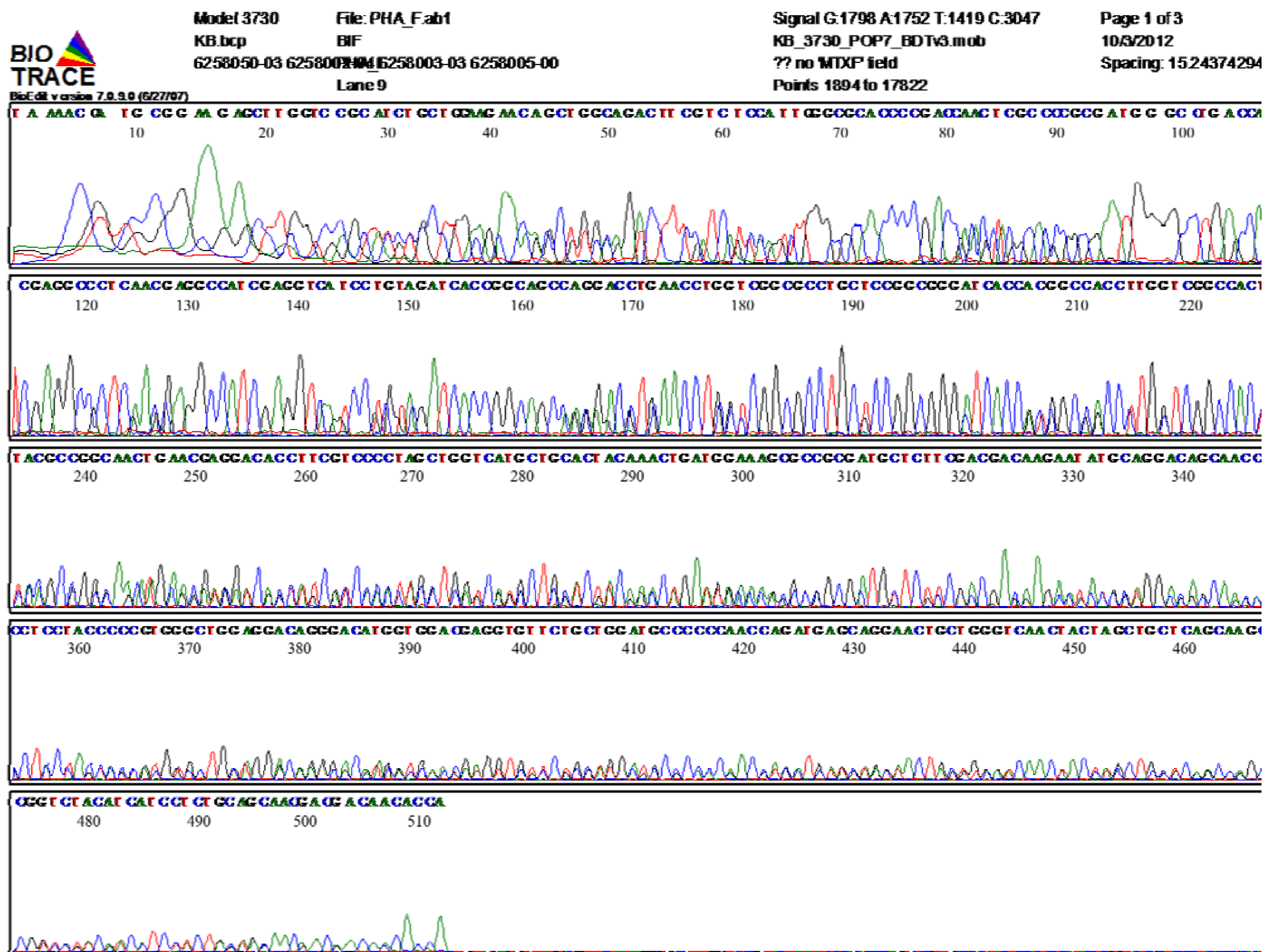

Figure 4 The result of trace metafile of sequences from forward primer for *phaC1* gene in *P. Putida*

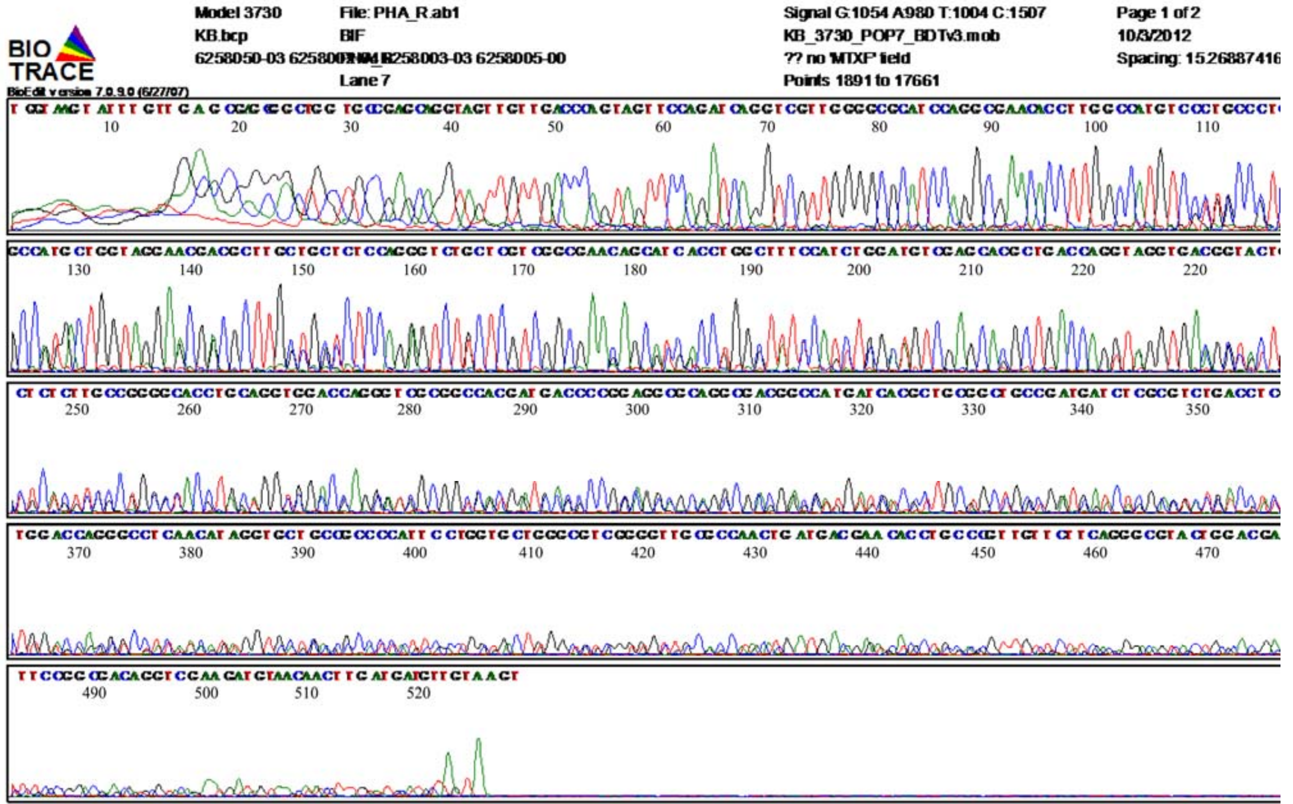

Figure 5 The result of trace metafile of sequences from reverse primer for *phaC1* gene in *P. putida*