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Molecular Structure of PCR Cloned PHA Synthase Genes of *Pseudomonas putida* KT2440 and Its Utilization for Medium-Chain Length Polyhydroxyalkanoate Production

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Abstract A new *phaC* gene cluster encoding polyhydroxyalkanoate (PHA) synthase I PHA depolymerase, and PHA synthase II was cloned using the touchdown PCR method, from medium-chain length (mcl-) PHA-producing strain *Pseudomonas putida* KT2440. The molecular structure of the cloned *phaC1* gene was analyzed, and the phylogenic relationship was compared with other *phaC1* genes cloned from *Pseudomonas* species. The cloned *phaC1* gene was expressed in a recombinant *E. coli* to the similar level of PHA synthase in the parent strain *P. putida* KT2440, but no significant amount of mcl-PHA was accumulated. The isolated *phaC1* gene was re-introduced into the parent strain *P. putida* KT2440 to amplify the PHA synthase I activity, and the recombinant *P. putida* accumulated mcl-PHA more effectively, increasing from 26.6 to 43.5%. The monomer compositions of 3-hydroxylalkanoates in mcl-PHA were also modified significantly in the recombinant *P. putida* enforcing the cloned *phaC1* gene.

Key words: *phaC* gene cluster, *phaC1*, *Pseudomonas putida* KT2440, mcl-PHA, touchdown PCR, PHA accumulation

Polyhydroxyalkanoates (PHAs) are the intracellular carbon and energy storage materials accumulated in numerous microorganisms under unbalanced growth conditions, such as rich carbon sources but limitated other nutrients. Utilization of PHAs as a commercial biopolymer has drawn much attention due to their biodegradability, and thermoplastic and elastomeric properties [38].

PHAs have been classified into two groups, depending on the length of carbon chain of β-hydroxy ester monomers; one is the short-chain length (scl-)PHAs (C_4-C_5) and the other is the medium-chain length (mcl-)PHAs (C_6-C_{14}) [9, 14]. Major scl-PHAs producers are *Ralstonia eutropha*, *Alcaligenes latus*, and *Azotobacter vinelandii* [15, 16, 27]. mcl-PHAs are accumulated only in a variety of fluorescent *Pseudomonas* species, including *Pseudomonas oleovorans*, *P. aeruginosa*, and *P. putida*, *etc.* mcl-PHAs show much lower crystallinity, but higher elasticity compared to scl-PHAs [8, 24].

PHA synthase is a key enzyme in the biosynthesis of PHAs, and catalyzes the conversion of 3-hydroxyacyl-CoAs to PHAs with the concomitant release of CoA. PHA synthase has been classified into four types based on substrate specificity and protein structure. Type I synthase encoded by the *phbC* gene of *R. eutropha* is the most representative one, and closely linked with the biosynthesis of scl-PHA [13, 27]. Type II synthase is consisted of the genes encoding two PHA synthases (*phaC1* and *phaC2*) associated with the mcl-PHA biosynthesis, separated by the PHA depolymerase gene (*phaZ*), found mainly in *Pseudomonas* species [6, 10, 11, 18, 20, 35, 40, 43]. Type III synthase is composed of two subunits encoded by the *phbE* and *phbC* genes, and found in *Chromatium vinosum*, *Thiocystis violace*, and *Syneceocystis* sp., playing a role in the biosynthesis of scl-PHA [26]. Recently, the type IV synthase cluster of the PHA synthase gene, whose functions need to be further clarified, has been isolated from *Bacillus megatrium* [19].

In our previous works, the *phbC* gene cloned from *R. eutropha* was re-introduced into the parent *R. eutropha* H16 to amplify PHB synthase activity [21, 22]. The transformant *R. eutropha* increased not only the biosyntheses of PHB and its copolymers, but the monomer compositions of 3HV in P(3HB-3HV) and 4HB in P(3HB-4HB) also increased [15, 23]. Similar results were also obtained from the transformant *A. latus* amplifying its own *phbC* gene [16, 31].

Fluorescent *Pseudomonas* species have been intensively investigated for overproduction of mcl-PHAs through batch and fed-batch cultivations using alkanoic acid and fatty acids as the carbon sources. The biosynthesis of mcl-PHA in the fluorescent *Pseudomonas* sp., including *P.*

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aeruginosa, *P. oleovorans*, *P. racinovorans*, and *P. putida* U. is known to be carried out by the cluster of 7 genes, composed of *phaC1* and *phaC2* (PHA synthase I and II), *phaZ* (PHA depolymerase), *phaD* (unknown protein), *phaF* and *phaI* (PHA granule-associated proteins), and ORF1 (unknown protein). PHA synthases, which are encoded by *phaC1* and *phaC2* genes, and polymerize C_6-C_{14} carbon monomers generated as the intermediates of fatty acid during the *de novo* biosynthesis or β-oxidation of fatty acids, have been isolated from *P. aeuroginosa* and *P. oleovorans* [6, 10, 11, 18, 20, 35, 38, 40, 43].

P. putida KT2440 has attracted much attention as a potent commercial producer of mcl-PHA, because of its versatile utilization of carbon sources including gluconate, various fatty acids, and oils [12, 25, 34]. However, the gene level research related to PHA biosynthesis has been rarely carried out, except for the identification of the *phaG* gene encoding 3-hydroxyacyl-acyl carrier protein-CoA transferase that produces 3-hydroxyacyl-CoA by transferring the acyl moiety of 3-hydroxyacyl-ACP to coenzyme A [26].

In this work, a new *phaC* gene cluster encoding mcl-PHA synthase I, PHA depolymerase, and mcl-PHA synthase II was cloned from *P. putida* KT2440 using touchdown PCR, and the molecular structure of the cloned gene cluster was investigated. The phylogenic relationship of a newly cloned *phaC1* gene was compared with other *phaC1* genes isolated from other *Pseudomonas* sp. The cloned *phaC1* gene was re-introduced into the parent *P. putida* KT2440 to amplify the PHA synthase, in order to overproduce mcl-PHA and to modulate various monomer compositions in mcl-PHA.

MATERIALS AND METHODS

Bacterial Strain, Plasmid, and Cultivation

P. putida KT2440 (ATCC 47054) was used as a source of gene cluster, and *E. coli* XL1-Blue was used as the host cell for PCR cloning of *phaC1* and *phaC2* genes. pGEM-T Easy vector (Promega Co, Milwaukee, U.S.A.) was used as the T-vector for the PCR cloning of *phaC1* and *phaC2* genes. pKT230 [1] was used as the shuttle vector for transformation of the cloned *phaC1* gene into the parent strain *P. putida* KT2440. The parent and recombinant *P. putida* were cultivated in NR medium at 30°C using kanamycin (100 μg/ml) as a selection marker. Recombinant *E. coli* was cultivated in LB medium at 37°C using ampicillin (50 μg/ml) as a selection marker.

Touchdown PCR for Cloning of *phaC* Gene Cluster of *P. putida* KT2440

Genomic DNA of *P. putida* KT2440 was extracted according to Mak and Ho's method [17], and then *phaC1* and *phaC2* genes were amplified from the genomic DNA using touchdown PCR [5], under the PCR conditions of the denaturation at 94°C for 30s, annealing for 1 min from 65°C to 55°C by decreasing 0.5 °C per cycle during 20 cycles, and elongation at 72°C for 4 min.

The PCR primers were designed considering the DNA sequences of the conserved regions among the *phaC* cluster gene of other *Pseudomonas* species [6, 10, 11, 18, 20, 35, 38, 40, 43]. The primers for *phaC1* and *phaC2* gene were:

The forward primer (*phaC1*-F) - 5'-ACAGCGGCCTGTTCACCTGGG-3'

The reverse primer (*phaC1*-R) - 5'-ACGATCAGGTGCAGGAACAGC-3'

The forward primer (*phaC2*-F) - 5'-CTACTGGCAGCTGTTCGC-3'

The reverse primer (*phaC2*-R) - 5'-ACGATCAGGCGCAGGAACAGC-3'.

Semi-nested PCR for Identification of *phaC1* and *phaC2* Genes

The *phaC1* and *phaC2* genes in the PCR product were confirmed by the semi-nested PCR method suggested by Solaiman *et al.* [33], who compared the *phaC1* and *phaC2* genes among various *phaC* genes of *Pseudomonas* sp. with a 540 bp of specific DNA fragment amplified using the specific primer set, composed of the forward primer (MCL-F): 5'-ACAGATCAACAAGTTCTACATCTTCGAC-3' and the reverse primer (MCL-R): 5'-GGTGTTGTCG TTGTTCCAGTAGAGGATGTC-3'.

Transformation of *phaC1* and *phaC2* Genes into *E. coli*

Each PCR product of the *phaC1* and *phaC2* genes was ligated with pGEM-T Easy vector to obtain the recombined plasmid pPHAC1 and pPHAC2, respectively. The constructed plasmids were transformed into *E. coli* XL1-Blue using the $CaCl_2$ method [28].

Nucleotide Sequence Analysis

Nucleotide sequencing of the cloned *phaC1* and *phaC2* genes was carried out according to the method of dideoxy chain termination described by Sanger *et al.* [29] using ABI PRISM 377 DNA sequencer (PE Applied Biosystems, Foster City, U.S.A.). The sequencing reaction was performed as described in the manual supplied with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystem).

Construction of Recombinant *P. putida* Harboring the *phaC1* Gene

To construct the recombinant *P. putida* re-introducing the *phaC1* gene to amplify the PHA synthase, 3.0 kb of *Not*I-fragment of the plasmid pPHAC1, including the *phaC1* gene, was inserted into the *Not*I-site of pKT230 to obtain the recombined plasmid pKPHAC, and then the constructed

plasmid was transformed into *P. putida* KT2440 by electrotransformation [28].

Measurement of mcl-PHA Synthase Activity

The transformed *E. coli* were suspended in 50 mM phosphate buffer (pH 7.0) and then disrupted by ultrasonication at 4°C. The activity of mcl-PHA synthase was determined using the modified method of Haywood *et al.* [9], who measured the absorbance of CoA liberated from (*R,S*)-3-hydroxybutyryl-CoA at 412 nm. The substrate used for measurement of mcl-PHA synthase activity, (*R,S*)-3-hydroxyoctanoyl-CoA, was synthesized from CoA and

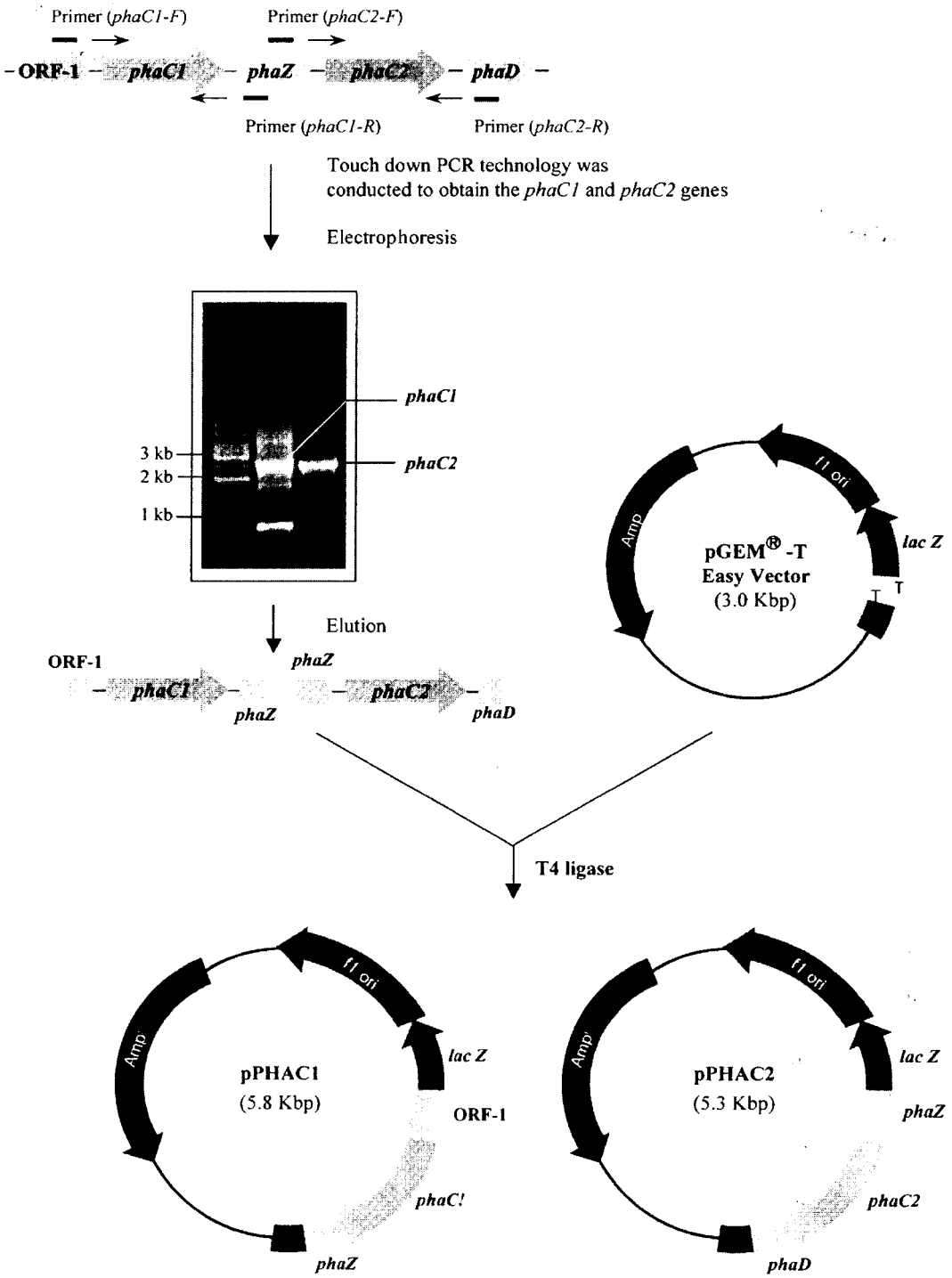

Fig. 1. Protocol for PCR cloning of *phaC1* and *phaC2* genes from *P. putida* KT2440.

(*S*)-3-hydroxyoctanoate (Sigma Co., St. Louis, U.S.A.) according to the method of Rehm *et al.* [26]. The intrinsic activity of PHA synthase was defined as the amount of enzyme releasing one mmol CoA per min per mg of soluble protein.

Cultivation of Recombinants for Accumulation of PHA

The recombinant *E. coli* harboring the cloned *phaC1* gene was cultivated in LB medium supplemented with sodium decanoate (0.5% w/v), ampicilin (50 μg/ml), and acrylic acid (0.2 mg/l) at 37°C for 72 h. The parent and recombinant *P. putida* were cultivated in MS medium [30] supplemented with oleic acid, decanoate, octanoate, and gluconate (0.5% w/v) as the carbon sources, respectively, and kanamycin (100 μg/ml) as a selective pressure for 48 h at 30°C.

Measurement of PHA Concentration

PHAs were extracted from lyophilized cells using chloroform, and then analyzed by gas chromatography after methanolysis [4]. The methyl esters of monomers were assayed using Perkin-Elmer 8420 GC system (PE Applied Biosystems), packed with HP-Innowax column (Hewlett-Packard Co., Palo Alto, U.S.A.). 3-Hydroxybutyrate, 3-hydroxyhexanoate, 3-hydroxyoctanoate, 3-hydroxydecanoate, and 3-hydroxydodecanoate (Sigma Co., St. Louis, U.S.A.) were used as standards.

Nucleotide Sequence Accession Number

The nucleotide sequence data were registered in the GenBank nucleotide sequence database under the accession number AY113181.

RESULTS AND DISCUSSION

Cloning of *phaC* Gene Cluster from *P. putida* KT2440 Using Touchdown PCR

The *phaC* cluster of *P. putida* KT2440 was cloned using touchdown PCR as illustrated in Fig. 1, to obtain a 2.8 kb of PCR product containing the *phaC1* gene and a 2.3 kb containing the *phaC2* gene. The *phaC1* and *phaC2* genes in the PCR product were confirmed by the semi-nested PCR method, observing the 540 bp DNA fragment in the conserved region of mcl-PHA synthase. Each PCR product containing the *phaC1* and *phaC2* genes was combined with the PCR cloning vector pGEM-T Easy, and they were transformed into *E. coli* XL1-Blue for DNA sequence analysis.

Molecular Structure of Cloned *phaC1* Gene of *P. putida* KT2440

The DNA sequence of the *phaC* gene cluster was deposited in GenBank under the accession number of AY113181. As shown in Fig. 2, the *phaC* gene cluster was comprised of 4,798 nucleotides encoding five ORFs including partial ORF1, *phaC1*, *phaZ*, *phaC2*, and partial *phaD* gene. The *phaC1* gene was consisted of 1,680 bp spacing from 409 bp to 2,088 bp, and the *phaC2* gene was consisted of 1,680 bp spacing from 3,027 bp to 4,709 bp. The *phaZ* gene consisted of 858 bp was also spaced from 2,151 to 3,008 bp. The total sequence and structure of the *phaC* gene of *P. putida* KT2440 could be elucidated, consequently.

The *phaC* gene cluster of *P. putida* KT2440 had the arrangement of *phaC1-phaZ-phaC2*, which is identical with other *Pseudomonas* species [6, 10, 11, 18, 20, 35, 40, 43]. The *phaC* gene cluster of *P. putida* KT2440 showed higher than 80% DNA sequence homology with those from other fluorescent *Pseudomonas* species. In particular, the *phaC1* gene of *P. putida* KT2440 exhibited the highest homology to that of *P. oleovorans*, having as high as 93%. The *phaC2* and *phaZ* also showed high homology to those of *P. oleovorans*, having as high as 90% and 95%, respectively.

The promoter region was identified at 408 bp upstream of the start codon, however, the promoter region for the *phaZ* and *phaC2* genes was not found throughout the whole sequence of the *phaC* gene cluster of *P. putida* KT2440. The *phaC* gene cluster of fluorescent *Pseudomonas* species is known to have only one promoter for expression of each *phaC* gene that positions in front of the *phaC1* gene [6]. Therefore, it can be postulated that the putative promoter region of the *phaZ* and *phaC2* genes is the same as that of the *phaC1* gene situated at 408 bp upstream. Generally, the *phaC1* gene has been known to encode the major enzyme for PHA synthesis in fluorescent *Pseudomonas*

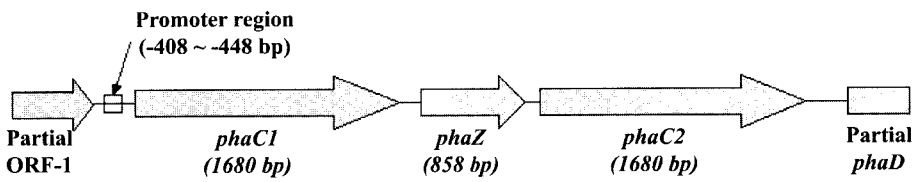

Fig. 2. *phaC* gene cluster locus of *P. putida* KT2440.
Partial ORF-1, partial ORF-1 lacking 5' end sequence; *phaC1*, PHA synthase I gene; *phaZ*, PHA depolymerase; *phaC2*, PHA synthase II; partial *phaD*, partial *phaD* lacking 3' end sequence. The nucleotide sequence was deposited in the GenBank nucleotide sequence database under the accession number AY113181.

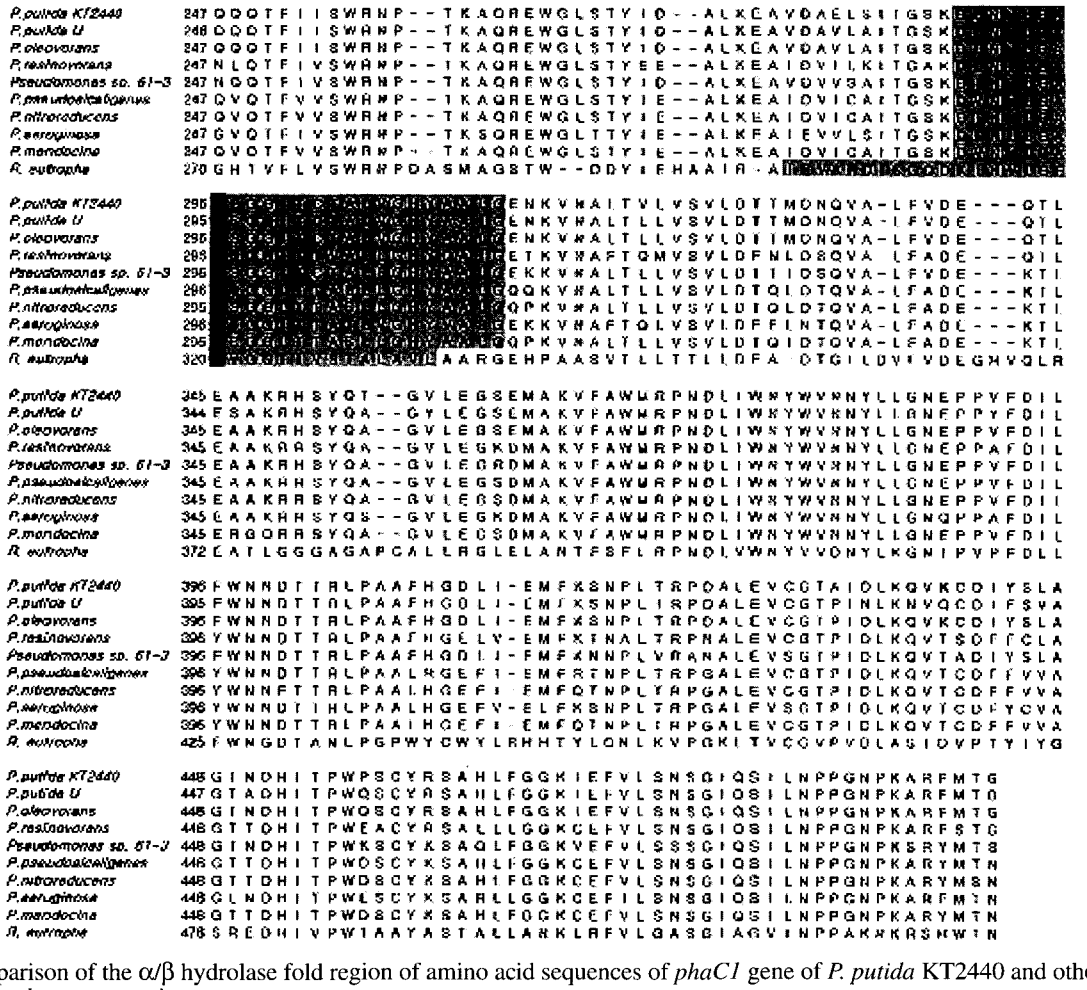

Fig. 3. Comparison of the α/β hydrolase fold region of amino acid sequences of *phaC1* gene of *P. putida* KT2440 and other mcl-PHA producing *Pseudomonas* species.

The gray box region indicates the transmembrane domain (TM) and the darker gray box region indicates a well-conserved cysteine residue in the active site of PHA synthases.

species [11, 18, 25], therefore, the molecular structure of the *phaC1* gene has been mainly analyzed in this work.

The newly isolated *phaC1* gene encoded a protein composed of 559 amino acids corresponding to 62,253 Da molecular weight. The α/β hydrolase fold region of the cloned *phaC1* gene was compared with other already identified PHA synthases I of *Pseudomonas* species, as shown in Fig. 3. PHA synthase I of *P. putida* KT2440 had a well-conserved α/β hydrolase fold region and cysteine residue at 296 which is essential for covalent catalysis of PHA synthase [26, 41]. All PHA synthases I isolated from *Pseudomonas* species showed a cysteine residue at 295-296 of their sequence [26, 41].

The transmembrane domain (TM) in the conserved α/β hydrolase fold region of PHA synthase I was modulated using the TMpred server program (www.chembinet.org), that predicts the membrane-spanning regions and their orientation [43]. The TM domain was composed of a 25 hydrophobic amino acids sequence, DLNNLGACSGGITCTALVGHYAALG,

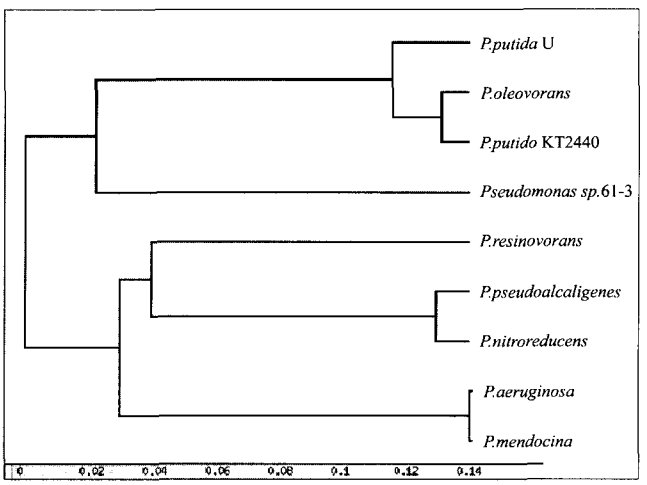

Fig. 4. Phylogenic tree of cloned *phaC1* genes of various *Pseudomonas* species.

The phylogenic tree was constructed based on the amino acid sequences of PHA synthase I using the Genebee server (www.genebee.msu.su). The bottom line indicates the percentage of divergence.

Table 1. Expression of PHA synthase and accumulation of mcl-PHA in recombinant *E. coli* harboring the *phaC1* gene of *P. putida* KT2440.

Strains	PHA synthase activity (U/mg protein)	PHA contents (%, w/w)	β-Hydroxyalkanoyl-methyl ester (mol %)[2]				
			HB	HH	HO	HD	HDD
E. coli XL1-Blue	0.04	N.D.[1]	N.D.	N.D.	N.D.	N.D.	N.D.
P. putida KT2440	1.26	36.00	N.D.	N.D.	98.37	1.63	N.D.
Recombinant *E. coli*	1.13	0.56	N.D.	0.20	87.70	11.10	0.89

[1]N.D.: Not detected.

[2]HB, 3-hydroxybutyrate; HH, 3-hydroxyhexanoate; HO, 3-hydroxyoctanoate; HD, 3-hydroxydecanoate; HDD, 3-hydroxydodecanoate.

E. coli was cultivated in LB medium containing 0.5% (w/v) of sodium octanoate at 37°C, 250 rpm for 72 h. *P. putida* KT 2440 was cultivated in MS medium containing 0.5% (v/v) of sodium octanoate at 30°C, 250 rpm for 48 h.

located at 289-313 amino acid residues, and it seems to play an important role in PHA synthesis catalysis as embedded state in PHA granule surrounding the membrane, as suggested by Wodzinska *et al.* [41].

Phylogenic Tree of *phaC1* Gene from *P. putida* KT2440 and Other *Pseudomonas* Species

Figure 4 illustrates the phylogenic tree of the amino acid sequences of PHA synthase I from *P. putida* KT2440 and other *Pseudomonas* species, being constructed using the Genebee server program (www.genebee.msu.su). In this work, all PHA synthases I of *Pseudomonas* species were divided into four subgroups, I, II, III, and IV, depending on the homology similarity of amino acid sequences. The newly cloned *phaC1* gene of *P. putida* KT2440 was classified as the subgroup I along with the PHA synthases I from *P. oleovorans* [10, 14] and *P. putida* U [6], however, phylogenic relationship was more similar with *P. oleovorans* than with *P. putida* U that belongs to the same species.

PHA synthase I of *Pseudomonas* sp. 61-3 was classified in the subgroup II in this work, and it mainly accumulates a copolymer consisting of random PHB and mcl-PHA unit [18]. PHA synthase I of *P. nitroreducens* was classified in the subgroup III, and accumulates mcl-PHAs from various fatty acids, especially accumulating a blend PHA polymer consisting of PHB and mcl-PHA from butyrate, used as a sole carbon source [42]. PHA synthase I of *P. aeruginosa*, classified in the subgroup IV, has accumulated mcl-PHAs from longer chain fatty acid higher than C_{18}, unlike that of other *Pseudomonas* species [2, 39]. However, the role of PHA synthases classified as subgroups III and IV in this work need to be further investigated to identify their differences.

Expression of Cloned *phaC1* Gene in Recombinant *E. coli*

The newly isolated *phaC1* gene was expressed in *E. coli*, and the level of expressed PHA synthase activity and the distribution of monomers in the accumulated PHA were measured as shown in Table 1. The *phaC1* gene was successfully expressed in recombinant *E. coli* as high as 1.13 U/mg protein of intrinsic PHA synthase activity, a nearly comparable level to the parent *P. putida* KT2440 with 1.26 U/mg protein.

However, only a very lower level of PHA less than 0.5% (w/w dry cell weight) was accumulated in the recombinant *E. coli*, similar to other previous works [25, 35, 43] who cultivated recombinant *E. coli* harboring PHA synthase I genes from other *Pseudomonas* species, mainly due to insufficient flux of substrates to the biosynthesis of PHA. The major monomers in accumulated mcl-PHA were relatively high molecular weight 3-hydroxyoctanoate (C_8) and 3-hydroxydecanoate (C_{10}) rather than lower 3-hydroxybutyrate (C_4), indicating that the PHA synthase I encoded by the cloned *phaC1* gene participates mainly in the biosynthesis of mcl-PHAs compared to scl-PHAs.

Construction of Transformant *P. putida* KT2440 Amplifying Its Own *phaC1* Gene

To investigate the effect of the amplified *phaC1* gene on the biosynthesis of mcl-PHA, the cloned *phaC1* gene was also transformed into the parent strain *P. putida* KT2440 after recombination in shuttle vector pKT230. Table 2 compares the intrinsic activity of PHA synthase, biomass, PHA concentration, and PHA contents in both the recombinant and parent *P. putida* KT2440. The PHA synthase activity was increased two-fold from 1.14 unit/mg to 2.27 unit/mg

Table 2. Expression of PHA synthase and accumulation of mcl-PHA in recombinant *P. putida* enforcing the *phaC1* gene of *P. putida* KT2440.

	PHA synthase (U/mg protein)	Biomass (g/l)	PHA concentration (g/l)	PHA contents (%, w/w)
Parent *P. putida* KT2440	1.14	0.97	0.26	26.6
Recombinant *P. putida*	2.27	1.24	0.54	43.5

Recombinant and parent *P. putida* were cultivated in MS medium supplemented with 0.5% oleic acid, at 30°C, 250 rpm for 48 h.

Table 3. Compositional modification of β-hydroxyalkanoates in mcl-PHA by the amplified *phaC1* gene of *P. putida* KT2440 at different carbon sources.

		PHA contents (%, w/w)	β-Hydroxyalkanoyl-methyl ester (mol %)[2]			
			HH	HO	HD	HDD
Gluconate (C_6)	Parent	24.0	4	26	63	6
	Recombinant	17.8	10	19	49	21
Octanoate (C_8)	Parent	10.2	N.D.[1]	51	32	17
	Recombinant	15.4	N.D.	31	32	37
Decanoate (C_{10})	Parent	27.6	N.D.	52	43	5
	Recombinant	35.4	N.D.	57	41	2
Oleic acid (C_{18})	Parent	26.6	N.D.	71	18	11
	Recombinant	43.5	N.D.	93	4	3

[1]N.D.: Not detected.
[2]HH, 3-hydroxyhexanoate; HO, 3-hydroxyoctanoate; HD, 3-hydroxydecanoate; HDD, 3-hydroxydodecanoate.
Parent and recombinant *P. putida* amplifying the *phaC1* gene were cultivated in MS medium supplemented with 0.5% (w/v) of different carbon sources at 30°C, 250 rpm for 48 h.

of protein in the recombinant *P. putida*. The content of PHA accumulated also increased significantly from 26.6% to 43.5% in the recombinant *P. putida* after enforcing the *phaC1* gene. The efficiency of mcl-PHA biosynthesis in *P. putida* could be increased significantly through the amplification of its own *phaC1* gene.

Content and Monomer Compositions of mcl-PHA in Recombinant *P. putida* Harboring Its Own *phaC1* Gene

Recombinant and parent *P. putida* were cultivated in various carbon sources, such as gluconate, octanoate, decanoate, and oleic acid, commonly used for the biosynthesis of mcl-PHA. As shown in Table 3, PHA content was elevated significantly after transformation of the cloned *phaC1* gene, increasing from 10.2 to 15.4% with octanoate, from 27.6 to 35.4% with decanoate, and from 26.6 to 43.5% with oleic acid. Meanwhile, an exceptionally small amount of mcl-PHA was accumulated with gluconate. This might have been due to limited flux of gluconate to the *de novo* pathway of fatty acid biosynthesis, which ultimately leads to the biosynthesis of mcl-PHA. The monomer compositions in mcl-PHA were significantly modified after amplification of PHA synthase activity; in particular, the molar fraction of 3-hydroxyoctanoate (C_8) cultured in octanoate, 3-hydroxydecanoate (C_{10}) in gluconate, and 3-hydroxyoctanoate (C_8) in both decanoate and oleic acid increased most noticeably.

Modulation of 3HV and 4HB molar fractions in scl-PHA has been attempted in a series of our earlier works [14, 22, 30, 31] using the recombinants *R. eutropha* and *A. latus* to transform its own *phbC* genes to amplify the PHB synthase activity. The 3HV monomer fraction in P(3HB-3HV) and 4HB fraction in P(3HB-4HB) have been found to be significantly modulated after transformation of its own *phbC* genes. This result also indicates that similar effect can be obtained even in the mcl-PHA producing strain *P. putida*, even though the detailed mechanism needs to be further investigated.

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