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Molecular Structure of the PHA Synthesis Gene Cluster from New mcl-PHA Producer *Pseudomonas putida* KCTC1639

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Abstract *Pseudomonas putida* KCTC1639 was newly identified as a potential producer of biodegradable medium chain length polyhydroxyalkanoates. It exhibited a carbon assimilation pattern quite different from other known *P. putida* strains, but a more similar pattern with *P. oleovorans*, which assimilates the carbon sources mainly through β-oxidation rather than the fatty acid biosynthesis pathway. The PHA synthesis gene cluster from *P. putida* KCTC1639 was composed of two gene loci; the PHA synthase gene locus and granule-associated gene locus, which were cloned and deposited in the GenBank under accession numbers AY286491 and AY750858 as a new nucleotide sequence, respectively. The molecular structure and amino acid homology of the new gene cluster were compared with those from *Pseudomonas* species, including other *P. putida* strains and *P. oleovorans*, and a higher than 90% homology was observed.

Key words: *P. putida* KCTC1639, mcl-PHA, PHA synthesis gene cluster, molecular structure, amino acid homology

The biodegradable polymer polyhydroxyalkanoates have been classified into two classes; short chain length PHA (scl-PHA) composed of C_4-C_5 and medium chain length PHA (mcl-PHA) of C_6-C_{14}. Only *Pseudomonas* species are known to accumulate mcl-PHAs through β-oxidation and the fatty acid biosynthesis pathway by *P. putida* and *P. aeruginosa*, and through β-oxidation for *P. oleovorans* [4, 14].

P. putida has been regarded as the major mcl-PHA-producing strain, and intensive researches have been carried out using this strain including cultivation of *P. putida* KT2440 [12], KT2442 [2], and BM01 [6] for overproduction of mcl-PHA, and *P. putida* U [1], 27N01 [15], and KCTC2407 [5] for biosynthesis of novel PHAs containing various functional phenoxy rings or carboxylic groups.

In our previous work [7], a new PHA synthase gene locus composed of *phaC1*, *phaZ*, *phaC2*, and *phaD* genes was cloned from *P. putida* KT2440, and it was deposited in the GenBank as a new gene sequence with accession number AY113181. The cloned *phaC1* gene encoding the PHA synthase I was transformed into the parent strain to achieve overproduction of mcl-PHA. The *phaC1* genes from *P. putida* KT2440 and the *phbC* gene from *R. eutorpha* H16 were cross-transformed to achieve *in vivo* blending of scl- and mcl-PHA [13].

The molecular structure of PHA synthase genes or clusters has to be determined not only for identification of the role of each gene on the biosynthesis of mcl-PHA but also for the metabolic engineering for strain improvement; nevertheless, only those of *P. putida* U [1] and *P. putida* KT2440 [7, 11] have been elucidated.

In this work, *P. putida* KCTC1639 was newly identified as a potential mcl-PHA-producing strain, and its biosynthesis capability and carbon utilization characteristics were studied. The PHA synthesis gene cluster composed of two gene loci (the PHA synthase gene locus and PHA granule-associated gene locus) was newly cloned, and the molecular structure and amino acid homology were compared with those from other *Pseudomonas* species.

Several prescreened *P. putida* strains were cultivated in an MS medium [17] supplemented with 0.5% (wt/vol) octanoate and 2.0% (wt/vol) gluconate as carbon sources at 30°C for 72 h. To identify the carbon assimilation characteristics, *P. putida* KCTC1639 was also cultivated in an MS medium supplemented with 2.0% (wt/vol) glucose, fructose, and gluconate as carbohydrates, and 0.5% (wt/vol) acetate, butyrate, hexanoate, octanoate, decanoate, dodecanoate, and tetradecanoate as fatty acids, respectively.

The composition and concentration of mcl-PHA were determined after extraction using chloroform from the lyophilized cell. The monomer units of mcl-PHA were

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Table 1. Comparison of various *Pseudomonas* species for accumulation of mcl-PHA.

Strains	Dry cell weight (g/l)		PHA contents (%, w/w)	
	Gluconate	Octanoate	Gluconate	Octanoate
P. putida KCTC2708	2.1	2.3	5.2	7.2
P. putida KCTC1644	2.3	2.1	6.5	7.4
P. putida KCTC1639	2.5	2.5	1.8	10.9
P. putida KT2440	2.6	2.0	8.8	10.3
P. oleovorans	2.1	2.2	0.9	8.8
P. aeuroginosa	2.0	2.4	7.5	10.3

Each strain was cultivated in MS medium supplemented with 2.0% gluconate and 0.5% octanoate as carbon sources at 30°C, 200 rpm, for 72 h.

methanolyzed using 15% acidified methanol at 100°C for 180 min, and then analyzed using a Perkin-Elmer 8420 GC system (PE Applied Biosystems, Foster City, CA, U.S.A.) packed with an HP-Innowax column (Hewlett-Packard Co., Palo Alto, CA, U.S.A.) [8].

The PHA synthesis gene cluster of *P. putida* KCTC1639 was amplified using a touchdown PCR; denaturation at 94°C for 30 sec, annealing from 65°C to 55°C for 1 min while decreasing 0.5°C per cycle during 20 cycles, and elongation at 72°C for 4 min. Three primer sets were designed considering the conserved regions from other known mcl-PHA-producing *Pseudomonas* species (*phaC1*-F: 5'-ACAGCGGCCTGTTCACCTGGG-3'; *phaC1*-R: 5'-GTG-CAGCCAGTGGATGCTGGT-3'; *phaC2*-F: 5'-CTACTG-GCAGCTGTTCGC-3'; *phaC2*-R: 5'-CGATCAGGTGCA-GGAACAGCC-3'; *phaDFI*-F: 5'-CGACCTGATCTGGA-ATTACTGG-3'; *phaDFI*-R: 5'-ATGAGGTACACAGCAT-GGCCAA-3').

The PCR fragments of the PHA synthesis gene cluster were ligated into the cloning vector pGEM-T easy (Promega Co. Milwaukee, WI, U.S.A.) to construct the recombinant plasmids pTPHA-C1, pTPHA-C2, and pTPHA-DFI, then transformed into *E. coli* DH5a for amplification. Each plasmid extracted from transformant *E. coli* were sequenced using an ABI PRISM 377 DNA sequencer (PE Applied Biosystems, Foster City, CA, U.S.A.).

The putative amino acid sequence of the PHA synthesis gene cluster of *P. putida* KCTC1639 was translated from the nucleotide sequence data by Translate tool in Expasy server program (http://kr.expasy.org/tools/dna.html), and compared with other *P. putida* strains [1, 7, 11], *P. oleovorans* [3], *Pseudomonas* sp. 61-3 [9, 10], and *P. aerugenosa* [16] using the Protein-Protein BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

The prescreened *P. putida* strains were cultivated in an MS medium containing gluconate and octanoate as carbohydrate and fatty acid, respectively, along with *P. oleovorans* and *P. aeruginosa.* As shown in Table 1, *P. putida* KCTC1639 showed the highest cell growth and PHA accumulation up with the content of 10.9% from octanoate, compared with other *P. putida* strains, as well as *P. oleovorans* and *P. aeruginosa.*

As shown in Table 2, it accumulated mcl-PHA more preferably from fatty acid that were catabolized through β-

Table 2. Effect of different carbon sources on dry cell weight, PHA contents, and monomer composition of *P. putida* KCTC1639.

Carbon sources	Dry cell weight (g/l)	PHA contents (%, w/w)	β-Hydroxylalcanoyl-methyl ester (mol %)[2]			
			C_6	C_8	C_{10}	C_{12}
Carbohydrate						
Glucose	1.0	2.5	5.2	1.2	21.1	72.5
Fructose	2.0	2.3	58.6	2.5	11.5	27.4
Gluconate	3.2	3.0	5.2	12.1	52.6	30.1
Fatty acid						
Acetate	1.0	0.4	100.0	N.D.	N.D.	N.D.
Butyrate	1.2	2.8	100.0	N.D.	N.D.	N.D.
Hexanoate	1.6	4.8	22.8	77.2	N.D.	N.D.
Octanoate	0.9	10.9	7.5	92.5	N.D.	N.D.
Decanoate	2.2	23.1	3.9	57.6	38.5	N.D.
Dodecanoate	1.5	7.1	5.4	28.7	29.5	36.3
Tetradecanoate	1.8	4.4	8.7	23.4	27.8	40.5

[1]N.D.: Not detected.
[2]C_6, 3-hydroxyhexanoate; C_8, 3-hydroxyoctanoate; C_{10}, 3-hydroxydocanoate; C_{12}, 3-hydroxydodecanoate *P. putida* was cultivated in MS medium supplemented with 2.0% carbohydrate and 0.5% fatty acid as carbon sources, as indicated, at 30°C, 200 rpm, for 72 h.

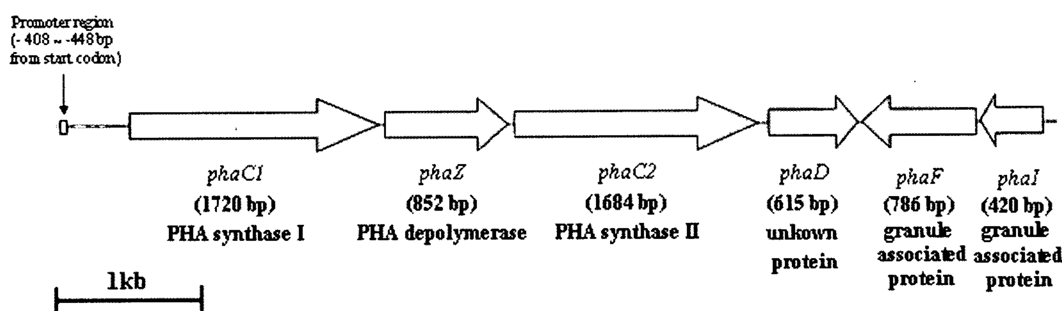

Fig. 1. Molecular structure of the PHA synthesis gene cluster locus from *P. putida* KCTC1639.
It was deposited in GenBank as a new PHA synthesis gene cluster under accession numbers AY286491 and AY750858.

oxidation, but not well from carbohydrate assimilated through the fatty acid biosynthesis pathway, showing carbon assimilation patterns quite different from those of other *P. putida* strains, which can simultaneously assimilate the carbohydrates and fatty acids [14]. *P. putida* KCTC1639 showed carbon assimilation characteristics more similar to *P. oleovorans*, which accumulates mcl-PHA mainly through β-oxidation of fatty acid [3]. Considering its higher PHA accumulation capability and unique carbon assimilation characteristics, *P. putida* KCTC1639 was selected as a new potential mcl-PHA producer.

The newly cloned PHA synthesis gene cluster of *P. putida* KCTC1639 was composed of 6,808 nucleotides encoding six ORFs: *phaC1*, *phaZ*, *phaC2*, *phaD*, *phaF*, and *phaI* genes. It was deposited in the GenBank under new accession numbers AY286491 for the *phaC1*, *phaZ*, and *phaC2* genes, and AY750858 for the *phaD*, *phaF*, and *phaI* genes. Figure 1 illustrates the molecular structure of the PHA synthesis gene cluster of *P. putida* KCTC1639, consisting of two loci; the PHA synthase gene locus consisted of *phaC1*, *phaZ*, *phaC2*, and *phaD* genes and the PHA granule-associated gene locus consisted of *phaF* and *phaI* genes. The above molecular arrangement was not different from those of *P. putida* KT2440 previously reported by current authors [7] and *P. putida* U [1] whose molecular structures have been so far identified.

The *phaC1* gene consisted of 1,720 bp sequencing from 409 to 2,128 bp containing 559 amino acids with MW of 62,199 Da, and the *phaC2* gene of 1,684 bp from 3,084 to 4,767 bp of 560 amino acids with MW of 62,809 Da. The *phaZ* gene was composed of 852 bp spanning from 2,191 to 3,042 bp, and the *phaD* gene of 615 bp from 4,781 to 5,395 bp. The putative promoter region for *phaC1* was located 408 bp upstream of the start codon; however, no such region was found in the *phaZ*, *phaC2*, and *phaD* genes, indicating that they seemed to be expressed by the putative promoter region in the *phaC1* gene, which is identical with *P. putida* U [1] and *P. putida* KT2440 [6], known to be controlled by a single promoter located in front of the *phaC1* gene.

The granule-associated *phaF* gene encoding protein PhaF consisted of 786 bp spanning from 5,546 to 6,331 bp containing 257 amino acids, corresponding to MW of 26,103 Da, whereas the *phaI* gene encoding protein PhaI consisted of 420 bp from 6,342 to 6,761 bp of 139 amino acids with MW of 15,446 Da. No identifiable putative promoter region was found in the PHA granule-associated gene locus.

As shown in Table 3, the homology of the putative amino acid sequence of the new PHA synthesis gene cluster from *P. putidia* KCTC1639 was compared with

Table 3. Amino acid sequence homology of the PHA synthesis gene cluster from *P. putidia* KCTC1639.

Gene product	Strains	Homology (%)
PHA synthase I	*P. putida* U	96
	P. putida KT2440	95
	P. oleovorans	96
	Pseudomonas sp. 61-3	82
	P. aeuroginosa	78
PHA synthase II	*P. putida* U	95
	P. putida KT2440	91
	P. oleovorans	90
	Pseudomonas sp. 61-3	73
	P. aeuroginosa	67
PHA depolymerase	*P. putida* U	97
	P. putida KT2440	91
	P. oleovorans	95
	Pseudomonas sp. 61-3	87
	P. aeuroginosa	84
PhaD	*P. putida* KT2440	90
	Pseudomonas sp. 61-3	74
	P. aeuroginosa	73
PhaF	*P. putida* KT2440	91
	P. oleovorans	90
	Pseudomonas sp. 61-3	71
	P. aeuroginosa	66
PhaI	*P. putida* KT2440	91
	P. oleovorans	95
	Pseudomonas sp. 61-3	64
	P. aeuroginosa	55

those of different *Pseudomonas* species that synthesize mcl-PHA. More than 90% total homology was found with those of two *P. putida* strains whose amino acid sequences have been identified. The amino acid sequence homologies of PHA synthase I, II, and PHA depolymerase were 95–96%, 91–95%, and 91–97%, respectively, compared with *P. putida* KT2440 [7] and *P. putida* U [1], showing even higher homology with *P. putida* U. The homology of the unknown function protein PhaD with *P. putida* KT2440 [7] was 90%. On the other hand, the PhaF and PhaI encoded in the PHA granule-associated gene locus showed 91% homologies with *P. putida* KT2440 [6].

It also showed a higher than 90% of total homology with *P. oleovorans* [3], but a relatively lower homology, ranging from 50% to 90%, compared with other mcl-PHA-producing *Pseudomonas* species, such as *Pseudomonas* sp. 61-3 [9, 10] and *P. aeuroginosa* [16] whose PHA synthesis gene cluster sequences are fully identified. *P. putida* KCTC1639 seems to have genetic characteristics of both *P. putida* and *P. oleovorans*, even though further research is needed to elucidate the detailed function of each gene in the PHA synthesis gene cluster from *P. putida* KCTC1639.

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