

# Identification and Analysis of Putative Polyhydroxyalkanoate Synthase (PhaC) in *Pseudomonas fluorescens*<sup>S</sup>

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*Pseudomonas fluorescens* KLR101 was found to be capable of producing polyhydroxyalkanoate (PHA) using various sugars and fatty acids with carbon numbers ranging from 2 to 6. The PHA granules consisted mainly of a poly(3-hydroxybutyrate) homopolymer and/or poly(3-hydroxybutyrate-co-3-hydroxyvalerate) copolymer. Genomic DNA of *P. fluorescens* was fractionated and cloned into a lambda library, in which a 5.8-kb fragment that hybridized to a heterologous *phaC* probe from *Ralstonia eutropha* was identified. In vivo expression in *Klebsiella aerogenes* KC2671 (pUMS), restriction mapping, Southern hybridization experiments, and sequencing data revealed that PHA biosynthesis by *P. fluorescens* relied upon a polypeptide encoded by a 1,683-bp non-operonal ORF, which was preceded by a possible -24/-12 promoter and highly similar to DNA sequences of a gene encoding PHA synthase in the genus *Pseudomonas*. In vivo expression of the putative PHA synthase gene (*phaC<sub>Pf</sub>*) in a recombinant *Escherichia coli* strain was investigated by using glucose and decanoate as substrates. *E. coli* (*phaC<sub>Pf</sub><sup>+</sup>*, pUMS) grown in medium containing glucose accumulated PHA granules consisting mainly of 3-hydroxybutyrate, whereas only a trace amount of 3-hydroxydecanoate was detected from an *E. coli* *fadR* mutant (*phaC<sub>Pf</sub><sup>+</sup>*) grown in medium containing decanoate. In vitro enzymatic assessment experiments showed that 3-hydroxybutyryl-CoA was efficiently used as a substrate of purified PhaC<sub>Pf</sub>, suggesting that the putative PHA synthase of *P. fluorescens* utilizes mainly short-chain-length PHA precursors as a substrate.

**Keywords:** *Pseudomonas fluorescens* KLR101, polyhydroxyalkanoate (PHA), putative PHA synthase gene, short-chain-length PHA precursors

## Introduction

Polyhydroxyalkanoate (PHA) is synthesized and accumulated as insoluble inclusions by various bacteria, when cells are cultivated in the presence of excess carbon sources along with a shortage of essential nutrients such as nitrogen, phosphate, oxygen, and/or sulfur ions [1]. PHA has received considerable interest as a biodegradable and biocompatible plastic with a wide range of potential applications [2]. Since the cloning of the PHA synthase operon of *Ralstonia eutropha* [3–5], numerous genes from a

wide range of bacteria have been cloned or identified [6, 7]. A total of 338 different PHA synthases from 214 species are available via Entrez protein search at the NCBI website (<http://www.ncbi.nlm.nih.gov/>). PHA synthases identified from members of the genus *Pseudomonas* contribute to the largest proportion (23% of total), which includes the recently identified *phaC* loci from two *P. fluorescens* strains obtained by full genome sequencing (GenBank No. CP000076 and CP000094). Likewise, accumulated evidence indicates that *P. fluorescens* strains are capable of PHA production [8–12]. However, their genetic backgrounds

have never been functionally evaluated. Prior investigations into the capacity of PHA accumulation by *P. fluorescens* KLR101 have reported the detection of poly(3-hydroxybutyrate) homopolymers and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) copolymers from cells grown in the presence of various carbon sources. A genomic fragment responsible for PHA production was obtained from a lambda library, and a PHA synthase gene (*phaC*) was identified by cloning, in vivo expression tests, and in vitro enzymatic assays.

## Materials and Methods

### Bacterial Strains and Plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. The *Pseudomonas fluorescens* and *Klebsiella aerogenes* strains were routinely propagated on nutrient agar at 28°C. The *Escherichia coli* strains were grown in LB medium at 37°C, and 100 µg/ml of ampicillin, 50 µg/ml of kanamycin, 50 µg/ml of chloramphenicol, and/or 15 µg/ml of tetracycline were added to the media, as indicated in Table 1.

### DNA Manipulation and Sequence Analyses

Primers I-179L and I-179R were used for PCR detection of the 540-bp *phaC* region [10]. Nucleotide sequencing analysis of the plasmids was performed using vector-borne primers or sequence-based internal primers. The nucleotide and deduced amino acid

sequences for PHA synthase of *P. fluorescens* KLR101 were compared with all sequences available in the GenBank database and aligned and edited using CLUSTAL\_X [13] and BIOEDIT software (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>). Phylogenetic trees were constructed using MEGA 3.1 software [14]. The GenBank/EMBL/DDBJ accession number for the newly determined *phaC* gene sequence of *P. fluorescens* is AY232443.

### Plasmid Construction

A genomic DNA library of *P. fluorescens* KLR101 was constructed using a Lambda DASH kit (Stratagene, USA). Plaque hybridization was performed with the 573-bp PstI fragment of the *phbC* gene from *Ralstonia eutropha* [15] by using a Genius kit (Boehringer Mannheim, USA). A 5.8-kb BglII fragment showing a positive signal was then cloned into the BamHI site of pBluescript II SK+ (Stratagene, USA). The resulting plasmid was used to transform *Klebsiella aerogenes* KC2671 (pUMS) [15] and then tested for in vivo expression. A 3.8-kb EcoRI fragment was then subcloned into pBluescript II SK+. The entire coding region for PHA synthase in *P. fluorescens* KLR101 was amplified from the 3.8-kb EcoRI fragment using forward primers (5'-GGAGTGTGGCATATGCGAGAAAG ACC-3' for pT7-7CPf and 5'-GGAGTGTGGATCCATGCGAGA AAGACC-3' for pRSET-ACPf) containing NdeI and BamHI enzyme sites, respectively (underlined), and reverse primers (5'-TGATCGCATATGTCAGCGCACTCGCA-3' for pT7-7CPf and 5'-TGATCGCCATGGTCAGCGCACTCGCA-3' for pRSET-ACPf) containing NdeI and NcoI enzyme sites, respectively (underlined). The amplified products were digested using the corresponding

**Table 1.** Bacterial strains and plasmids used in this study.

Bacterial strain or plasmid	Relevant feature(s)	Reference
<b>Bacterial strains</b>		
<i>Pseudomonas fluorescens</i> KLR101	Wild type; <i>phaC</i>	This study
<i>Klebsiella aerogenes</i> KC2671	<i>hutC515 recA3011 Δ[bla]-2</i>	[15]
<i>Escherichia coli</i>		
BL21(DE3) pLysS	F- <i>ompT hsdSB(rB-mB)- gal dcm</i> (DE3) pLysS (Cam <sup>r</sup> )	Invitrogen
LS5218	<i>fadR601 atoC</i> (Con)2	[37]
<b>Plasmids</b>		
pBluescript II SK+	Phagemid vector; <i>Plac</i> promoter; f1 ori; ColE1 ori; Amp <sup>r</sup>	Stratagene
pT7-7	T7 expression vector; ColE1 ori; Amp <sup>r</sup>	[39]
pUMS	<i>phbA<sub>Re</sub>; phbB<sub>Re</sub>; p15A</i> ori; Tet <sup>r</sup>	[16]
pCR2.1	PCR cloning vector; f1 ori; pUC ori; Amp <sup>r</sup> ; Kan <sup>r</sup>	Invitrogen
pKK223-3	<i>Ptac</i> expression vector; pBR322 ori, Amp <sup>r</sup>	[38]
pREP4	<i>lacI<sup>q</sup></i> ; p14A ori; Km <sup>r</sup>	Qiagen
pRSET-A	T7 expression vector; N-terminal 6-His tag; ColE1 ori; Amp <sup>r</sup>	Invitrogen
pT7-7CPf	pT7-7 carrying <i>phaC<sub>Pf</sub></i>	This study
pCR2.1CPf	pCR2.1 carrying <i>phaC<sub>Pf</sub></i>	This study
pKK223-3CPf	pKK223-3 carrying <i>phaC<sub>Pf</sub></i>	This study
pRSET-ACPf	pRSET-A carrying <i>phaC<sub>Pf</sub></i>	This study

enzymes and ligated to polylinker regions of T7 expression vectors, pT7-7 and pRSET-A, resulting in pT7-7CPf and pRSET-ACPf, respectively. An Aval site located 570 bp downstream from the start codon was used to confirm the correct direction of the *phaC* ORF on pT7-7CPf. Polylinker regions of the recombinant plasmids were sequenced for confirmation of in-frame cloning. Plasmids pT7-7CPf and pRSET-ACPf were then transformed into *E. coli* BL21 (DE3) pLysS. After the overexpression of the appropriate polypeptide was confirmed by SDS-PAGE experiments, *E. coli* BL21(DE3) pLysS (pT7-7CPf) was transformed using pUMS [16] for the in vivo expression test.

Plasmid pKK223-3CPf was constructed as follows. The ORF for PHA synthase was amplified using forward (5'-ATGCGAGAAAGACCCGTGACG-3') and reverse (5'-TCAGCGCACTCGCACATAGGT-3') primers, and cloned into the pCR2.1 vector (Invitrogen, USA), from which a 1.7-kb EcoRI fragment containing the *phaC* ORF was excised and cloned into the *tac* expression vector pKK223-3. In-frame cloning was confirmed by sequencing of a polylinker region. The plasmid pKK223-3CPf was then used to transform *E. coli* LS5218, which was subsequently transformed by the LacI expression vector pREP4 (Qiagen, USA). Overexpression of putative PhaC in the strain LS5218 (pKK223-3CPf and pREP4) by IPTG induction was confirmed by SDS-PAGE experiments. Plasmid maps were drawn using CloneMap software (CGC Scientific, USA).

#### Overexpression of PHA Synthase

Overnight cultures of recombinant *E. coli* strains were harvested and washed, and a portion of the cell suspension was inoculated into 100 ml of freshly prepared of LB medium. Protein production was induced by the addition of IPTG (0.4 mM) for 2 h, followed by purification and separation on a 10% SDS-PAGE gel using the CBS Lite slab-gel system (CBS Scientific, USA). Protein bands were visualized by staining using Coomassie brilliant blue R-250 (Bio-Rad, USA), and the gels were dried using gel-drying films (Promega, USA).

#### In Vivo Expression of PHA Synthase and Detection of PHA

*P. fluorescens* and recombinant *K. aerogenes* were cultivated in a reciprocal shaker for 72 h in standard nutrient liquid (SNL) medium [17] supplemented with carbon sources. *E. coli* BL21(DE3) pLysS (pT7-7CPf and pUMS) was incubated in LB medium containing 1% (w/v) glucose for 48 h. *E. coli* LS5218 (pKK223-3CPf and pREP4) was incubated in LB medium containing 0.2% (w/v) decanoate in the presence of 0.2 mg/ml of acrylate [18], to which glucose (1% (w/v)) was added for cell growth. A stock solution of decanoate was prepared by dissolving in 0.5% (w/v) Brij58 [19]. IPTG was added to LB medium at a concentration of 0.2 mM every 12 h. PHA granules were selectively stained with Nile blue A (Sigma, USA) according to Ostle and Holt [20] and examined under a confocal microscope. Cells were harvested, washed, lyophilized, and subjected to methanolysis according to Brandl *et al.* [21]. Gas chromatographic analysis was performed

using an HP-Innowax crosslinked PEG column (60 m × 0.32 mm × 0.25 μm) with the HP 6890 GC system (Agilent, USA).

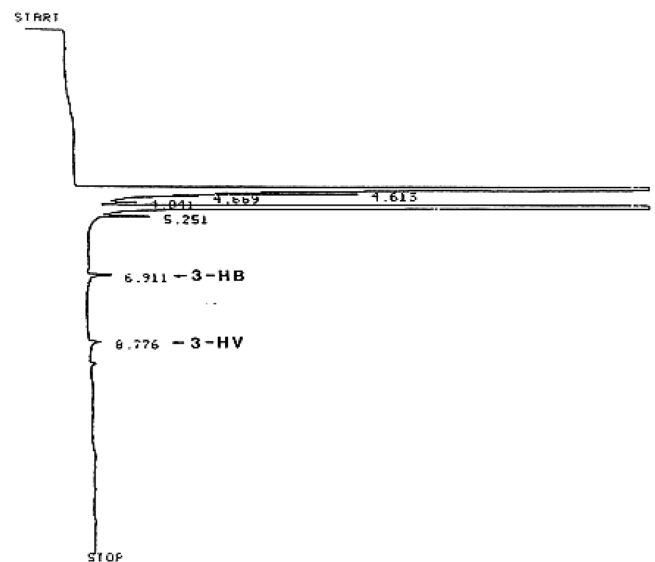
#### In Vitro Enzymatic Assessment of Soluble PHA Synthase

The enzymatic activity of soluble PHA synthase was determined using either purified enzyme or crude extracts. His-tagged protein was purified from an overexpressed cell culture of *E. coli* BL21(DE3) pLysS (pRSET-ACPf) using the ProBond purification system (Invitrogen, USA), in which a purified fraction was concentrated and reconstituted with KPi buffer (143 mM KPi, pH 7.2, and 5% (v/v) glycerol) using a 30,000 MW cutoff filter, Centricon YM-30 (Merk, USA). A portion of the purified fraction was examined by SDS-PAGE. Soluble cell extracts of *E. coli* BL21(DE3) pLysS (pT7-7CPf) were prepared according to Qi *et al.* [22]. The enzyme assay was performed based on the off-line Ellman method [22–26] using 0.5 mM DL-β-hydroxybutyryl-CoA (Sigma, USA) as a substrate. The relative concentration of free thiols was calculated according to the equation  $C_o = A/\epsilon \times D$  [9], where  $C_o$  is the original concentration of CoA thioester,  $A$  is the absorbance at 412 nm,  $\epsilon$  is the extinction coefficient (13.7 mM/cm), and  $D$  is the dilution factor. Nonspecific hydrolysis of CoA thioester was monitored using a negative control.

## Results and Discussion

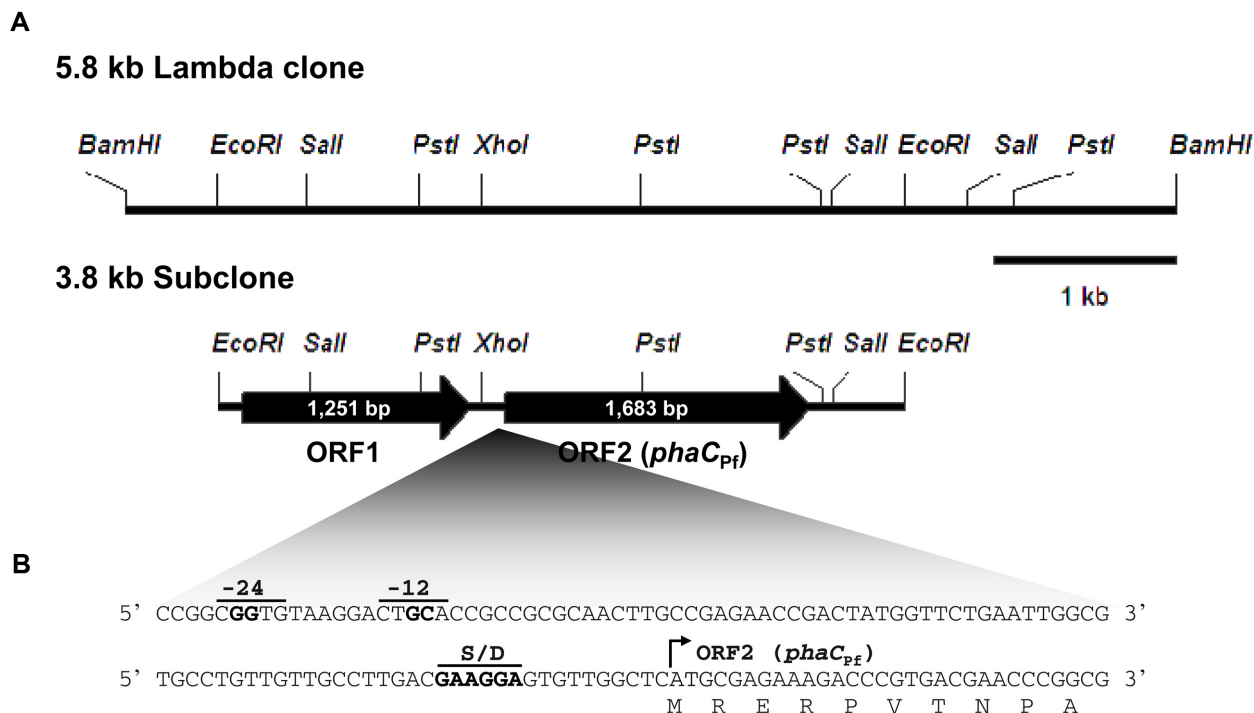
#### Available Carbon Sources for PHA Production by *P. fluorescens* KLR101

*P. fluorescens* KLR101 was capable of accumulating PHA granules when cells were grown in SNL medium



**Fig. 1.** Gas chromatogram of *Pseudomonas fluorescens* grown in 1% (w/v) glucose and 10 mM propionate.

3-HB and 3-HV indicate 3-hydroxybutyrate and 3-hydroxyvalerate, respectively.



**Fig. 2.** Restriction maps of the clones harboring *phaC<sub>pf</sub>* (A) and the 5' UTR sequence of ORF2 (B).

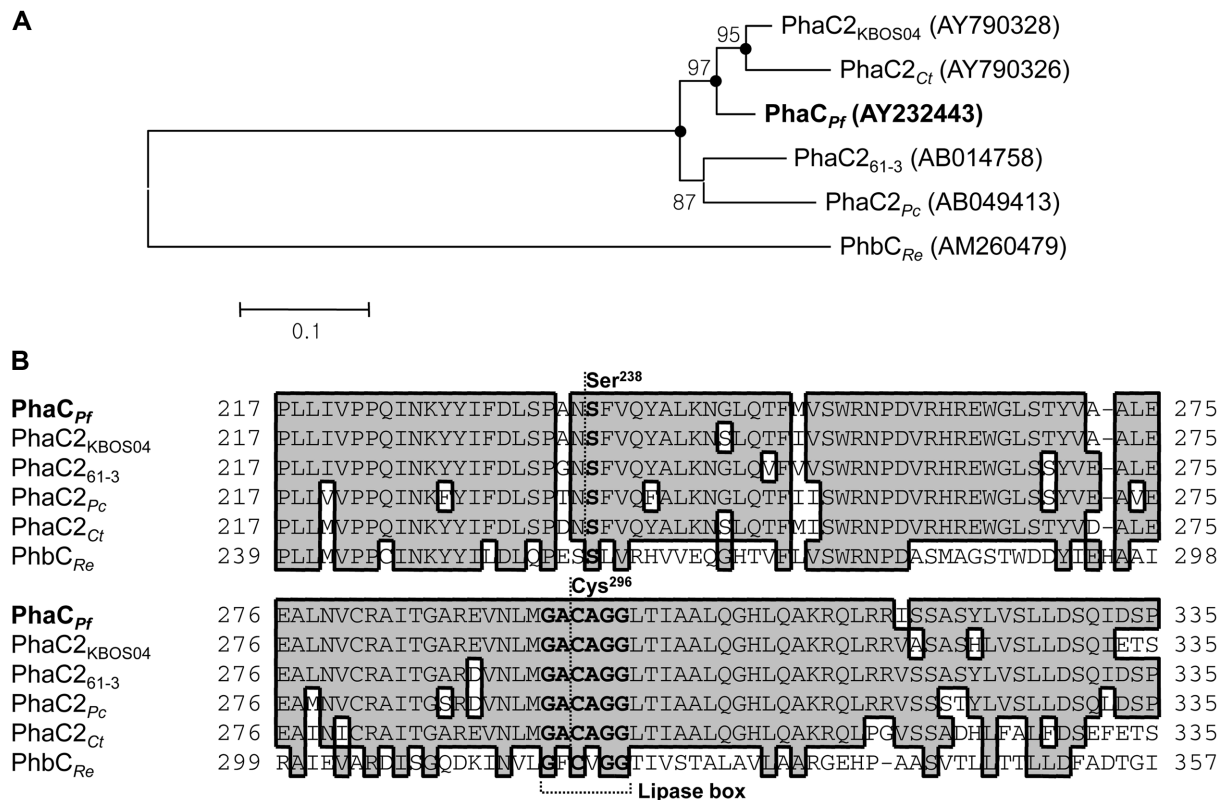
A possible -24/-23 promoter and a Shine-Dalgarno (S/D) sequence are represented on the 5' UTR sequence of ORF2 (B).

supplemented with various carbon sources, such as glucose (1% (w/v)), sucrose (1% (w/v)), sodium acetate (0.1% (w/v)), propionate (10 mM), gluconate (1% (w/v)), sodium citrate (0.1% (w/v)), or benzoate (5 mM). Gas chromatographic analyses showed that the predominant monomer unit was 3-hydroxybutyrate. When the strain was grown in 1% (w/v) glucose and 10 mM propionate, production of a poly(3-hydroxybutyrate-co-3-hydroxyvalerate) copolymer was also observed (Fig. 1). Confirmation that *P. fluorescens* KLR101 possesses an intrinsic PHA biosynthesis pathway was carried out by amplification and sequencing analysis of the 540-bp internal region of the *phaC* gene encoding PHA synthase [10] (data not shown). Members of the genus *Pseudomonas* are well-known medium-chain-length (mcl)-PHA producers [27]. However, the profile of utilizable carbon sources revealed that the *in vivo* substrate range of strain KLR101 was mainly short-chain-length (scl)-PHA precursors.

#### Genetic Organization of Two ORFs

A 5.8-kb genomic fragment of the strain was identified from the lambda clone library using the heterologous gene probe from *R. eutropha* [15] (Fig. S1). As shown in Fig. 2A, the *phaC* gene was detected in a 3.8-kb *EcoRI* fragment, which

was subcloned and sequenced (GenBank No. AY232443). A sequence similarity search was conducted, and two possible ORFs of approximately 1.25 and 1.68 kb in the 3.8-kb fragment were detected, which was consistent with the restriction mapping results (Fig. 2A). Of particular note, ORF2 was markedly matched with the *phaC2* gene of *Pseudomonas* sp. KBOS 04 (GenBank No. AY790328) with 88% sequence similarity. Moreover, a potential ribosome-binding site [28] and possible -24/-12 promoter region were identified in ORF2 [29, 30] (Fig. 2B). ORF1 consisted of 1,251 bp with its own promoter (possible -35/-10 promoter), and the deduced product was highly similar to the amino acid sequence of PhhR, a transcriptional activator of the phenylalanine hydroxylase gene cluster [31]. Despite the high sequence similarity with type II PHA synthase, the arrangement of the putative PHA synthase gene was slightly different from those of typical type II synthase genes. The *phaC2* genes are hitherto organized as an operonal arrangement known as *phaC1ZC2* [30, 32-35], of which gene expression is generally controlled by the -24/-12 and/or -35/-10 promoter preceding *phaC1* [7, 30, 33]. Some *phaC2* genes have been reported to have their own -35/-10 promoters [33, 35], which is considered as bicentricity, offering flexibility in response to environmental



**Fig 3.** Phylogenetic relationship between the PHA synthase from *P. fluorescens* KLR101 and those from closely related strains. (A) Bootstrap values (using 1,000 replications) are indicated at the branch points. Filled circles demonstrate that the corresponding nodes were also recovered in maximum-parsimony trees [40]. Accession numbers are shown in parentheses. Bar, 0.1 expected nucleotide substitutions. (B) Partial sequence alignment is shown. Identical amino acids are shaded, and highly conserved motifs are indicated. PhaC<sub>Pf</sub>, PhaC from *P. fluorescens* KLR101; PhaC2<sub>KBOS04</sub>, PhaC2 from *Pseudomonas* sp. KBOS 04; PhaC2<sub>61-3</sub>, PhaC2 from *Pseudomonas* sp. 61-3; PhaC2<sub>Pc</sub>, PhaC2 from *P. chlororaphis* IFO 3521; PhaC2<sub>Ct</sub>, PhaC2 from *Comamonas testosteroni* KBOS 01; PhbC<sub>Re</sub>, PhbC from *Ralstonia eutropha* H16.

changes. In order to determine whether or not ORF2 is a gene for PHA production, a functional expression system using the *K. aerogenes* KC2671 (pUMS) strain, which is unable to produce PHA, was employed. When in vivo expression of the 5.8-kb lambda fragment was conducted in this strain, PHB granule formation was only observed under nitrogen-limiting conditions (Fig. S2A), indicating that the -24/-12 promoter preceding ORF2 could actively respond to environmental changes. Thus, the putative *phaC* gene is considered as a non-operonal gene and may have specific effects on PHA production in strain KLR101.

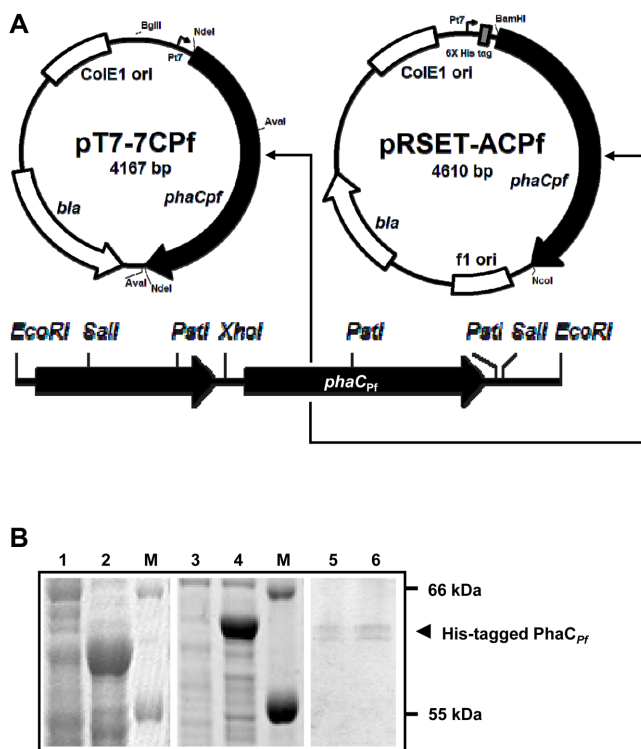
#### Phylogenetic Analysis and Conserved Residues of Putative PhaC<sub>Pf</sub>

A polypeptide, designated as PhaC<sub>Pf</sub>, consisting of 560 amino acids with an isoelectric point of 9.12 and a calculated MW of 62.6 kDa, was deduced from ORF2, which is in agreement with well-known properties of PHA

synthases [2]. In a phylogenetic tree constructed by the neighbor-joining algorithm [36], PhaC<sub>Pf</sub> formed a coherent cluster with PHA synthases identified from members of the genera *Pseudomonas* and *Comamonas* (Fig. 3A). Functionally important residues for enzymatic activity were conserved in the lipase box (Gly-X-Cys<sup>296</sup>-X-Gly-Gly) [2, 30], in which Cys<sup>296</sup> is known as a catalytic thiol covalently bonded to a growing polymer chain [25] (Fig. 3B). The possible aspartate and histidine residues that participate in the catalytic triad were identified as Asp<sup>329</sup>, Asp<sup>401</sup>, His<sup>453</sup>, and His<sup>480</sup> (data not shown). Highly conserved Ser<sup>238</sup>, which is known as a target of post-translational 4-phosphopantetheinylation [25], was also detected in the primary structure of PhaC<sub>Pf</sub> (Fig. 3B).

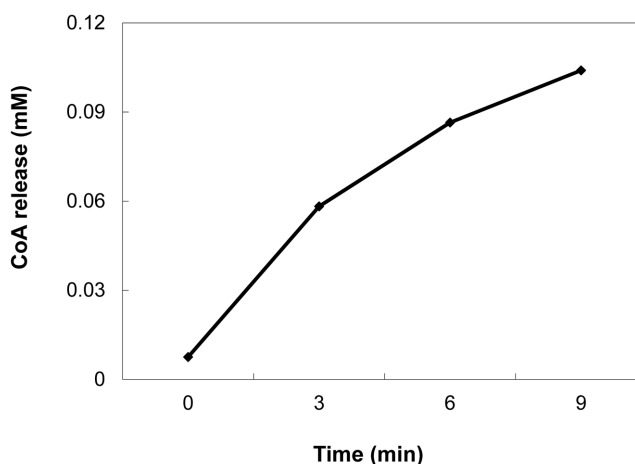
#### Overexpression and Functional Expression of Putative PhaC<sub>Pf</sub>

The entire region of ORF2 was amplified using NdeI-site



**Fig. 4.** Plasmid construction and overexpression of PhaC<sub>Pf</sub>. (A) ORF2 was cloned non-directionally and directionally into pT7-7 and pRSET-A, respectively, via tailed PCR cloning. *Ava*I sites, which were used as orientation markers, are indicated on pT7-7CPf. (B) Overexpression of the two plasmids was visualized on a 10% SDS-PAGE gel. Lanes 1 and 2, crude lysates of pT7-7CPf clones without and with IPTG, respectively; lanes 3 and 4, crude lysates of pRSET-ACPF clones without and with IPTG, respectively; lanes 5 and 6, 10- and 20- $\mu$ l portions of the purified fraction of pRSET-ACPF-clone lysate, respectively; M, Mark12 Unstained standard (Invitrogen, USA).

containing primers and cloned into expression vector pT7-7, resulting in pT7-7CPf (Fig. 4A). Using sequencing, the start codon of ORF2 was confirmed to be preceded by a ribosome-binding site and a T7 promoter without an internal start codon. Overexpression test of pT7-7CPf in *E. coli* BL21(DE3) pLysS showed that a polypeptide of around 60 kDa was overproduced upon IPTG induction (Fig. 4B, lanes 1 and 2). Recombinant *E. coli* was then transformed using the plasmid pUMS harboring *phbA* (3-ketothiolase gene) and *phbB* (acetoacetyl-CoA reductase gene). Cells grown in 1% (w/v) glucose and 0.4 mM IPTG accumulated PHA granules that consisted mainly of 3-hydroxybutyrate (Fig. S2), indicating that PHA synthase from *P. fluorescens* KLR101 was functionally expressed under in vivo conditions. Decanoate as a substrate was also tested using the *E. coli fadR atoC* (Con) mutant LS5218 [37].



**Fig. 5.** Reaction kinetics of His-tagged PhaC<sub>Pf</sub> for 3-HB-CoAs. The reaction was initiated by addition of substrate, and the time course of CoA release was monitored at 412 nm with 5,5'-dithiobis-2-nitrobenzoic acid [23].

To reduce the possibility that the  $\beta$ -oxidation pathway was not fully activated in the fatty acid-adapted cell portion, *E. coli* LS5218 was employed as a host strain. ORF2 was cloned into a *tac* promoter vector, pKK223-3 [38], and the resulting plasmid pKK223-3CPf was transformed into strain LS5218, which was then co-transformed with pREP4 as the IPTG control. Recombinant *E. coli* showed an overexpression profile similar to that of *E. coli* BL21(DE3) pLysS (pT7-7CPf) (data not shown). The strain was cultivated with 0.2% (w/v) decanoate in the presence of 1% (w/v) glucose for cell growth and mcl-PHA production was induced by the addition of acrylate [18]. However, only a trace amount of 3-hydroxydecanoate was detected by GC analysis (Fig. S2B).

To demonstrate the in vitro enzymatic activity of soluble PhaC<sub>Pf</sub>, ORF2 was cloned into pRSET-A (Fig. 4A). Sequencing of a polylinker region of the resulting plasmid (pRSET-ACPF) confirmed that a polypeptide translated from the plasmid was in-frame with a leader peptide (36 amino acids) containing a hexahistidyl sequence, which was consistent with the overexpression profile of pRSET-ACPF (Fig. 4B, lanes 3 and 4). His-tagged PhaC<sub>Pf</sub> was then purified and visualized on a SDS-PAGE gel (Fig. 4B, lanes 5 and 6). The purified protein was used for enzymatic assays as follows.

#### Enzymatic Activity of the Putative PHA Synthase

The reaction kinetics of the purified enzyme with 3-hydroxybutyryl-CoA over a short period demonstrated a proportional relationship (Fig. 5), indicating a high

substrate preference of His-tagged PhaC<sub>pf</sub> for the scl-PHA monomer. The soluble cell lysate of *E. coli* BL21(DE3) pLysS (pT7-7CPf) showed similar reaction kinetics (data not shown), which indicates no difference in enzyme activity between the wild-type and fusion proteins. Thus, it appears that polymerization was initiated immediately after substrate addition without any lag period, and the reaction was sufficiently efficient to release free CoA thioesters at a rate of 10.7  $\mu\text{M}/\text{min}$ .

Taken together, the phylogenetic analyses indicate that the putative PHA synthase identified from *P. fluorescens* KLR101 had high sequence similarity with type II PHA synthase PhaC2. However, the synthase was found to be different owing to its unique gene structure and much higher substrate specificity for scl-PHA precursors over mcl-PHA precursors. Therefore, the PHA synthase from *P. fluorescens* KLR101 could be categorized as a type I synthase.

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## Conflict of Interest

The authors have no financial conflicts of interest to declare.

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