Specific Identification of (*R*)-3-hydroxyacyl-ACP: CoA Transacylase Gene from *Pseudomonas* and *Burkholderia* Strains by Polymerase Chain Reaction

应用聚合酶链式反应快速特异鉴定假单胞菌和伯克霍尔德氏菌(*R*)-3-羟基酯酰载酯蛋白-辅酶 A 转酰基酶基因

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摘 要 聚羟基脂肪酸酯(PHA)是一类具有广泛应用前景的可降解生物塑料。因其可以以葡萄糖等廉价底物直接发酵生产 PHA 而日益受到重视。目前的研究表明在积累中长链 PHA 的假单胞菌中,由 *phaG* 基因编码的(*R*)-3-羟基酯酰载酯蛋白-辅酶 A 转酰基酶(PhaG)起关键作用,但目前为止对该蛋白还知之甚少。通过聚合酶链式反应(PCR)建立了一种快速、特异鉴定 *phaG* 基因的方法,应用该方法成功地从两株积累不同 PHA 的假单胞菌 *Pseudomonas stutzeri* 1317 和 *Pseudomonas nitroreducens* 0802 中分别克隆得到 *phaG* 基因,并在 *phaG* 基因突变株 *Pseudomonas putida* $PHAG_N$-21 中表达成功。同时,还首次报道了从非假单胞菌菌株 *Burkholderia caryophylli* AS 1.2741 中鉴定得到 *phaG* 基因,提示 PhaG 介导的中长链 PHA 合成途径作为一种通用的代谢模式在细菌中广泛存在,为进一步实现从廉价的非相关底物合成中长链 PHA 提供了必要的分子生物学基础。

关键词 聚羟基脂肪酸酯,(*R*)-3-羟基酯酰载酯蛋白-辅酶 A 转酰基酶,聚合酶链式反应,*Pseudomonas stutzeri*(施氏假单胞菌),*Pseudomonas nitroreducens*(硝基还原假单胞菌),*Burkholderia caryophylli*(麝香石竹伯克霍尔德氏菌)

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Abstract Polyhydroxyalkanoates (PHA) were biodegradable thermoplastics. Due to their broad applications, direct biosynthesis of PHA from inexpensive substrates, such as carbohydrates, is actively pursued. It has been recently revealed that (*R*)-3-hydroxyacyl-ACP:CoA transacylase (PhaG) played an important role in this pathway. In this study, a polymerase chain reaction (PCR) protocol was developed for the rapid and specific identification of *phaG* gene from various bacteria. Using the PCR strategy, the complete open reading frames of two *phaG* genes from *Pseudomonas stutzeri* 1317 and *Pseudomonas nitroreducens* 0802 were cloned from the genomic DNA and functionally expressed in *Pseudomonas putida* $PHAG_N$-21. Furthermore, this strategy was successful applied in non-*Pseudomonas* strains, such as *Burkholderia*. These results suggest that PhaG-mediated pathway of medium-chain-length polyhydroxyalkanoates was widespread among bacteria.

Key words Polyhydroxyalkanoates, (*R*)-3-hydroxyacyl-ACP:CoA transacylase, Polymerase chain reaction, *Pseudomonas stutzeri*, *Pseudomonas nitroreducens*, *Burkholderia caryophylli*

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1 INTRODUCTION

Polyhydroxyalkanoates (PHA) are produced by various microorganisms as intracellular carbon and energy storage material under nutrient-limiting conditions[1-3]. Most fluorescent *Pseudomonas* strains belonging to the rRNA homology group I containing γ subdivision Proteobacteria are able to accumulate PHA consisting of various 3-hydroxyalkanoates (3HA) with carbon-chain-length ranging from 6 to 14 carbon atoms (medium-chain-length, MCL)[4]. PHA are expected to become alternatives for petrochemical-based plastics because they are biodegradable thermoplastics[5], and are also considered as new tissue engineering materials due to their excellent biocompatibility[6-7].

Composition of PHA depends on the synthase, the carbon source, and the metabolic routes involved[1-4]. It has been reported that several fluorescent *Pseudomonas* accumulate MCL PHA (PHA_{MCL}) from carbon sources non-related to PHA structure by employing a fatty acid *de novo* biosynthesis pathway[1-4]. Via this route, (*R*)-3-hydroxyacyl-ACPs, the intermediates of fatty acid *de novo* biosynthesis pathway, were converted to their corresponding CoA derivatives to serve as the substrates of PHA synthase. This conversation can be mediated in a one step reaction catalyzed by an (*R*)-3-hydroxyacyl-ACP: CoA transacylase (PhaG), encoded by *phaG* gene[8]. Interestingly, heterologous expression of *phaG* in *Escherichia coli* led to extracellular accumulation of 3-hydroxydecanoic acid[9], with the assistant of thioesterase II[10].

However, due to the complexity of the bacterial genome, *phaG* genes were only isolated from four *Pseudomonas* strains by complex heterologous-probe-hybridization method to date, including *Pseudomonas putida*[8], *Pseudomonas aeruginosa*[11], *Pseudomonas oleovorans*[12], and *Pseudomonas* sp. 61-3[13], though a high efficiency identification method has been pursued for a while.

In this study, a rapid and convenient polymerase chain reaction (PCR) strategy, utilizing short conserved stretches of published *phaG* genes, was performed to clone the *phaG* gene from two PHA synthesis *Pseudomonas* strains that synthesize PHA. Complete *phaG* genes of these microorganisms were directly cloned and identified from their genomes for the first time. Furthermore, the strategy was also successful in cloning *phaG* gene from a non-*Pseudomonas* PHA-synthesis strain *Burkholderia caryophylli* AS 1.2741, which belongs to the rRNA homologous group II containing β subdivision Proteobacteria.

2 MATERIALS AND METHODS

2.1 Bacterial strains, plasmids, and growth conditions

Bacterial strains and plasmids used in this study are summarized in Table 1. *Pseudomonas stutzeri* 1317, *Pseudomonas nitroreducens* 0802, and *B. caryophylli* AS 1.2741 were isolated from oil-contaminated soil samples in north China and identified by the Institute of Microbiology, Academic Sinica in Beijing, China. All strains were cultured at 30℃ and 200 r/min overnight in Luria-Bertani (LB) medium. 1 ml inoculum from the overnight-cultured broth was added to fresh mineral salt (MS) medium and grown at 30℃ on a rotating shaker (NBS Series 25D, New Brunswick, USA) at 200 r/min for 48 h[10]. Carbon source, such as glucose, sodium octanoate, and lauric acid, was added at the beginning of the fermentation, and the pH was adjusted to 7.0. If necessary, 50 mg/L kanamycin was added to the medium to maintain the stability of the plasmids.

2.2 Cellular dry weight and PHA analysis

To determine the cellular dry weight (CDW), liquid cultures were centrifuged at 10 000 × g for 15 min, the cells were washed twice and lyophilized for 24 h. Determination of the intracellular PHA content and polymer composition by gas chromatography were performed as described previously[10].

2.3 DNA manipulations

Ex Taq DNA polymerase were purchased form TaKaRa (Dalian, China); T4 DNA ligase and pGEM®-T vector system I were purchased from Promega (Madison, WI, USA); restriction endonucleases were purchased from New England Biolabs (Beverly, MA, USA). All primers used in this study (P1: 5′-CCC CAA CAT ATG AGG CCA GAA AT-3′; P2: 5′-ACC CAG GAA TTC AGA TGG CAA AT-3′; P3: 5′-CGC TCT AGA ACT AGT GGA TCC CCC GGG CAA GTA CCT-3′; P4: 5′-TTC CTG CAG CAT ATG TTG ATT GTC TCT CTG CCG TCA-3′) were supplied from BioAsia company (Shanghai, China). The conjugation technique was conducted as described by Simon *et al*.[14], in which *Escherichia coli* S17-1 was employed as a donor strain. All other genetic techniques were performed following either standard procedures[15] or the instructions from the manufactures.

Touchdown PCR technology (employing the P1-P2 primer pair) was applied to obtain the preferred product and minimize artifacts. Briefly, after the initial 2 min of denaturation at 95℃, the annealing temperature was changed from 65℃ (5 cycles) to 60℃ (5 cycles) and then to 55℃ (25 cycles). Elongation was at 72℃ for 2 min, with the last step for 5 min at 68℃.

2.4 Nucleotide Sequence Accession Number

The nucleotide sequences of *phaG* genes identified here will appear in the GenBank databases under accession numbers: AF516488 ($phaG_{Ps}$ from *P. stutzeri* 1317), AY039839 ($phaG_{Pn}$ from *P. nitroreducens* 0802), and AY039840 ($phaG_{Bc}$ from *B. caryophylli* AS 1.2741).

Table 1 Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant characteristic(s)*	Source or reference
Strains		
Escherichia coli S17-1	*recA*; harbors the tra genes of plasmid RP4 in the chromosome; *proA thi*-1	[14]
Pseudomonas putida PHAG$_N$-21	PhaG-negative mutant of *P. putida* KT2440	[8]
Pseudomonas stutzeri 1317	rRNA homology group I, non-fluorescent pigment produced, PHA-producing	Lab collection
Pseudomonas nitroreducens 0802	natural relationship is tentative, fluorescent pigment produced, PHA-producing	Lab collection
Burkholderia caryophylli AS 1.2741	rRNA homology group II, PHA-producing	Lab collection
Plasmids		
pGEM$^{®}$-T	*Ap*(*r*); *lacPOZ'*; T-vector	Promega Co.
pBluescript SK$^-$	*Ap*(*r*); *lacPOZ'*	[15]
pBBR1MCS-2	*Kn*(*r*); broad-host-range vector	[16]
pBHR68	pBluescript SK$^-$ containing *Wautersia eutropha* polyhydroxybutyrate synthesis operon downstream of *lac* promoter	[17]
pLZZH07	pBluescript SK$^-$ containing *R. eutropha* PHB-synthesis-locus's promoter with a *Nde* I site at the exactly translate start point	This study
pLZZH13	pBBR1MSC-2 containing *R. eutropha* PHB-synthesis-locus's promoter with a *Nde* I site at the exactly translate start point	This study
pLZZG06	pLZZH13 containing *phaG* gene cloned from *P. stutzeri* 1317	This study
pLZZG08	pLZZH13 containing *phaG* gene cloned from *P. nitroreducens* 0802	This study
pLZZG09	pLZZH13 containing *phaG* gene cloned from *B. caryophylli* AS 1.2741	This study

* *Ap*(*r*), ampicillin resistant gene; *Kn*(*r*), kanamycin resistant gene; gene *phaG* encodes (*R*)-3-hydroxyacyl-ACP:CoA transacylase.

Table 2 Growth and PHA accumulation of *Pseudomonas* and *Burkholderia* strains cultivated on various carbon sources[a]

Bacteria strain	Carbon source[b]	CDW[c]/(g/L)	PHA/CDW/(%, W/W)	Composition of PHA (mol %)[c]				
				3HB	3HHx	3HO	3HD	3HDD
P. stutzeri 1317	G	1.47 ± 0.07	20.55 ± 4.50	2.53	1.51	18.81	67.43	9.72
	O	1.37 ± 0.08	9.34 ± 0.95	ND[d]	6.68	76.38	12.67	4.27
	L	1.22 ± 0.08	34.24 ± 1.17	ND	3.67	39.47	46.57	10.29
P. nitroreducens 0802	G	0.16 ± 0.02	ND	–	–	–	–	–
	O	1.59 ± 0.12	52.11 ± 1.83	100	ND	ND	ND	ND
	L	1.06 ± 0.02	34.32 ± 1.28	97.39	ND	0.19	1.86	0.59
B. caryophylli AS 1.2741	G	1.65 ± 0.11	4.44 ± 0.52	2.71	1.00	8.57	55.21	32.51
	O	1.69 ± 0.09	31.48 ± 2.58	ND	4.68	89.96	3.78	1.58
	L	2.46 ± 0.15	35.37 ± 1.32	0.26	4.20	45.30	40.12	10.12

[a] Cells were cultivated on MS medium containing various carbon sources. After 48 h cultivation at 30℃ and 200 r/min, cells were harvested and detected. Results were obtained from three separate fermentation experiments;

[b] G: 20 g/L glucose; O: 2.5 g/L sodium octanoate; L: 5 g/L lauric acid;

[c] CDW, cellular dry weight; 3HB, 3-hydroxybutyrate; 3HHx, 3-hydroxyhexanoate; 3HO, 3-hydroxyoctanoate; 3HD, 3-hydroxydecanoate; 3HDD, 3-hydroxydodecanoate;

[d] ND, not detectable.

3 RESULTS

3.1 Growth and PHA accumulation of *Pseudomonas* and *Burkholderia* strains cultivated on various carbon sources

As a typical PHA$_{MCL}$-producing strain, *P. stutzeri* 1317 utilized both glucose and fatty acids to synthesize PHA, consisting of 3-hydroxyoctanoate (3HO) and 3-hydroxydecanoate (3HD) as the major monomers (Table 2). *P. nitroreducens* 0802 only accumulated large amounts of PHA when fatty acids were provided as carbon sources; glucose supported almost no PHA accumulation. Interestingly, as the chain-length of the fatty acid substrates increased from octanoic acid (C_8) to lauric acid (C_{12}), PHA synthesized by *P. nitroreducens* 0802 shifted from 3-hydroxybutyrate homopolymer (PHB) to various monomers containing longer side chains (Table 2).

B. caryophylli AS 1.2741 was also able to utilize both

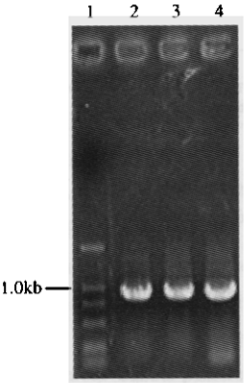

Fig.1 Polymerase chain reaction (PCR) amplification of *phaG* genes. Purified genomic DNA and *Ex Taq* DNA polymerase were used in the reactions

1: DNA size marker DL 2000 (TaKaRa, Dalian, China); 2: genomic DNA of *P. stutzeri* 1317 was used as the template; 3: genomic DNA of *P. nitroreducens* 0802 was used as the template; 4: genomic DNA of *B. caryophylli* AS 1.2741 was used as the template

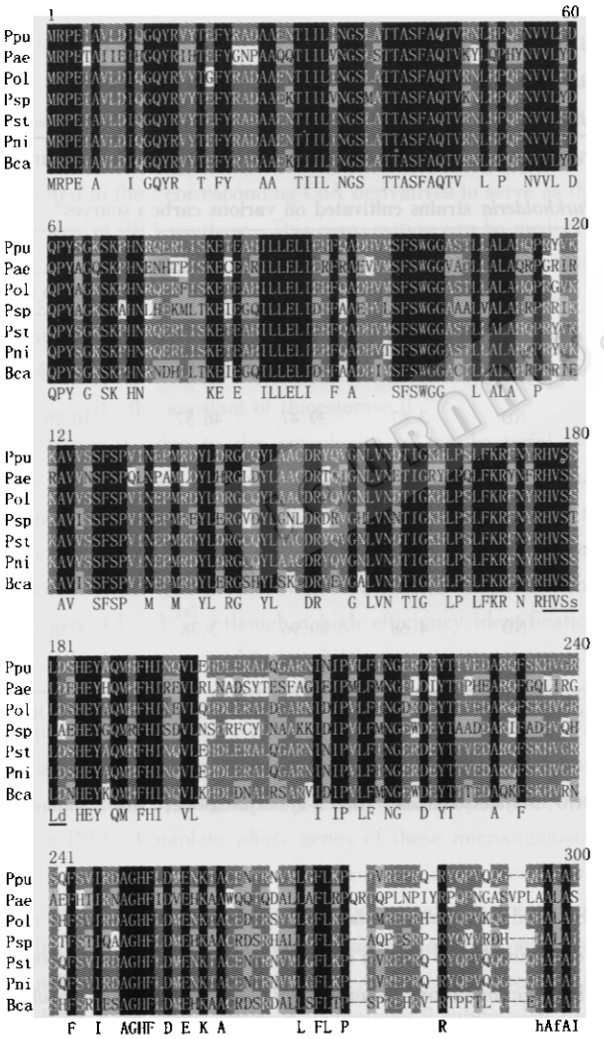

Fig.2 Alignment of the deduced amino acid sequences of various bacterial *phaG*

Ppu: *P. putida*; Pae: *P. aeruginosa*; Pol: *P. oleovorans*; Psp: *Pseudomonas* sp. 61-3; Pst: *P. stutzeri* 1317; Pni: *P. nitroreducens* 0802; Bca: *B. caryophylli* AS 1.2741. Conserved amino acid residues were listed. The HX_4D motif is underlined

glucose and fatty acids as a carbon source. Many monomers of PHA were synthesized when cultivated on both structural non-related and related carbon sources (Table 2), distinguishing this strain from all other *Burkholderia* strains, which are reported to synthesize only PHB[18].

3.2 PCR cloning and molecular analysis of *phaG* genes

The coding regions of known *Pseudomonas phaG* genes display great homology, especially in both terminal sequences. Highly conserved sequences of these coding regions were used as primers for the PCR screening protocol. Purified genomes were used as templates, and touchdown PCR technology (employing the P1-P2 primer pair) was applied to obtain the preferred product and minimize the artifacts. A distinct PCR product of approximately 900-bp was obtained, in good agreement with the length of *phaG* genes (Fig. 1).

Subsequently, the PCR products were separately cloned into pGEM®-T and sequenced. These sequences were analyzed, and the complete open reading frames (ORFs) were identified by computer analysis (Vector NTI, InforMax, USA). The corresponding protein sequences of the new isolated DNA fragments processed high similarity with the PhaG sequences of known *Pseudomonas* strains (identity 47.3%, positives 96.3%). Furthermore, the conserved HX_4D motif, which has been proposed to play an important role in enzymatic catalysis[11-12], was also found in all these cases (Fig. 2).

3.3 Construction of plasmids to heterologous expression of *phaG* genes

To evaluate the function of the translational products of these new isolated DNA fragments, a broad-rang-host expression vector pLZZH13 was constructed. It is a derivative of vector pBBR1MCS-2 and contains the promoter of *Wautersia eutropha* (formerly as *Ralstonia eutropha*) *phb* operon with an *Nde* Ⅰ site. Briefly, the 1.6-kb *Xba* Ⅰ-*Pst* Ⅰ fragment containing the *W. eutropha* PHB-synthesis operon with an *Nde* Ⅰ site inside was amplified from plasmid pBHR68 using primers P3 and P4, and then subcloned into the respective sites of plasmid pBluescript SK⁻. The resulting plasmid, pLZZH07, was hydrolyzed with *Eco*R Ⅰ and *Sma* Ⅰ, and the 1.6kb fragment was inserted into the *Eco*R Ⅰ and *Hinc* Ⅱ sites of pBBR1MCS-2, resulting in the vector pLZZH13 (Fig. 3). The *phaG* genes of *P. stutzeri* 1317, *P. nitroreducens* 0802, and *B. caryophylli* AS 1.2741 were subcloned into *Nde* Ⅰ and *Eco*R Ⅰ sites of pLZZH13 under the control of a *W. eutropha* PHB-synthesis promoter, leading to pLZZG06, pLZZG08, and pLZZG09, respectively (Fig.3).

3.4 Analysis of PHA synthesis in *P. putida* $PHAG_N$-21 carrying the new isolated *phaG* genes

Plasmids pLZZG06, pLZZG08 and pLZZG09 were introduced into the PhaG-negative strain *P. putida* $PHAG_N$-21 by

conjugation. Since PhaG mediates PHA_{MCL} synthesis using a fatty *de novo* biosynthetic pathway and non-related carbon sources in *P. putida*, recombinant strains carrying the *phaG* gene were able to synthesize PHA_{MCL} when cultivated on glucose as sole carbon source (Table 3). All these PhaGs mediated PHA_{MCL} accumulation consisting of 3HO and 3HD as the mainly monomers (Table 3), similar to the PHA produced by wild type *P. putida* (3HHx 1.6%, 3HO 32.1%, 3HD 62.7, and 3HDD 3.6%)[11]. Thus, $PhaG_{Ps}$, $PhaG_{Pn}$, and $PhaG_{Bc}$ functionally replaced $PhaG_{Pp}$.

4 DISCUSSION

As the key player in direct production of PHA_{MCL}[8, 11-13] and HA_{MCL}[9-10] from carbohydrates, PhaG presented a favorable chance in direct biosynthesis of HA_{MCL}[9, 18]. Unfortunately, due to the complexity of the bacterial genome, only four *phaG* genes were identified employing complex heterologous-probe-hybridization method, even this protein interested a lot of researches.

To obtain more information of this useful protein, a PCR protocol was developed in this study to clone *phaG* gene from a typical PHA-producing strain *P. stutzeri* 1317 (Fig. 1). This gene, which is under the control of the promoter for the *W. eutropha* PHB-synthesis-operon, enabled strong PHA_{MCL} synthesis in the PhaG-negative mutant host *P. putida* $PHAG_N$-21 grown in glucose as sole carbon source (Table 3). This indicated that the newly isolated DNA fragment exerted the same functional role as demonstrated for *phaG* of *P. putida* encoding a (*R*)-3-hydroxyacyl-ACP: CoA transacylase. This strategy was also facilitated cloning of *phaG* gene from other two *Pseudomonas* strains belong to rRNA homology group I: *Pseudomonas pseudoalcaligenes*, and *Pseudomonas mendocina* (GenBank accession nos. AF396832 and AY338498, data not shown).

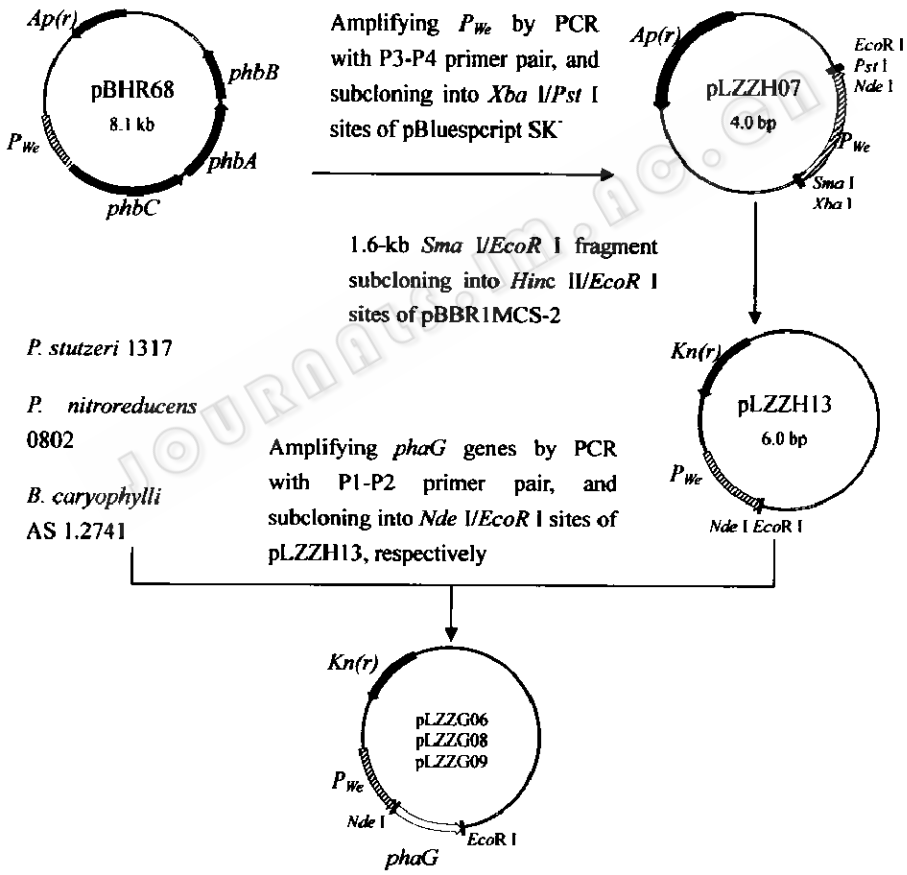

Fig.3 Construction of the plasmids used for the heterologous expression of *phaG* genes cloned

Ap(r), ampicillin resistant gene; *Kn(r)*, kanamycin resistant gene; P_{We}, promoter region of *phbCAB* operon of *Wautersia eutropha*; *phaG* genes presented in pLZZG06, pLZZG08, and pLZZG09 were isolated by polymerase chain reaction from *P. stutzeri* 1317, *P. nitroreducens* 0802, and *B. caryophylli* AS 1.2741, respectively

Interestingly, *phaG* gene also presented in the genomic DNA of *P. nitroreducens* 0802 (Fig. 1). Additionally, it mediated PHA_{MCL} producing in *P. putida* $PHAG_N$-21 when expressed heterologously (Table 3), although its function was not observed in the wild type. On the other hand, this strain only accumulated PHA from fatty acids, but not from carbohydrate (Table 2). Similar phenomena were also observed in *P. oleovorans*[12]. The cryptic character of *P. nitroreducens* 0802 *phaG* was presumably due to inefficient transcription; similar to the *phaG* of *P. oleovorans*[12].

Table 3 PHA in *P. putida* PHAG$_N$-21 harboring plasmids containing various *phaG* from various strains grown in glucose[a]

Plasmid Harboring	CDW[b] /(g/L)	PHA/CDW /(% [W/W])	Composition of PHA (mol %)[b]				
			3HB	3HHx	3HO	3HD	3HDD
pLZZH13	1.08 ± 0.08	1.35 ± 0.26	ND[c]	ND	10.29	58.39	31.32
pLZZG06	0.85 ± 0.08	7.72 ± 0.62	ND	2.31	16.01	72.65	9.03
pLZZG08	0.99 ± 0.04	6.21 ± 0.72	ND	2.16	20.07	63.77	14.01
pLZZG09	0.99 ± 0.10	8.73 ± 0.81	ND	2.43	15.01	72.37	10.18

[a] Cells were grown in MS medium containing 20 g/L glucose, 50 mg/L kanamycin. After 48 h at 30℃ and 200 rpm, the cells were harvested and analyzed. Results were from four separate fermentation experiments;

[b] CDW, cellular dry weight; 3HB, 3-hydroxybutyrate; 3HHx, 3-hydroxyhexanoate; 3HO, 3-hydroxyoctanoate; 3HD, 3-hydroxydecanoate; 3HDD, 3-hydroxydodecanoate;

[c] ND, not detectable.

New isolated strain *B. caryophylli* AS 1.2741, which belongs to the rRNA homologous group II, accumulated PHA$_{MCL}$ from both glucose and fatty acids (Table 2), unlike other *Burkholderia* strains[19]. A functional *phaG* gene was cloned from this strain (Fig. 1 and Table 3). *B. caryophylli* is the first non-*Pseudomonas* strain found to contain a *phaG* gene.

In conclusion, this study demonstrated that the PCR cloning protocol described above is a rapid, simple and specific method for isolation *phaG* genes from most PHA$_{MCL}$-producing microorganisms. High homology of *phaG* genes (Fig. 2) also indicated that the gene is quite conservative. The results shown above support the hypothesis[12] that the PhaG-mediated pathway represented a general principle of PHA$_{MCL}$ synthesis and could be widespread among bacteria, including *Pseudomonas* spp. that can or cannot accumulate PHA$_{MCL}$ from non-related carbon sources, and even non-*Pseudomonas* strains.

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REFERENCES

[1] Anderson AJ, Dawes EA. Occurrence, metabolism, metabolic role and industrial uses of bacterial polyhydroxyalkanoates. *Microbiol Rev*, 1990, **54**: 450 - 472

[2] Madison LL, Huisman GW. Metabolic engineering of poly(3-hydroxyalkanoates): from DNA to plastic. *Microbiol Mol Biol Rev*, 1999, **63**: 21 - 53

[3] Steinbüchel A, Lütke-Eversloh T. Metabolic engineering and pathway construction for biotechnological production of relevant polyhydroxyalkanoates in microorganisms. *Biochem Eng J*, 2003, **16**: 81 - 96

[4] Huisman GW, de Leeuw O, Eggink G *et al*. Synthesis of poly-3-hydroxyalkanoates is a common feature of fluorescent pseudomonads. *Appl Environ Microbiol*, 1989, **55**: 1949 - 1954

[5] Jendrossek D, Schirmer A, Schlegel HG. Biodegradation of polyhydroxyalkanoic acids. *Appl Microbiol Biotechnol*, 1996, **46**: 451 - 463

[6] Zheng Z, Deng Y, Lin XS *et al*. Induced production of rabbit articular cartilage-derived chondrocytes collagen II on polyhydroxyalkanoates blend. *J Biomat Sci Polym E*, 2003, **14**: 615 - 624

[7] Williams SF, Martin DP, Horowitz DH *et al*. PHA application: addressing the price performance issue I. *Tissue engineering*. *Int J Biol Macromol*, 1999, **25**: 111 - 121

[8] Rehm BHA, Krüger N, Steinbüchel A. A new metabolic link between fatty acid de novo synthesis and polyhydroxyalkanoic acid synthesis. *J Biol Chem*, 1998, **273**: 24044 - 24051

[9] Zheng Z, Zhang MJ, Zhang G *et al*. Production of 3-hydroxydecanoic acid by recombinant *Escherichia coli* HB101 harboring *phaG* gene. *Antonie van Leeuwenhoek*, 2004, **85**: 93 - 101

[10] Zheng Z, Gong Q, Liu T *et al*. Thioesterase II of *Escherichia coli* plays an important role in 3-hydroxydecanoic acid production. *Appl Environ Microbiol*, 70 (7): 3807 - 3813

[11] Hoffmann N, Steinbüchel A, Rehm BHA. The *Pseudomonas aeruginosa phaG* gene product is involved in the synthesis of polyhydroxyalkanoic acid consisting of medium-chain-length constituents from non-related carbon sources. *FEMS Microbiol Lett*, 2000, **184**: 253 - 259

[12] Hoffmann N, Steinbüchel A, Rehm BHA. Homologous functional expression of cryptic *phaG* from *Pseudomonas oleovorans* establishes the transacylase-mediated polyhydroxyalkanoate biosynthetic pathway. *Appl Microbiol Biotechnol*, 2000, **54**: 665 - 670

[13] Matsumoto K, Matsusaki H, Taguchi S *et al*. Cloning and characterization of Pseudomonas sp. 61 - 3 *phaG* gene involved in polyhydroxyalkanoate biosynthesis. *Biomacromolecules*, 2001, **2**: 142 - 147

[14] Simon R, Priefer U, Pühler A. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram-negative bacteria. *Bio/Technology*, 1983, **1**: 784 - 791

[15] Sambrook J, Russel DW. *Molecular cloning: a laboratory manual*. 3rd ed, New York: Cold Spring Harbor Laboratory Press, 2001

[16] Kovach ME, Elzer PH, Hill DS *et al*. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carry different antibiotic-resistance cassettes. *Gene*, 1995, **166**: 175 - 176

[17] Spiekermann P, Rehm BHA, Kalscheuer R *et al*. A sensitive, viable-colony staining method using Nile red for direct screening of bacteria that accumulate polyhydroxyalkanoic acids and other lipid storage compounds. *Arch Microbiol*, 1999, **171**: 73 - 80

[18] Zheng Z, Gong Q, Chen GQ. A novel method for production of 3-hydroxydecanoic acid by recombinant *Escherichia coli* and *Pseudomonas putida*. *Chinese J Chem Eng*, 2004, **12** (4): 550 - 555

[19] Timm A, Steinbüchel A. Formation of polyesters consisting of medium-chain-length 3-hydroxyalkanoic acids from gluconate by *Pseudomonas aeruginosa* and other fluorescent pseudomonads. *Appl Environ Microbiol*, 1990, **56**: 3360 - 3367