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Cloning and Characterisation of (*R*)-3-hydroxyacyl-acyl Carrier Protein-coenzyme A Transferase Gene (*phaG*) from *Pseudomonas* sp. USM 4-55

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Abstrak: Enzim (*R*)-3-hydroxyacyl-ACP-CoA transferase merupakan enzim pemangkin penukaran (*R*)-3-hydroxyacyl-ACP kepada terbitan (*R*)-3-hydroxyacyl-CoA yang berfungsi sebagai substrat untuk pempolimeran polyhydroxyalkanoat (PHA) daripada substrat tidak berkaitan dalam pseudomonads. PhaG merupakan enzim yang bertanggungjawab menyalurkan substrat untuk enzim polyhydroxyalkanoat (PHA) sintase melalui laluan biosintesis *de novo* asid lemak apabila karbohidrat seperti glukosa atau glukonat digunakan dalam kultur pertumbuhan. Gen *phaG* telah diklon daripada *Pseudomonas sp.* USM 4-55 menggunakan kaedah prob homolog. Gen *phaG* terletak di dalam rantaian DNA *Sal* I bersaiz 3660 bp (nombor capaian GenBank EU305558). *Open reading frame* (ORF) *phaG* ialah 885 bp DNA yang mengekod 295 asid amino. Berat molekul anggaran ialah 33251 Da dan ia menunjukkan 62% identiti terhadap PhaG daripada *Pseudomonas aeruginosa*. Aktiviti enzim PhaG daripada *Pseudomonas* sp. USM 4-55 disahkan melalui ujikaji komplementasi. Plasmid pBCS39 yang mengandungi rantaian DNA *Sal* I 3660 bp menunjukkan aktiviti enzim PhaG apabila dimasukkan ke dalam sel perumah *phaG-mutant strain Pseudomonas putida* $PhaG_N$-21. *P. putida* $PhaG_N$-21 yang membawa plasmid pBCS39 menghasilkan PHA sehingga 18% berat kering sel (CDW). *P. putida* $PhaG_N$-21 yang membawa vektor (PBBR1MCS-2) hanya menghasilkan 0.6% CDW PHA.

Kata kunci: *Pseudomonas* sp. USM-455, *phaG*, Polyhydroxyalkanoate

Abstract: The (*R*)-3-hydroxyacyl-ACP-CoA transferase catalyses the conversion of (*R*)-3-hydroxyacyl-ACP to (*R*)-3-hydroxyacyl-CoA derivatives, which serves as the ultimate precursor for polyhydroxyalkanoate (PHA) polymerisation from unrelated substrates in pseudomonads. PhaG was found to be responsible for channelling precursors for polyhydroxyalkanoate (PHA) synthase from a *de novo* fatty acid biosynthesis pathway when cultured on carbohydrates, such as glucose or gluconate. The *phaG* gene was cloned from *Pseudomonas* sp. USM 4-55 using a homologous probe. The gene was located in a 3660 bp *Sal* I fragment (GenBank accession number EU305558). The open reading frame (ORF) was 885 bp long and encoded a 295 amino acid protein. The predicted molecular weight was 33251 Da, and it showed a 62% identity to the PhaG of *Pseudomonas aeruginosa.* The function of the cloned *phaG* of *Pseudomonas* sp. USM 4-55 was confirmed by complementation studies. Plasmid pBCS39, which harboured the 3660 bp *Sal* I fragment, was found to complement the PhaG-mutant heterologous host cell, *Pseudomonas putida* $PhaG_N$-21. *P. putida* $PhaG_N$-21, which harboured pBCS39, accumulated PHA that accounted for up to 18% of its cellular dry weight (CDW).

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P. putida $PhaG_N$-21, which harboured the vector alone (PBBR1MCS-2), accumulated only 0.6% CDW of PHA.

Keywords: *Pseudomonas* sp. USM-455, *phaG*, Polyhydroxyalkanoate

INTRODUCTION

Polyhydroxyalkanoates (PHA) is a kind of bioplastic that has decent potential to replace fossil-based thermoplastics, because it is biodegradable. PHAs are also used in product applications, such as latex paints (van der Walle *et al*. 2001) and medical applications (Williams & Martin 2005), such as scaffolding material for tissue engineering (Williams *et al.* 1999). PHAs accumulate in various microorganisms as intracellular carbon and energy storage material under nutrient-limiting conditions (Steinbüchel & Fuchtenbusch 1998; Madison & Huisman 1999). For example, almost all pseudomonads synthesise mcl-PHA when cultured on alkanes, organic acids, glucose or many other carbon sources (Fiedler *et al*. 2000, Matsusaki *et al.* 2000). Steinbüchel (2001) reported that there are approximately 150 different hydroxyalkanoic acids that are known to be constituents of bacterial storage polyester (PHA).

Huijberts *et al*. (1994) and Rehm *et al.* (1998) found that there are at least three different metabolic routes in *P. putida* for the synthesis of 3-hydroxyacyl coenzyme A, which is the substrate of the PHA synthase to synthesise PHA. They are 1) the beta oxidation pathway, 2) the fatty acid *de novo* biosynthesis pathway and 3) the chain elongation reaction pathway (Kessler *et al*. 1998).

Further investigation on PHA synthesis by the fatty acid *de novo* pathway revealed that (*R*)-3-hydroxyacyl-acyl-carrier protein-Coenzyme A transferase (PhaG) was the enzyme that was responsible for channelling substrates from the fatty acid *de novo* biosynthesis pathway to PHA synthase (Madison & Huisman 1999; Fiedler *et al*. 2000) in order to accumulate PHA in *P. putida* (Rehm *et al*., 1998). The evidence shows that PhaG catalyses the conversion of (*R*)-3-hydroxyacyl-ACP into (*R*)-3-hydroxyacyl-CoA derivatives, which serve as the ultimate precursors for PHA polymerisation from unrelated substrates.

The organism used in this study is a Gram-negative soil bacterium, *Pseudomonas* sp. USM 4-55, which is able to accumulate two types of polymer simultaneously, which are P(3HB) and mcl-PHA (Sudesh *et al.* 2004). Here, we describe the cloning of *phaG* from *Pseudomonas* sp. USM 4-55 as well as its functional expression in a *phaG* mutant *P. putida* $PhaG_N$-21.

MATERIALS AND METHODS

Bacterial Strains and Growth of Bacteria

The bacterial strains and plasmids that were used in this study are listed in Table 1. Pseudomonads were grown at 30°C in either Luria-Bertani (LB) or E medium (Kroumova *et al*. 2002) with 1.5% (w/v) sodium gluconate.

When needed, kanamycin (50 mg/l) and ampicillin (50 mg/l) were added to the medium for plasmid maintenance purposes.

Table 1: Bacterial strains and plasmids.

Strains and plasmid	Characteristics	Source or reference
Strain:		
Escherichia coli		
JM109	E14-(mcrA), recA1, gyrA96, thi-1, hsdR17(rk-, mk+), supE 44, relA1, D(lac-proAB), [F' traD36, proAB, $lacI^qZ\Delta M15$]	Stratagene
S17-1	recA and tra genes of plasmid RP4 integrated into chromosome; auxotrophic for proline and thiamine	Simon *et al.* (1983)
Pseudomonas sp. USM4-55	Wild type	Isolated in this lab
Pseudomonas putida $PhaG_N$-21	PhaG-negative mutant of *P. putida* KT2440	Rehm *et al.* (1998)
Plasmid:		
pBluescript II KS(+)	2961bp Phagemid, derived from pUC19, lacPOZ, Ap^r, T3 and T7 promoters, blue/white colour selection	Stratagene
pCE660	pBluescript II KS(+) derivative containing 660bp PCR product of *phaG* of *P*. sp USM 4-55	This study
pP1	pBluescript II KS(+) derivative containing *Sal* I fragment from positive plaque carrying *phaG* of *P*. sp USM 4-55	This study
pBBR1MCS-2	Km^r, broad host range, lacPOZ'	Kovach *et al.* (1995)
pBCS39	pBBR1MCS-2 derivative containing the *Sal* I fragment harbouring *phaG* of *P*. sp. USM 4-55 with putative promoter	This study
Lambda FIX®II / *Xho 1* Partial Fill-in.	Vector was digested with *Xho 1* and filled in with dCTP and dTTP, Spi/P2 selection, T3 and T7 promoters	Stratagene

DNA Manipulations

The isolation of total genomic DNA and plasmid, the digestion of DNA with restriction endonucleases, agarose gel electrophoresis, and the transformation of *E. coli* JM 109 were carried out by standard procedures (Sambrook *et al.* 1989).

Transfer of Plasmid

Plasmids were transferred into *E. coli* according to the established heat shock method (incubate at 42°C for 90 s) (Sambrook *et al*. 1989). The transfer of the plasmid into the *P. putida* $PhaG_N$-21 *phaG*-negative mutant was performed by conjugation. Conjugation was conducted as described by Simon *et al*. (1983) and employed *E. coli* S17-1 as the donor strain.

Nucleotide Sequence Analysis

The DNA fragments to be sequenced were cloned into pBluescript II KS(+) (Stratagene, California). A primer walking strategy was applied in order to get the full length sequence (Fig. 1). The sequencing reaction was performed according to the instructions in the ABI PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (PE Applied Biosystems, California). The reaction mixture was then loaded onto the ABI Prism 310 for sequence analysis. The nucleic acid sequence was analyzed using MacDNAsis and Genbank BLAST.

The sequence data has been submitted to the GenBank nucleotide database (Accession No. EU305558).

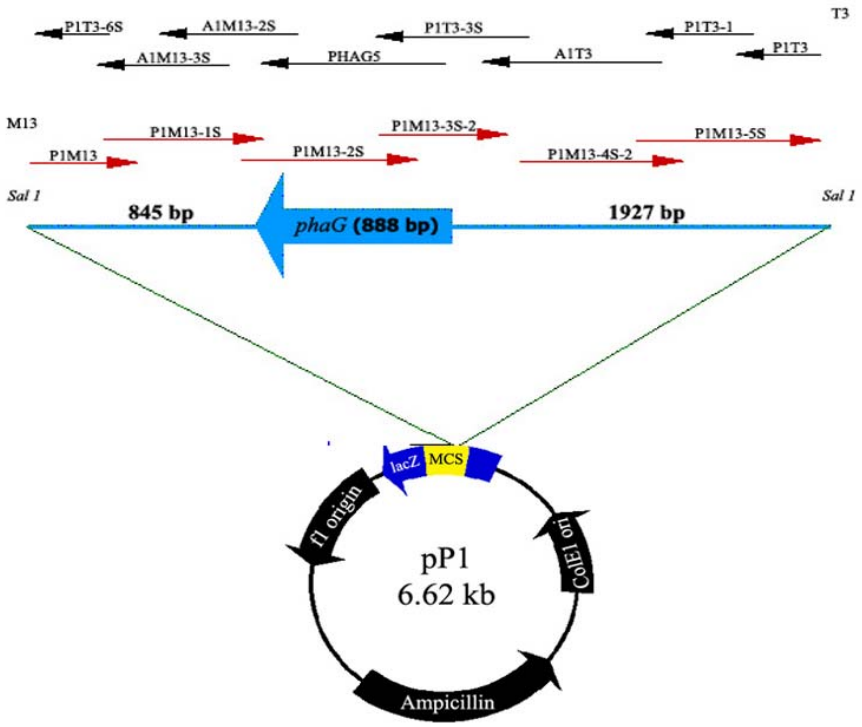

Figure 1: Sequencing strategy of pP1.

Construction of Genomic Library of *Pseudomonas* sp. USM4-55

The genomic library of *Pseudomonas* sp. USM 4-55 was constructed using λ FIX 11, according to the cloning kit manufacturer's protocol (Stratagene, California).

Plasmid Construction

A partial *phaG* gene was amplified from *Pseudomonas sp*. USM 4-55 genomic DNA using primers that were designed based upon the conserved region of the *phaG* genes of *Pseudomonas oleovorans*, *P. aeruginosa* and *P. putida*. The primers were 5'-ACCACRGCGTCSTTCGCCCAG-3' and 5'-CTTGTSCTCGACGTCGAKGAAGTGG-3'. The PCR product and the subsequent subcloned *Sal* I fragment were cloned into pBluescript II KS(+), which resulted in plasmid pCE 660 and pP1 respectively. The *Sal* I fragment was also cloned into the broad-host range plasmid pBBR1MCS-2, which resulted in plasmid pBCS39. The map of plasmid pBCS39 is shown in Figure 2.

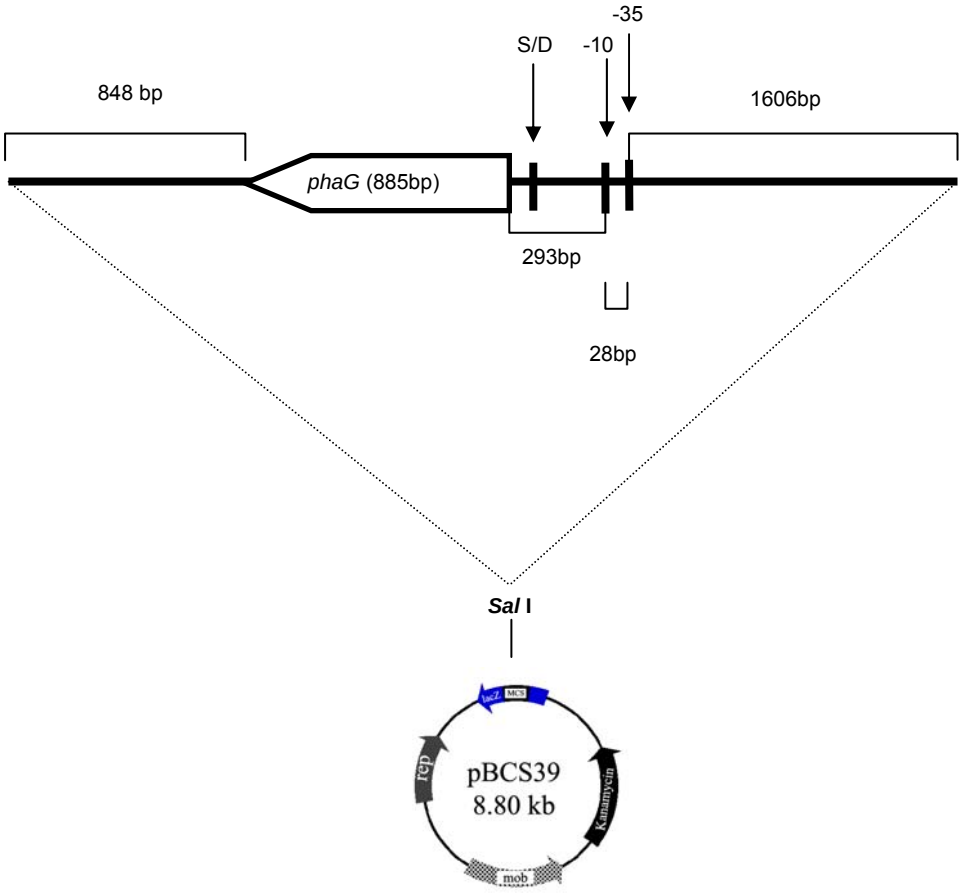

Figure 2: Map of plasmid pBCS39.

Functional Expression of The *phaG* Gene

Functional expression of the *phaG* in pBCS39 was confirmed by complementation of the *phaG*-negative mutant, *P. putida* $PhaG_N$-21. The resulting recombinant bacterium was cultivated on E medium (Kroumova *et al.* 2002) plus 1.5% (w/v) sodium gluconate, and, after 48 h of incubation at 30°C, the PHA content in the lyophilised cell was analysed. PHA accumulation from gluconate indicates the *in vivo* activity of PhaG.

Gas Chromatography Analysis of Polyester in Cell

PHA was qualitatively and quantitatively analysed by gas chromatography (GC). Liquid cultures were centrifuged at 5000 rpm for 10 min. The cells were then washed twice with sterile distilled water and lyophilised overnight. Twenty-five milligrams of lyophilised cell material was subjected to methanolysis, as described by Braunegg *et al*. (1978), in the presence of 15% (v/v) sulphuric acid. GC analysis was performed by injecting 0.2 μl of the sample into a Shimadzu-60B GC using capillary column BP-1.

RESULTS

Identification and Cloning of The *phaG* Gene from *Pseudomonas* sp. USM 4-55

The cloning strategy that was selected to clone the *phaG* gene from *Pseudomonas* sp. USM 4-55 was through a screen of the total genomic DNA library using a homologous probe. The homologous probe was prepared by PCR using the genomic DNA of *Pseudomonas sp* USM 4-55 as the template. Based on the conserved regions of the *phaG* genes of *P. putida*, *P. oleovorans* and *P. aeruginosa*, the PHAG5 and PHAG6 primers were designed. A positive recombinant lambda that was isolated by hybridisation screening was analysed by southern hybridisation (Fig. 3(a)). A positive *Sal* I fragment (Fig. 3(b)) was cloned into pBluescript II KS(+), which was subsequently named pP1.

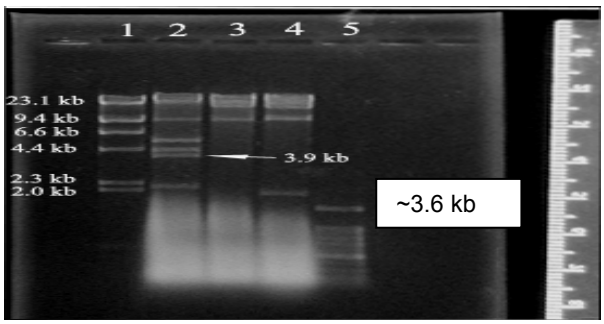

Figure 3(a): Restriction analysis of Lambda carrying the *Sal* I fragment. Lane 1 λ: *Hind* III marker, Lane 2: *Sal* I digested λ, Lane 3: *Sac* I digested λ, Lane 4: *Xba* 1 digested λ and Lane 5: 100bp DNA ladder marker.

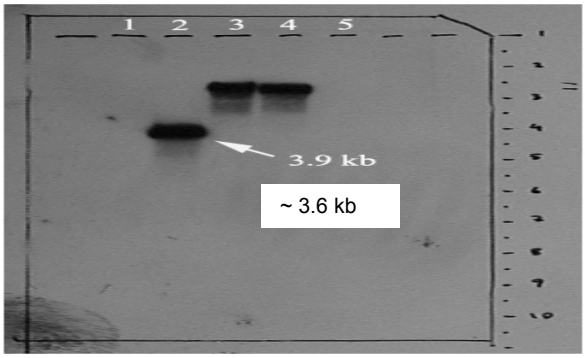

Figure 3(b): Autoradiograph (Southern blot analysis) of gel on Figure 3(a). The estimated size of the positive DNA fragment in Lane 2 is 3.6 kb, when compared to the λ *Hind* III marker, and the fragments in Lane 3 and Lane 4 were 22 kb.

Nucleotide Sequence of The *phaG* Gene Locus

Sequence analysis revealed that the size of the fragment is 3660 bp (GenBank accession number EU305558). BlastX analysis of the sequence revealed that there were five ORFs within the fragment. ORF 4 (nucleotides 1928 to 2812) contained an amino acid sequence that exhibited significant identity with the PhaG proteins from *P. aeruginosa* (AF209711, 62%), *P. putida* (AF052507, 56%), *Burkholderia caryophylli* (AY039841.1, 57%), *Pseudomonas nitroreducens* (AY039839.1, 56%), *P. oleovorans* (AF169252.1, 55%), *Pseudomonas fluorescens* (ZP_00084908.1, 53%), *Pseudomonas pseudoalcaligenes* (AF396832, 55%), *Pseudomonas sp*. 61-3 (AB047080.1, 55%) and *Pseudomonas syringae* (AE016853.1, 55%). A reliable Shine-Dalgarno consensus sequence was detected at base position 1915. The DNA sequence upstream of the start codon (1928 bp) was analysed by the GeneTyx software to identify any homology with unknown prokaryote control regions. A putative control sequence was detected, which included a possible σ70 promoter (TTGCAC) at base position 1607 and a possible -24/12 promoter at base position 1630 (TTGAAT). This ORF encoded a putative protein that was composed of 295 amino acid residues with a calculated molecular mass (M_W) of 33251 Da. The deduced amino acid sequence of the ORF revealed high homologies (62%) to genes that encode the (*R*)-3-hydroxyacyl-ACP-CoA acyltransferases of *P. aeruginosa* (Fig. 4).

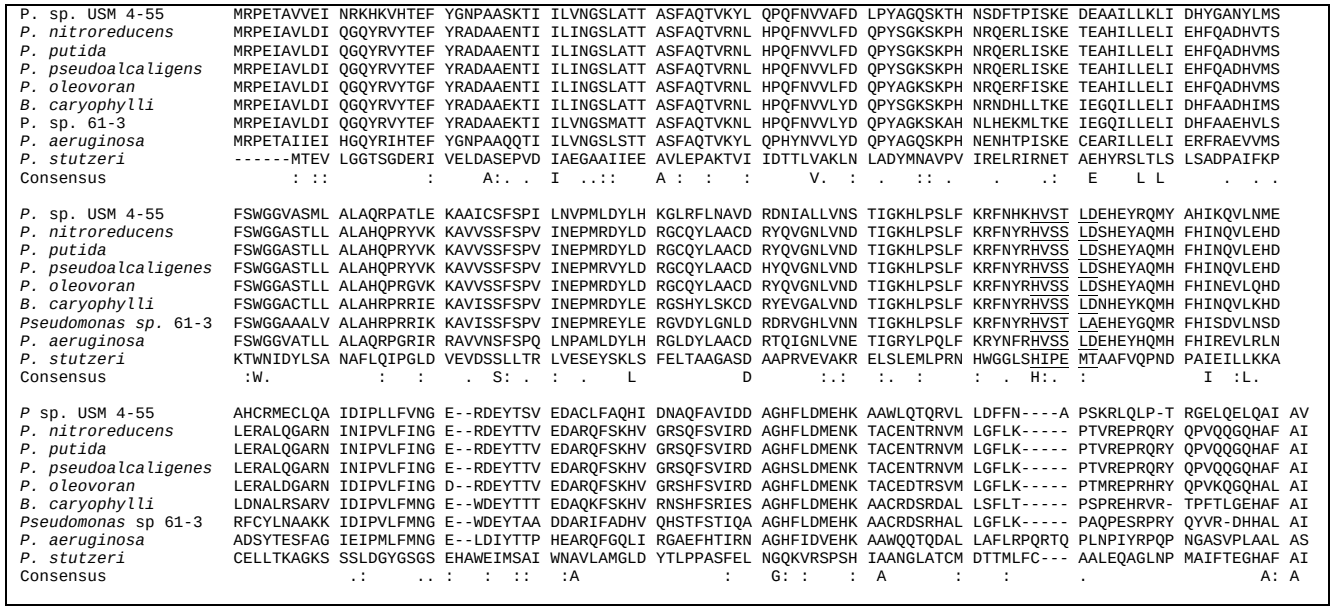

Figure 4: Alignment using Clustal (Larkin *et al*. 2007) of the deduced amino acid sequences of PhaG from *Pseudomonas* sp USM 4-55, *P. nitroreducens, P. putida, P. pseudoalcaligens, P. oleovoran, P. Caryophylli, Pseudomonas* sp. 61-3, *P. aeruginosa* and *P. stutzeri*. The HX_4D motif is underlined.

The conserved HX_4D motif, which has been proposed to play an important role in enzymatic catalysis, was also found in the amino acid sequence that was deduced from the *phaG* of *Pseudomonas* sp. USM 4-55 (Fig. 4), and its sequence is HVSTLD.

Heterologous Complementation of *phaG*

In order to confirm the identity of the *Sal* I fragment, heterologous expression study was carried out in the *phaG*-negative mutant strain of *P. putida* $PhaG_N$-21. The expression plasmid was constructed by cloning the *Sal* I fragment into the *Sal* I site of a broad-host range plasmid (pBBR1MCS-2), which was designated as pBCS39. The plasmid construct (Fig. 2) was then transformed into the *phaG*-negative mutant strain of *P. putida* $PhaG_N$-21 by conjugation. The resulting transconjugants were grown on mineral medium that contained 1.5% sodium gluconate as the sole carbon source.

GC analysis confirmed that the CS39 fragment did indeed show *PhaG* activity by complementing the mutated *phaG* gene in *P. putida* $PhaG_N$-21. The GC results from four transconjugants (C3 series) were averaged and summarised in Table 2. As compared to the controls, which were the *P. putida* $PhaG_N$-21 mutant and CMCS-2, the C3 cells showed an increased ability to synthesise PHA, which was, on average, 45-fold increase in the PHA content.

Table 2: Complementation of *P. putida* mutant $PhaG_N$-21 by pBCS39 harbouring the 3.6 kb *Sal I* fragment of *P.* sp. USM 4-55.

Strain	DCW (g/l)	PHA content (wt% of DCW)	PHA composition (mol%)							
			3HB (C_4)	3HHx (C_6)	3HO (C_8)	3HD (C_{10})	3HDD (C_{12})	3H5DD (C_{12-1})	3H7TD (C_{14})	TOTAL %
P. utida $PhaG_N$-21	1.0	0.3	N/D	N/D	18	47	6	11	N/D	100
CMCS-2	0.8	0.6	N/D	N/D	14	50	11	18	N/D	100
C3	0.7	17.7	1	9	25	61	2	2	TR	100

Cells were grown at 30°C for 48 hours in mineral medium, containing 1.5% (w/v) sodium gluconate as the carbon source. PHA monomers content were analysed by GC. 3HB: 3-hydroxybutyrate, 3HHx: 3-hydroxyhexanoate; 3HO: 3-hydroxyoctanoate, 3HD: 3-hydroxydecanoate, 3HDD: 3-hydroxydodecanoate, 3H5DD: 3-hydroxy-cis-5-dodecanoate, 3H7TD: 3-hydroxy-cis-7-tetradecenoate, N/D: None detectable, TR: trace, CMCS-2: *P. putida* $PhaG_N$-21 harbouring plasmid pBBR1MCS-2, C3: *P. putida* $PhaG_N$-21 harbouring pBCS39.

DISCUSSION

In this study, the *phaG* gene from *Pseudomonas* sp. USM 4-55 was successfully cloned and characterised. The gene was isolated by screening a genomic library of *Pseudomonas* sp. USM 4-55 using a homologous probe that was prepared by amplification of a section of the *phaG*. The primers were designed based upon the conserved regions of the *phaG* genes from *P. putida, P. aeruginosa* and

P. oleovorans. The genomic DNA of *Pseudomonas* sp. USM 4-55 was used as the template.

Southern analysis of gDNA of *Pseudomonas* sp. USM 4-55 using the homologous probe (DCE660) indicated the presence of a single copy of the *phaG* gene, which is consistent with the report by Hoffmann *et al.* (2000a). For further analysis, a 3660 bp *Sal* I fragment (CS39) was subcloned into pBluescript II KS vector, which formed plasmid pP1.

BLASTX analysis of the *Sal* I fragment revealed an 885 bp ORF that had a high similarity to the PhaG of several *Pseudomonas* species that were deposited in the GenBank Database. The ORF translates into a predicted polypeptide of 295 residues and is proposed to be the putative *phaG* of *Pseudomonas sp*. USM 4-55. This putative PhaG protein is most closely related to the PhaG protein of *P. aeruginosa* (62% identity). Other regions of the *Sal* I fragment, aside from the *phaG* gene, showed a high similarity to the published *P. putida* KT2440 genome.

The molecular organisation of *phaG* in three strains of *Pseudomonas* is compared in Figure 5. Overall, the organisation of *phaG* in *Pseudomonas sp*. USM 4-55 seems to be closely related to that of *P. putida* KT2440. BLASTX analysis showed that, besides *phaG*, the 3660 bp *Sal* I fragment could possibly encode a partial GGDEF domain protein, which is a conserved hypothetical protein of *P. putida* (PP1410), and the ribosomal small subunit pseudouridine synthase (RSSPS). Again, similar to *phaG*, the RSSPS shows the highest identity to *P. aeruginosa* PAO1 (61%), but its location mirrors that of *P. putida* KT2440 (Stover *et al.* 2000).

In congruence with the report by Rehm *et al*. (1998), this study also found that the adjacent DNA sequences of *phaG* in *Pseudomonas sp*. USM 4-55 were not related to genes involved in PHA metabolism. In *P. putida* KT2440, *phaG* is separated from *phaC1* by about 2 Mbp (Nelson *et al*. 2002).

Heterologous complementation was carried out in mineral medium that contained sodium gluconate as the sole carbon source. The provision of gluconate ensures that the substrate for PHA synthesis is derived via the fatty acid *de novo* synthesis pathway. *P. putida* $PhaG_N$-21 (Rehm *et al.* 1998) is known to have a defective *phaG*, although it can grow normally on any carbon source. It was able to synthesise PHA from fatty acids, but gluconate or glucose as the carbon source results in either a very low or nonexistent PHA synthesis. This made it an ideal host for a complementation assay to confirm the identity of the putative *phaG* of *Pseudomonas* sp. USM 4-55. For this purpose, the 3660 bp *Sal* I fragment was cloned into a PBBRMCS-2 plasmid and transferred into *P. putida* $PhaG_N$-21 by conjugation. The resulting transconjugant provided evidence of a functional *phaG* gene. The PhaG mutant, *P. putida* $PhaG_N$-21, produces very little PHA, which amounts to less than 1% of DCW, and does not accumulate C_4, C_6 and C_{14} monomers. The putative *pha*G gene that is present in the 3660 bp *Sal* I fragment of *Pseudomonas* sp. USM 4-55 conferred upon *P. putida* $PhaG_N$-21 the ability to accumulate PHA up to 17.7% of DCW when grown on gluconate as the sole carbon source. An earlier study on homologous complementation of *P. putida* $PhaG_N$-21 resulted in a higher PHA accumulation of up to 50% DCW (Rehm *et al.* 1998). Heterologous complementation of

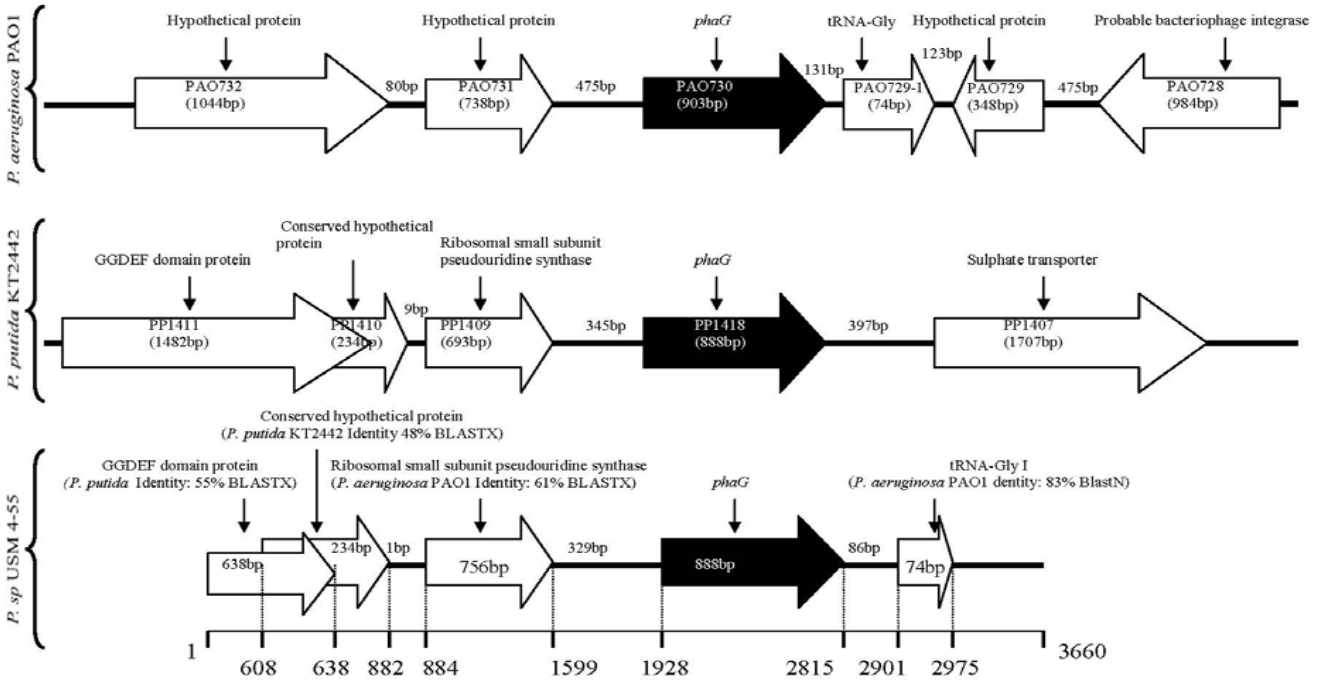

Figure 5: Molecular organisation of *phaG* in *P. aeruginosa* PAO1 (AE004091/GI:12057214), *P. putida* KT2442 (NC_002947/GI: 26986745) and *P.* sp USM 4-55. The *phaG* gene is indicated by the dark shaded arrow.

P. putida $PhaG_N$-21 has also been reported by Hoffmann *et al.* (2000b) using *PhaG* of *P. oleovorans*, which resulted in PHA accumulation of 36.7% of DCW. Although the accumulated PHA from this study was lower than previously mentioned complementation studies (Rehm *et al*. 1998; Hoffmann *et al.* 2000a, b), the results demonstrate that the putative *phaG* in the 3660 bp *Sal* I fragment of *Pseudomonas* sp. USM 4-55 was indeed the *phaG* gene of *Pseudomonas* sp. USM 4-55.

Heath and Rock (1998) reported that the HX_4D motif was conserved in a variety of glycerolipid acyltransferases. Matsumoto *et al.* (2001) also reported that the histidine is the most important residue and is essential at that position for PhaG activity. These proteins share a highly conserved domain that all contain an indispensable histidine and an aspartic acid residue that are separated by four or fewer conserved residues. This motif is also found in *phaG* of *Pseudomonas* sp. USM 4-55, located at amino acid positions 177 to 182, with the sequence HVSTLD.

CONCLUSION

In this study, the *phaG* gene from *Pseudomonas* sp. USM 4-55 was successfully cloned and characterised. An 885 bp ORF was identified, and it contained a predicted polypeptide of 295 amino acids and a calculated molecular mass (M_r) of 33251 Da. Functional activity of the PhaG of *Pseudomonas* sp. USM 4-55 was confirmed by a complementation test of the 3660 bp *Sal* I fragment in a *phaG* mutant, *P. putida* $PhaG_N$-21.

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