CHARACTERIZATION OF VARIANTS OF POLYHYDROXYALKANOATE SYNTHASE OF *CUPRIAVIDUS NECATOR* GENERATED BY ERROR-PRONE POLYMERASE CHAIN REACTION

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by

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LIST OF ABBREVIATIONS

bp	base pair
PCR	polymerase chain reaction
EP-PCR	error-prone polymerase chain reaction
RE	restriction enzyme
PHA	polyhydroxyalkanoate
P(3HB)	poly-(3-hydroxybutyrate)
3HB	3-hydroxybutyrate
3HV	3-hydroxyvalerate
4HB	4-hydroxybutyrate
P(3HB-*co*-4HB)	poly-(3-hydroxybutyrate-*co*-4-hydroxybutyrate)
P(3HB-*co*-3HO)	poly-(3-hydroxybutyrate-*co*-3-hydroxyoctanoate)
v/v	volume/volume
w/v	weight/volume
w/w	weight/weight
$PhaC_{Cn}$	polyhydroxyalkanoate synthase enzyme from *Cupriavidus necator*
$phaC_{Cn}$	polyhydroxyalkanoate synthase gene from *Cupriavidus necator*
Wild-type $PhaC_{Cn}$	PHA synthase from *Cupriavidus necator* that did not carry any point mutation
Wild-type $phaC_{Cn}$ gene	*phaC* gene from *Cupriavidus necator* that did not carry any point mutation

PENCIRIAN VARIAN POLIHIDROKSIALKANOAT SINTASE DARIPADA *CUPRIAVIDUS NECATOR* YANG DIJANA MELALUI REAKSI BERANTAI POLIMERASE CENDERUNG RALAT

ABSTRAK

Dalam kajian ini, PCR cenderung ralat digunakan untuk memperkenalkan mutasi rawak terhadap polihidroksialkanoate sintase jenis I daripada *Cupriavidus necator* untuk menyiasat kepentingan penggantian asid amino dari segi fungsi, yang sehingga kini masih belum ketahui strukturnya. Melalui pendekatan ini, mutan H8 yang dijana yang mempunyai lima amino acid terganti I71T, I172T, P339L, N514S dan S522R, telah diperoleh. Mutan H8 menunjukkan penghasilan P(3HB) yang rendah (0.1% Berat Kering Sel (BKS)) berbanding dengan jenis liar (11.3% BKS). Dari segi lokasi di dalam $PhaC_{Cn}$, mutasi I71T terletak di kawasan N-terminal yang tinggi variasinya, mutasi I172T terletak antara kawasan N-terminal dan kawasan terpelihara, manakala mutasi P339L dan S522R terletak di dalam kawasan lipatan α/β hidrolase yang sangat terpelihara dalam semua kelas PHA sintase. Setiap mutasi ini dipencil dan diperkenalkan ke dalam $PhaC_{Cn}$ jenis liar, menghasilkan varian $PhaC_{Cn}$ yang mempunyai mutasi tunggal. P(3HB) yang dihasilkan oleh varian $PhaC_{Cn}$ yang mempunyai mutasi tunggal I71T, I172T dan P339L masing-masing ialah 1.2%, 3.5% dan 1.9%. Berbanding dengan mutan G4D yang menghasilkan P(3HB) hingga 22.5% BKS, varian $PhaC_{Cn}$ yang mempunyai mutasi tunggal (I71T, I172T and P339L) menunjukkan penghasilan P(3HB) yang rendah dengan ketara (1.2-3.5% BKS) berbanding dengan jenis liar (11.3% BKS). Sebanyak tiga varian $PhaC_{Cn}$ dengan mutasi berganda dua iaitu G4D/I172T, G4D/P339L and G4D/S522R juga

berjaya dihasilkan dengan nilai P(3HB) masing-masing ialah 15.3%, 15.8% dan 6.8%. Seperti yang dijangka dalam kajian mutasi tunggal, mutasi kedua telah mengurangkan keupayaan mutan G4D menghasilkan P(3HB). Kesimpulannya, varian PhaC_{Cn} yang membawa mutasi tunggal dan berganda yang dihasilkan menunjukkan kesan yang besar kepada kandungan P(3HB) dalam sel. Mutasi tunggal dalam N-terminal dan rantau yang terpelihara ini menunjukkan pengurangan dalam pengumpulan P(3HB) secara mendadak. Ini menunjukkan kepentingan asid amino ini dalam mentadbir keberkesanan PhaC_{Cn} dari segi penghasilan polimer.

CHARACTERIZATION OF VARIANTS OF POLYHYDROXYALKANOATE SYNTHASE OF *CUPRIAVIDUS NECATOR* GENERATED BY ERROR-PRONE POLYMERASE CHAIN REACTION

ABSTRACT

In this study, error-prone PCR of type I polyhydroxyalkanoate synthase ($PhaC_{Cn}$) from *Cupriavidus necator* was carried out to introduce random mutations in $PhaC_{Cn}$ to investigate the functional importance of the substituted amino acids of $PhaC_{Cn}$, which to date, is structurally unknown.. Via this approach, mutant H8, which harboured five amino acid substitutions (I71T, I172T, P339L, N514S and S522R) was obtained. The H8 mutant exhibited low P(3HB) accumulation (0.1% CDW (Cell dry weight)) when compared to the wild-type (11.3% CDW). In terms of their positions in $PhaC_{Cn}$ sequence, mutation I71T is located at the highly variable N-terminal domain of the $PhaC_{Cn}$, the mutation I172T is located in between the N-terminal and the conserved region, while mutations N514S, P339L and S522R were located in the α/β hydrolase fold region, which is highly conserved in all classes of PHA synthases. Each of the mutation was individually isolated and inserted into the wild-type creating single mutation $PhaC_{Cn}$ variants. The P(3HB) accumulation of the single mutation $PhaC_{Cn}$ variants (I71T, I172T and P339L) were 1.2%, 3.5% and 1.9%, respectively. In contrast to mutant G4D, which accumulated P(3HB) up to 22.5% CDW, the single mutation $PhaC_{Cn}$ variants (I71T, I172T and P339L) showed significantly lower P(3HB) accumulation (1.2 – 3.5% CDW) when compared to the wild type (11.3% CDW). A total of three double mutation $PhaC_{Cn}$ variants, namely G4D/I172T, G4D/P339L and G4D/S522R were also created and their

P(3HB) accumulation values were 15.3%, 15.8% and 6.8%, respectively. As expected from before, the second mutation on the G4D mutant was found to lower the P(3HB) accumulation ability of the G4D mutant. It can be concluded that The $PhaC_{Cn}$ variants generated which carried single and double mutations showed significant effect to the P(3HB) content accumulated in cells. Single mutations in the N-terminus and the conserved region of $PhaC_{Cn}$, have dramatically reduced P(3HB) accumulation, suggesting the importance of those amino acids in governing the efficacy of $PhaC_{Cn}$ in polymer production.

1.0 INTRODUCTION

Poly-(*R*)-3-hydroxyalkanoates (PHAs) are linear polyesters composed of 3-hydroxy fatty acid monomers (3HA). The molecular masses of PHAs range from 50 kDa to 1000 kDa (Madison & Huisman, 1999). PHAs are biodegradable which make them to be potential polymers to replace petrochemical plastics. They exhibit rather high molecular weights, thermoplastic and elastomeric features, and some other interesting physical and material properties which make them suitable for use in many commercial applications (Rehm, 2003).

PHAs are accumulated in cells under excess carbon and nutrient-limiting conditions, such as nitrogen, phosphorus or oxygen (Suriyamongkol, *et al*., 2007). They form insoluble inclusions with diameters ranging from 0.2 to 0.5 μm in the cytoplasm of bacterial cells (Suriyamongkol, *et al*., 2007). PHAs act as intracellular storage material and are generally believed to play a role as a carbon sink and reducing equivalents in PHA-producing microorganisms (Madison & Huisman, 1999).

The most abundant PHA is poly-(*R*)-3-hydroxybutyrate [P(3HB)] (Merrick, 2002). It is the most common type of short-chain-length PHA homopolymer which consists of 3-hydroxybutyric acid (3HB) (Suriyamongkol, *et al*., 2007). In a natural producer, P(3HB) is synthesised through a three-step pathway by two successive 3HB monomer-supplying enzymes, β-ketothiolase (PhaA) and acetoacetyl-CoA (PhaB) and lastly by the polymerisation enzyme, PHA synthase (PhaC) (Taguchi & Doi, 2004). The model P(3HB) producer is *Cupriavidus necator* (formerly known as *Ralstonia eutropha*) (Fukui, *et al*., 2009). This bacteria produces type I PHA synthase ($PhaC_{Cn}$) which has specificity for short-chain-length CoA thioesters with 3-5 carbon atoms. The function of $PhaC_{Cn}$ is to polymerise (*R*)-3-hydroxybutyryl-CoA monomers into P(3HB) polymer. $PhaC_{Cn}$ is a key enzyme in influencing the production level, molecular weight and polydispersity of the PHA polymer synthesised and the compositional variation of the

polymer upon copolymerization (Taguchi, *et al*., 2001). Hence, modification of $PhaC_{Cn}$ enzyme will bring about changes in these aspects related to the production and properties of the polymer.

One of the methods that have proved to be successful in modifying $PhaC_{Cn}$ enzyme with improved properties is through directed evolution. Directed evolution is a method which combines random mutagenesis with an efficient screening for mutants (Taguchi & Doi, 2004). Random mutagenesis is generated through error-prone PCR (EP-PCR) where random mutation(s) are introduced into the gene of interest during PCR by reducing the fidelity of DNA polymerase (Fuji & Hayashi, 2004). Previously, modifications of *phaC* gene were tried and tested through directed evolution and this method has proved to be highly successful in generating improved PhaC variants even without the information on the tertiary structure of the enzyme (Tsuge, *et al*., 2004). This is highly beneficial as one can rely on the use of directed evolution approach to generate PhaC variants with improved characteristics without knowing the crystal structure of the enzyme which to date, has not been solved.

In a previous study, directed evolution was applied in PHA synthases from *R. eutropha* (Normi, *et al*., 2005), *A. caviae* (Kichise, *et al*., 2002) and *Pseudomonas* sp. 61-3 (Matsusaki, *et al*., 1998). These PhaCs showed improved properties in their enzyme. Between these PhaCs, PhaC1 from *Pseudomonas* sp. 61-3 showed higher polymerisation activity toward C4 monomer than the other type II enzymes. The substitution of amino acid glutamine at position 481 had enhanced the activity for P(3HB) synthesis (Tsuge, *et al*., 2004). In another study by Taguchi *et al*., the mutant F420S was also generated through directed evolution (Taguchi, *et al*., 2001a). This mutant exhibited 2.4-fold increase in activity towards 3HB-CoA as compared to wild-type.

A successful case study of directed evolution by Normi *et al*, at year 2005, a beneficial PhaC mutant, the G4D $PhaC_{Cn}$ variant was generated, which harboured a

substitution of glycine to aspartate at the position 4 (G4D). This mutant showed an increase in $PhaC_{Cn}$ concentration and subsequently increase the level of P(3HB) accumulated in recombinant *Escherichia coli* JM109 cells (Normi, *et al*., 2005).

The above studies indicated the great potential of directed evolution on PHA synthase from *Cupriavidus necator*. A single amino acid change, for example, G4D can show significant increase in P(3HB) accumulation (Normi, *et al*., 2005). This study was to investigate the structure-function relationship of $PhaC_{Cn}$. To achieve this, three objectives was set for the project:

1. To create random mutations in $PhaC_{Cn}$
2. To isolate and study the lone effects of these mutations
3. To study the effect of the isolated mutation points in combination of G4D.

2.0 LITERATURE REVIEW

2.1 History of Poly-(*R*)-3-hydroxyalkanoates (PHAs) – the discoveries and technologies

Early last century, PHA inclusions were first observed as lipid-like inclusions present in *Azotobacter chroococcum*. These inclusions were soluble in chloroform. Later in year 1925, inclusions with similar chemical composition were identified in *Bacillus megaterium* and termed as poly(3-hydroxybutyric acid) [P(3HB)] by Maurice Lemoigne (Lemoigne, 1926). By the end of 1950s, evidence was gathered through studies on *Bacillus* genus suggesting that P(3HB) functions as intracellular reserve of carbon and energy (Doudoroff & Stanier, 1959).

However, at this stage, 3-hydroxybutyrate (3HB) unit was thought to be the only constituents of PHA. It was almost two decades later, in year 1974, that Wallen and Rohwedder reported the identification of 3-hydroxyvalerate (3HV) and 3-hydroxyhexanoate (3HHx) from activated sewage sludge (Wallen & Rohwedder, 1974) and this marked the starting point of more discoveries of different components of 3-hydroxyalkanoate (3HA) in the polymer. In early 1980s, it was reported that batch grown *Bacillus megaterium* accumulated polymer consisted of 95% 3-hydroxybutyrate (3HB), 3% 3-hydroxyheptanoate (3HHp), 2% of an 8-carbon hydroxyalkanoate (HA) and trace amount of 3 other HA compound (Findlay & White, 1983). Consecutively, 3-hydroxyocatanoate (3HO) was discovered when *Pseudomonas oleovorans* was cultivated in *n*-octane (De Smet, *et al*., 1983). By the end of 1980s, numerous other hydroxyalkanoate (HA) constituents were discovered, including 4- and 5-hydroxyalkanoate (4HA and 5HA) (Kunioka, *et al*., 1988; Doi, *et al*., 1987). Co-polymer of P(3HB-*co*-4HB) which was produced by *Cupriavidus necator* (formerly known as

Ralstonia eutropha) was also discovered by Doi and co-worker at the same time (Doi, *et al*., 1987).

By year 1990, it was clear that these polymer inclusions are not just synthesised by Gram-negative bacteria only, but also by a wide range of Gram-positive bacteria, aerobic and anaerobic photosynthetic bacteria (i.e. cyanobacteria and non-sulfur and sulfur purple bacteria) as well as some archaebacteria (Anderson & Dawes, 1990).

In the late 1970s, molecular biology revolution brought in new tools for biological research which is useful in deciphering genetic information and this helped to understand further the principles of PHA biosynthesis at the genetic level (Sudesh, *et al*., 2000). By the end of 1980s, Slater and co-workers (Slater, *et al*., 1988) successfully cloned the genes encoding enzymes involved in PHA biosynthesis from *C. necator* into *Eschericia coli*. Detailed studies on *C. necator* revealed that only three enzymes were involved in biosynthesis of P(3HB) and the PHA synthase enzyme which carries out the polymerisation reaction was identified as the key enzyme (Sudesh, *et al*., 2000). By year 1999, 38 PHA synthase structural genes have been cloned from more than 32 different bacteria and studies showed that these enzymes can be categorized into three different groups based on their primary structures and substrate specificities (Rehm & Steinbüchel, 1999).

At present, the research trend is heading towards protein engineering of PHA synthases with the purpose to obtain PHA synthase variants with improved properties which will eventually be beneficial for the production of the environmentally friendly polymers (Sudesh, *et al*., 2000).

2.2 Introduction to PHA

2.2.1 PHA as intracellular compounds

PHAs are polyester synthesised naturally by various microorganisms in response to an imbalance in growth conditions brought about by nutrient limitations (Anderson & Dawes, 1990; Sudesh, *et al*., 2000). Cells accumulate PHA when there is excess supply of carbon source but with limitation of nutrients such as magnesium, sulfur, nitrogen, phosphorus and oxygen (Sudesh, *et al*., 2000). By polymerizing PHA, cells do not undergo changes at its osmotic state and leakage of valuable compounds from the cells, especially carbon, is thus prevented (Senior and Dawes, 1973; Madison and Huisman, 1999). Hence, PHA acts as an ideal carbon and energy storage material in cells which accumulate it. It also serves as a sink for reducing power and therefore acts as a redox regulator within the cells (Senior & Dawes, 1971; Sudesh, *et al*., 2000). Studies showed that when oxygen or nitrogen limitation occurs, PHA synthesis served as an electron sink for generating and regulating reducing power (Senior & Dawes, 1971; McDermott, *et al*., 1989; Sudesh, *et al*., 2000).

PHAs are stored as insoluble spherical inclusions in the cells' cytoplasm (Figure 2.1). This can reach to as high as 90% of the cell dry weight (Grage, *et al*., 2009; Madison & Huisman, 1999). Bacterial cells synthesize PHA typically from 10^3 to 10^4 monomers with diameter of 0.2 – 0.5 μm (Suriyamongkol, *et al*., 2007). Generally, the molecular mass of PHA range from 50,000 to 1,000,000 Da depending on the microorganism which produces it, as well as the cultivation conditions (Madison & Huisman, 1999).

PHA can be degraded by intracellular or extracellular PHA depolymerases secreted from microorganisms (Jendrossek, *et al*., 1996). These PHA depolymerases

hydrolyse PHA into water-soluble oligomers and monomers that can be used as nutrient by other living cells (Baei, *et al*., 2009).

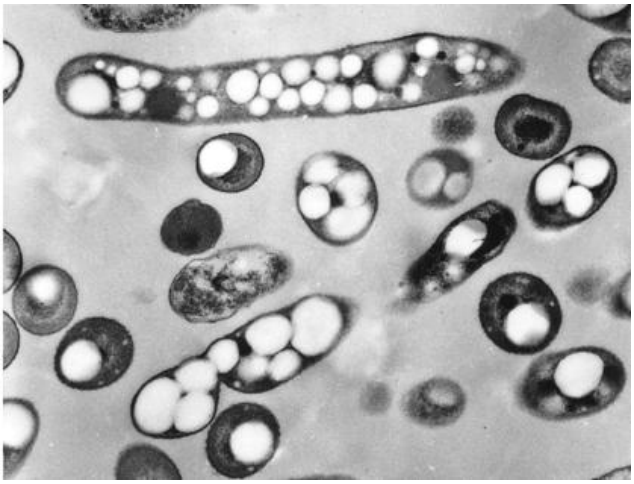

Figure 2.1 TEM image of PHA granules in Cupriavidus *necator*.
(Figure source: Tian, *et al*., 2005).

2.2.2 The chemistry of PHA

PHA is linear, head-to-tail polyesters which composed of 3-hydroxy fatty acid monomers (Figure 2.2). The polymerisation reaction of 3-hydroxy fatty acids occur when carboxyl group (COOH-) of one monomer forms an ester bond with the hydroxyl group (OH-) of the neighboring monomer and this is catalysed by the cell's enzyme, PHA synthase (PhaC) (Figure 2.3) (Madison & Huisman, 1999; Verlinden, *et al*., 2007). Due to the stereospecificity of this enzyme, all the hydroxyl-substituted carbon atoms in the 3-hydroxy fatty acids are of the *R*-configuration (Sudesh, *et al*., 2000), except in one rare case where S-configuration was detected (Haywood, *et al*., 1991). At the β position of the same hydroxyl-substituted carbon, an alkyl group varying from methyl to tridecyl is positioned (Table 2.1).

Table 2.1 The general structure of PHA

No. of repeating units	R group	Type of PHAs
n = 1	R = hydrogen	Poly(3-hydroxypropionate)
	R = methyl	Poly(3-hydroxybutyrate)
	R = ethyl	Poly(3-hydroxyvalerate)
	R = propyl	Poly(3-hydroxyhexanoate)
	R = pentyl	Poly(3-hydroxyactanoate)
	R = nonyl	Poly(3-hydroxydodecanoate)
n = 2	R = hydrogen	Poly(4-hydroxybutyrate)
n = 3	R = hydrogen	Poly(5-hydroxyvalerate)

PHA consist of an R group which refers to the side group and it varies from methyl (C_1) to tridecyl (C_{13}) while "n" refers to number repeating units.
(Table source: Lee, 1996).

This side chain, however, is not necessarily saturated; it can be unsaturated, aromatic, halogenated, epoxidized and branched. Interestingly, the position of the hydroxyl group can also be variable, other than 3-hydroxyl acid, position 4-, 5- and 6-hydroxyl acids can also be incorporated. These variations make the PHA polymer family suitable for various applications (Madison & Huisman, 1999; Lee, 1996).

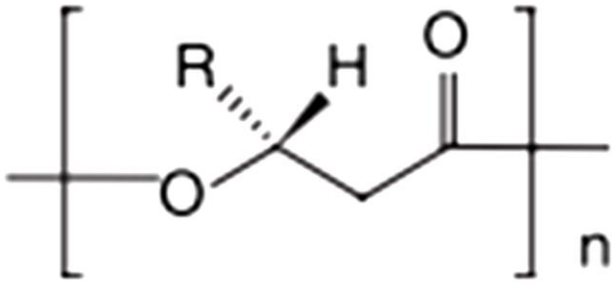

Figure 2.2 Chemical structure of poly(3-hydroxyalkanoate).

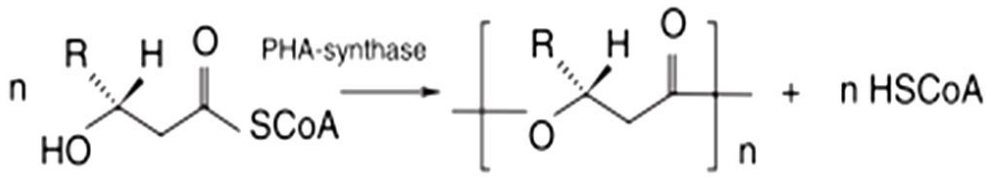

Figure 2.3 Synthesis of PHAs in bacteria using hydroxyacyl-CoA thioesters as precursor. (Figure source: Verlinden, *et al*., 2007).

2.2.3 Types of PHA

Bacteria synthesize a wide range of PHA. To date, about 150 types of different monomers have been identified as constituents of PHA depending on the substrate specificity of the PHA synthases (Steinbüchel & Valentin, 1995). All of these PHA can be classified into three broad classes according to the size of the monomers. PHA with monomer containing up to five carbons (C5) are classified as short-chain-length PHA (short-chain-length-PHA) while PHA with monomer containing six to fourteen carbons (C6-C14) are classified as medium-chain-length PHA (medium-chain-length-PHA) (Table 2.2) (Suriyamongkol, *et al*., 2007).

The most well studied short-chain-length-PHA producer is *Cupriavidus necator* (formerly termed *Ralstonia eutropha*) which accumulates poly(3-hydroxybutyrate) [P(3HB)] homopolymer when grown in glucose (Lee, *et al*., 2009). *Pseudomonas oleovorans* is an example of an medium-chain-length-PHA. producer. It accumulates poly(3-hydroxyoctanoate) [P(3HO)] when grown in octanoic acid (Durner, *et al*., 2000).

The third class of PHA is the hybrid short-chain-length-medium-chain-length PHA (short-chain-length-medium-chain-length-PHA) which consist of short-chain-length- and medium-chain-length-HA with number of carbons ranging from C3-C14. Example of hybrid short-chain-length-medium-chain-length-PHA is the random P(3HB-*co*-3HO) copolymer. One of the bacteria which produce hybrid short-chain-length-medium-chain-length-PHA is *Pseudomonas* sp. 61-3 (Table 2.2) (Matsusaki, *et al*., 1998).

Table 2.2 Classification of PHA based on monomer size

Type of PHA	Number of C atoms	Example	Representative bacteria
Short-chain-length PHA	C3-C5	P(3HB)	*Cupriavidus necator*
Medium-chain-length PHA	C6-C14	P(3HO)	*Pseudomonas* sp.
Short-chain-length-medium-chain-length PHA	C3-C14	P(3HB-co-3HO)	*Pseudomonas* sp. 61-3

Polymers with short-chain-length monomer have thermoplastic properties but are generally more brittle, while polymers with medium-chain-length monomer have elastomeric properties. Short-chain-length-medium-chain-length-PHA copolymers consist of properties from both short-chain-length-PHA and medium-chain-length-PHA depending on the ratio of short-chain-length and medium-chain-length monomer. Thus, short-chain-length-medium-chain-length-PHA copolymers have wide arrays of usage (Nomura, *et al*., 2005).

2.3 P(3HB)

Among all the PHA, the most common type is poly-(*R*)-3-hydroxybutyrate, P(3HB). It was the first PHA which was identified in year 1925 by Lemoigne, therefore it is the most well studied PHA (Doi, 1990). P(3HB) homopolymer contains repeating units of C4 (*R*)-3-hydroxybutyrate (3HB) monomer where its side chain is a methyl group (Figure 2.4) (McCool & Cannon, 2001). P(3HB) has similar physical and thermal properties with the conventional polypropylene plastic; thus, making it suitable for use in certain applications, for example in packaging and paint industry (Anderson & Dawes, 1990). In addition to these characteristics, its biodegradable property has drawn much attention from scientists in developing it as an environmentally-friendly replacement to petrochemical-based plastics.

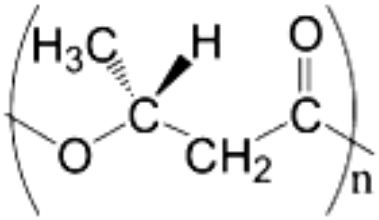

Figure 2.4 The chemical structure of poly-(*R*)-3-hydroxybutyrate, P(3HB).

2.3.1 The physical and thermal properties of P(3HB)

PHA have various molecular weights depending on the bacterial strains and the carbon sources used to produce them (Table 2.3) (Doi, 1990). Their thermal and mechanical properties are expressed in terms of glass-to-rubber transition temperature (T_g) at its amorphous stage and melting temperature (T_m) at its crystalline phase (Anderson & Dawes, 1990).

Table 2.3 Compositions and Molecular Weight of PHA produced by different bacterial strains [a,b]

		Polyester Composition (mol %)[c]			Molecular Weight[d]	
Strain	Carbon Source	3HB	3HV	4HB	M_n x 10^{-3}	M_w / M_n
Alcaligenes eutrophus	Fructose	100	0	0	737	1.9
	Butyric acid	100	0	0	432	2.1
	Pentanoic acid	21	79	0	254	2.0
	γ-Butyrolactone	83	0	17	369	1.8
Bacillus megaterium	Glucose	100	0	0	166	2.9
Zoogloea ramigera	Glucose	100	0	0	542	2.5
Protomonas extorquens	Methanol	67	33	0	437	2.3

[a] At 30°C, pH 7.5.
[b] PHA samples were extracted from bacterial strains with hot chloroform.
[c] Determined from H-NMR spectra.
[d] Determined by GPC.
(Table source: Doi, 1990).

For P(3HB) homopolymer, the weight-average molecular weight (M_w) produced by bacteria is in the range of 1 x 10^4 to 3 x 10^6 g/mol which is similar to conventional plastics especially polypropylene (Sudesh, *et al*., 2000; Madison & Huisman, 1999). In addition, P(3HB) has high melting temperature (T_m = 180°C) and low glass transition temperature (T_g = 4°C) which are also similar to polypropylene (T_m = 176°C and T_g = -10°C, respectively) (Table 2.4). Thus, P(3HB) also behave as a thermoplastic similar to polypropylene. Mechanically, the stiffness (expressed in Young's modulus) and the tensile strength of P(3HB) (3.5 GPa and 40 MPa) are also

closely similar to that of polypropylene (1.7 GPa and 38 MPa). However, P(3HB) is more brittle compared to polypropylene. The extension to break for P(3HB) is 5% which is markedly lower than that of polypropylene (400%) (Sudesh, *et al*., 2000).

The physical and mechanical properties of P(3HB) was found to be improved through genetic engineering of the PHA synthase (*phaC*) genes. Kusaka and co-workers (1998) successfully cloned a recombinant *E. coli* XL-1 Blue (pSYL105) harbouring modified biosynthesis genes *phbCAB* gene from *C. necator* which was able to produce P(3HB) homopolymer with ultra-high molecular weight. This P(3HB) was found to have higher extension to break (58%) and high tensile strength (62 MPa) respectively (Kusaka, *et al*., 1998).

Introduction of other 3HA monomer into P(3HB) polymer will also influence the physical and thermal properties of the polymer produced. For example, the incorporation of 3-hydroxyvalerate (3HV) monomer into P(3HB) resulted in a poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)] copolymer which is less stiff and brittle than P(3HB) homopolymer (Madison & Huisman, 1999). The copolymer of P(3HB-*co*-3HV) with 20 mol% 3HV showed decreased stiffness and tensile strength; but increased elongation to break (Table 2.4). This copolymer also showed lower melting and glass-transition temperatures (Madison & Huisman, 1999; Sudesh, *et al*., 2000).

In addition, the incorporation of 6 mol% of medium-chain-length 3HA i.e 3-hydroxydecanoate, 3-hydroxydodecanoate, 3-hydroxyoctanoate and 3-hydroxy-*cis*-5-dodecanoate into P(3HB) polymer resulted in copolymers with lower melting and glass-transition temperatures, Young's modulus and tensile strength. However, the copolymers have very high flexibility where the percentage of elongation to break was very high (Table 2.4) (Sudesh, *et al*., 2000). This indicated that the introduction of medium-chain-length-3HA into P(3HB) polymer will improve the physical properties of the polymer, making it softer, more elastic and flexible.

Table 2.4 Comparison of the properties of P(3HB) homopolymer and copolymers with common plastics

Sample	Melting temperature (^{o}C)	Glass-transition temperature (^{o}C)	Young's modulus (GPa)	Tensile strength (MPa)	Elongation to break (%)
P(3HB)	180	4	3.5	40	5
P(3HB-co-20 mol% 3HV)	145	-1	0.8	20	50
P(3HB-co-6 mol% 3HA) [a]	133	-8	0.2	17	680
Polypropylene	176	-10	1.7	38	400
Low-density polyethylene	130	-30	0.2	10	620

[a] 3HA units: 3-hydroxydecanoate (3 mol%), 3-hydroxydodecanoate (3 mol%), 3-hydroxyoctanoate (<1 mol%), 3-hydroxy-*cis*-5-dodecanoate (<1 mol%).
(Table source: Sudesh, *et al*., 2000).

2.3.2 The biological properties of P(3HB)

The unique property of PHA is their biodegradability in natural environments such as soil, sea water and sewage. Biodegradation of PHA occurs through an enzymatic mechanism. Many microorganisms in various environments produce intra- or extracellular PHA depolymerase which hydrolyses PHA into water-soluble oligomers and monomers, which are subsequently utilised as nutrients. The degradation reaction can take time from as short as a few months to years (Suriyamongkol, *et al*., 2007). The rate of biodegradation of PHA depends on environmental factors and also the PHA materials themselves. The environmental factors include temperature, moisture level, pH, ultra-violet radiation and nutrient supply; while the material factors of PHA include the composition, crystallinity, additives and surface area of the polymer (Sudesh, *et al*., 2000; Shangguan, *et al*., 2006).

The biodegradation rate of P(3HB) is strongly dependent on the concentration of the P(3HB) depolymerase enzyme. P(3HB) is a solid polymer which act as a water-insoluble substrate, while P(3HB) depolymerase is a water-soluble enzyme. Thus, the biodegradation of P(3HB) is a heterogeneous reaction which involves two steps which are adsorption and hydrolysis. Adsorption happens when the depolymerase enzyme binds

to the surface of the P(3HB) polymer through the binding domain of the enzyme. Then, hydrolysis of the polymer chain happens through the active site of the enzyme. The resulting products are the water-soluble monomers and oligomers (Sudesh, *et al*., 2000). The degradation rate of P(3HB) ranges from a few months (in anaerobic sewage) to years (in seawater) (Madison & Huisman, 1999).

2.4 Biosynthesis of PHA

2.4.1 Metabolic pathways of PHA biosynthesis

PHAs can be accumulated naturally by various microorganisms through various metabolic pathways with various carbon sources. The carbon sources can be differentiated into structurally related and unrelated carbon sources. Structurally related carbon sources, acting as monomers will generate PHAs with chemical structures related to the carbon sources used while structurally unrelated carbon sources will generate PHAs with chemical structures which are entirely different or unrelated to the chemical structure of the carbon sources used.

There are three well-known naturally occurring pathways for biosynthesis of PHA (Fig. 2.5). The most well studied is the P(3HB) biosynthetic pathway (Pathway I). It involves only three steps. The first step involves condensation of two acetyl-CoA molecules (which are intermediates from the tricarboxylic acid cycle) into acetoacetyl-CoA by β-ketothiolase enzyme (Park & Lee, 2003). Acetoacetyl-CoA will then be converted into (*R*)-3-hydroxybutyryl-CoA by acetoacetyl-CoA-reductase (Park & Lee, 2003). In the final step, (*R*)-3-hydroxybutyryl-CoA will be polymerised by PHA synthase into poly-(*R*)-3-hydroxybutyrate (Park & Lee, 2003). PHA generated from pathways II and III are medium-chain-length-PHA in which the monomers are the intermediates from the two fatty acid metabolic pathways. In pathway II, enoyl-CoA and 3-ketoacyl-CoA

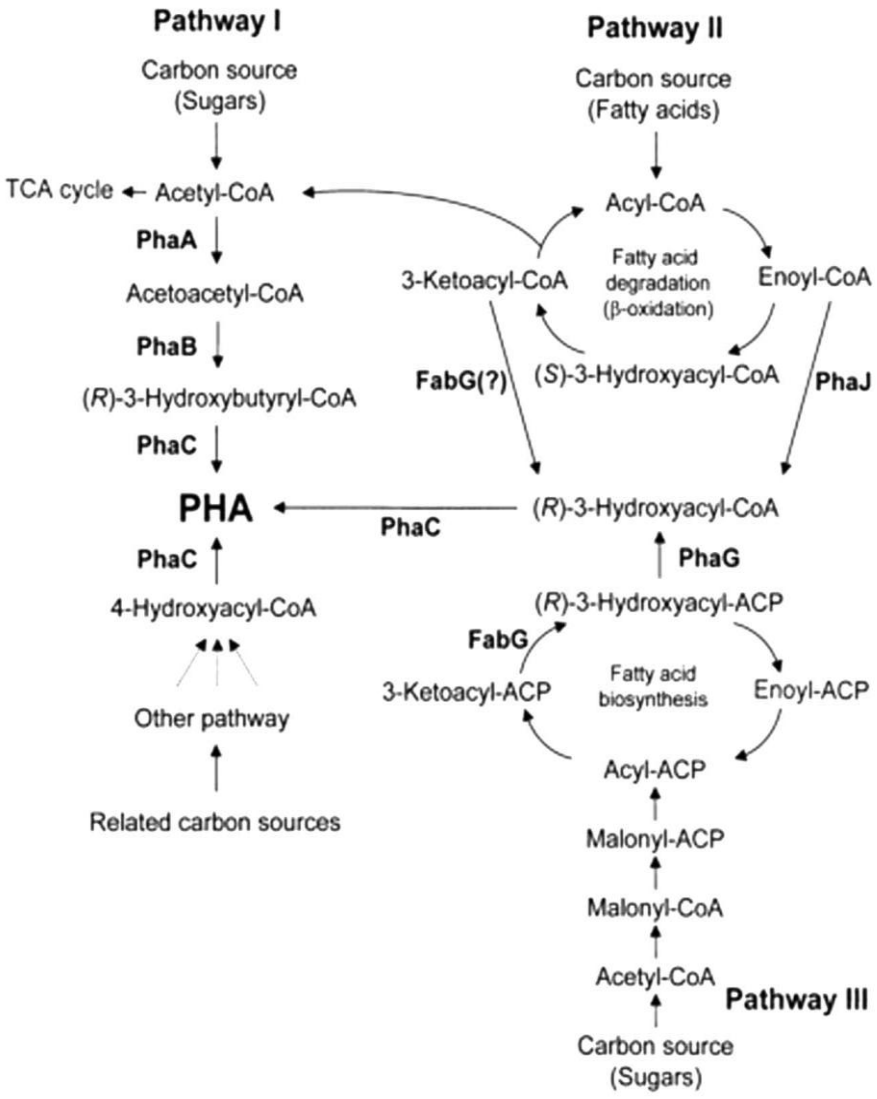

Figure 2.5 Natural occurring PHA biosynthesis pathways. PhaA is 3-ketothiolase; PhaB is NADPH-dependent acetoacetyl-CoA reductase; PhaC is PHA synthase; PhaG is 3-hydroxyacyl-ACP-CoA transferase; PhaJ is (*R*)-specific enoyl-CoA hydratase; FabG is 3-ketoacyl-ACP reductase.
(Figure source: Tsuge, 2002).

(which are both intermediates from fatty acid β- oxidation pathway) act as substrates for PHA biosynthesis. They are converted into (*R*)-3-hydroxyacyl-CoA by (*R*)-specific enoyl-CoA hydratase and 3-ketoacyl-ACP reductase enzymes respectively (Park & Lee, 2003). In the pathway III, the intermediates from fatty acid *de novo* biosynthesis pathway which is (*R*)-3-hydroxyacyl-ACP is converted by 3-hydroxyacyl-ACP-CoA transferase into (*R*)-3-hydroxyacyl-CoA (Rehm, *et al*., 1998). The (*R*)-3-hydroxyacyl-CoA generated from both pathways II and III will be polymerised into PHA by PHA synthase. Examples of bacteria which utilise these pathways are *Pseudomonas oleovorans* (uses the β-oxidation pathway) and *Pseudomonas putida* (uses the *de novo* pathway) (Rehm, *et al*., 1998).

It is important to note that there is another naturally-occurring pathway which facilitates various related carbon sources to form 4-hydroxyacyl-CoA monomers which are subsequently converted into PHA by PHA synthase.

2.4.2 Genes involve in PHA biosynthesis

PHA are formed through several different pathways in which each of the pathways were evolved and optimised based on the ecological niche of the PHA producer itself (Madison & Huisman, 1999). Each pathway includes genes which encode for the proteins involve in PHA synthesis. These genes are often clustered in the bacterial genomes (Rehm and Steinbuchel, 1999). Terminologically, the genes coding for PHA biosynthesis proteins are referred in alphabetical order for example *phaA*, *phaB*, *phaC*, *phaG*, *phaJ* and etc. (Rehm & Steinbüchel, 1999).All these genes encode for enzymes involved in PHA biosynthesis from monomer supplying to polymerisation and inclusion formation.

There are several monomer supplying genes. Examples are *phaA* and *phaB* which encode for β-ketothiolase and acetoacetyl-CoA-reductase, respectively. They are

involved in the monomer supplying steps in biosynthesis of short-chain-length P(3HB). Meanwhile, *phaJ* gene which encodes for (*R*)-specific-enoyl-CoA hydratase and *phaG* gene which encodes for 3-hydroxyacyl-ACP-CoA transacylase are involved in the monomer supplying steps for medium-chain-length-PHA biosynthesis pathways which utilise monomers from fatty acid metabolism. They convert enoyl-CoA to (*R*)-3-hydroxyacyl-CoA and (*R*)-3-hydroxyacyl-ACP to 3-hydroxyacyl-ACP-CoA respectively, to be polymerised into medium-chain-length-PHA in the subsequent metabolic step.

There is only one polymerising enzyme termed PHA synthase which is further classified into three different classes. Class I PHA synthase contains only one subunit, PhaC, which is encoded by *phaC* gene (Kozhevnikov, *et al*., 2010). Class II PHA synthase contains two subunits which is encoded by *phaC1* and *phaC2* (Kozhevnikov, *et al*., 2010). Class III and class IV PHA synthase also contains two subunits: a PhaC which is encoded by *phaC* gene and a PhaE which is encoded by *phaE* in class III while PhaR which is encoded by *phaR* in class IV PHA synthase, respectively (Kozhevnikov, *et al*., 2010).

Other genes related to PHA are those encoding for granule-associated proteins. The most found granule-associated protein, Phasins, is encoded by *phaP* gene. Phasin is the dominant protein found on the surface of PHA inclusions and is involved in the formation of a network on the surface of the inclusions in *C. necator* (York, *et al*., 2001). Another granule-associated protein, PhaD, which is encoded by *phaD* gene serves to stabilize PHA granules. However, it is not the major granule-associated protein (Klinke, *et al*., 2000). PhaD act by an unknown mechanism involving PhaI protein encoded by *phaI* gene. PhaI protein is also a PHA granule-associated protein (Klinke, *et al*., 2000). Another granule-associated protein is PHA depolymerase which is encoded by *phaZ*. PHA depolymerase will hydrolyse PHA to yield β-hydroxybutyric acid oligomers or monomers (Chou, *et al*., 2009).

Many of the genes associated to PHA biosynthesis have been cloned, sequenced and their genes arrangements elucidated to date. Examples are shown in Figure 2.6.

2.4.3 Biosynthesis pathways and genes in *C. necator* – the model P(3HB) producer

The PHA biosynthesis pathway in *C. necator* involved three steps of reaction which are catalysed by successive 3HB monomer-supplying enzymes ($PhaA_{Cn}$ and $PhaB_{Cn}$) and a polymerisation enzyme ($PhaC_{Cn}$) (Taguchi & Doi, 2004).

The first step of the reaction is catalysed by enzyme β-ketothiolase (PhaACn) which is encoded by $phaA_{Cn}$ gene. This enzyme condenses two molecules of acetyl-CoA to form acetoacetyl-CoA which is then reduced by acetoacetyl-CoA reductase ($PhaB_{Cn}$) which is encoded by $phaB_{Cn}$ gene to form (*R*)-3-hydroxybutyryl-CoA. Lastly, the polymerisation enzyme, PHA synthase ($PhaC_{Cn}$) which is encoded by $phaC_{Cn}$ gene will polymerise (*R*)-3-hydroxybutyryl-CoA to form P(3HB) (Normi, *et al*., 2005) (Fig. 2.7). P(3HB) which is synthesised via the above pathway only contain short-chain-length monomers. However, through alteration in the type and the relative quantity of the carbon source in the growth medium, a wide range of PHAs can also be synthesised (Steinbüchel & Schlegel, 1991; Kim & Lenz, 2001). For example, the addition of propionic acid or valeric acid in growth medium containing glucose can lead to accumulation of P(3HB-co-3HV) co-poymer (Fig. 2.7). This came from the changes in the pathway where one of the propionyl-CoAs and one acetyl-CoA were condensed by 3-ketothiolase encoded by bktB to form 3-ketovaleryl-CoA. This molecules were then reduced to (*R*)-3-hydroxyvaleryl-CoA catalysed by acetoacetyl-CoA-reductase.The (*R*)-3-hydroxyvaleryl-CoA molecules were subsequently polymerised into P(3HB-co-HV) by PHA synthase (Poirier, *et al*., 2002).

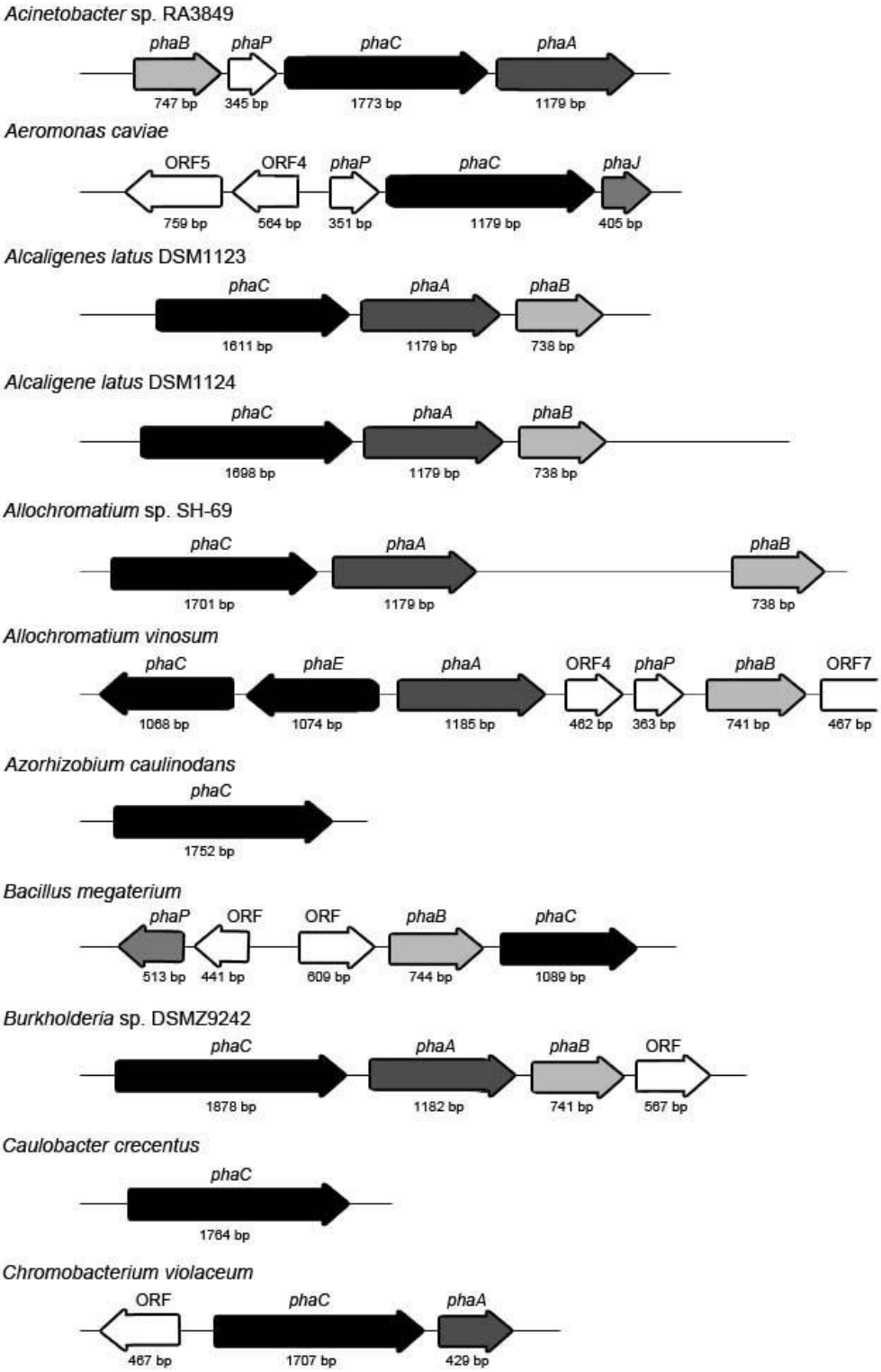

Figure 2.6 Organization of genes involved in PHA synthesis in different bacteria.

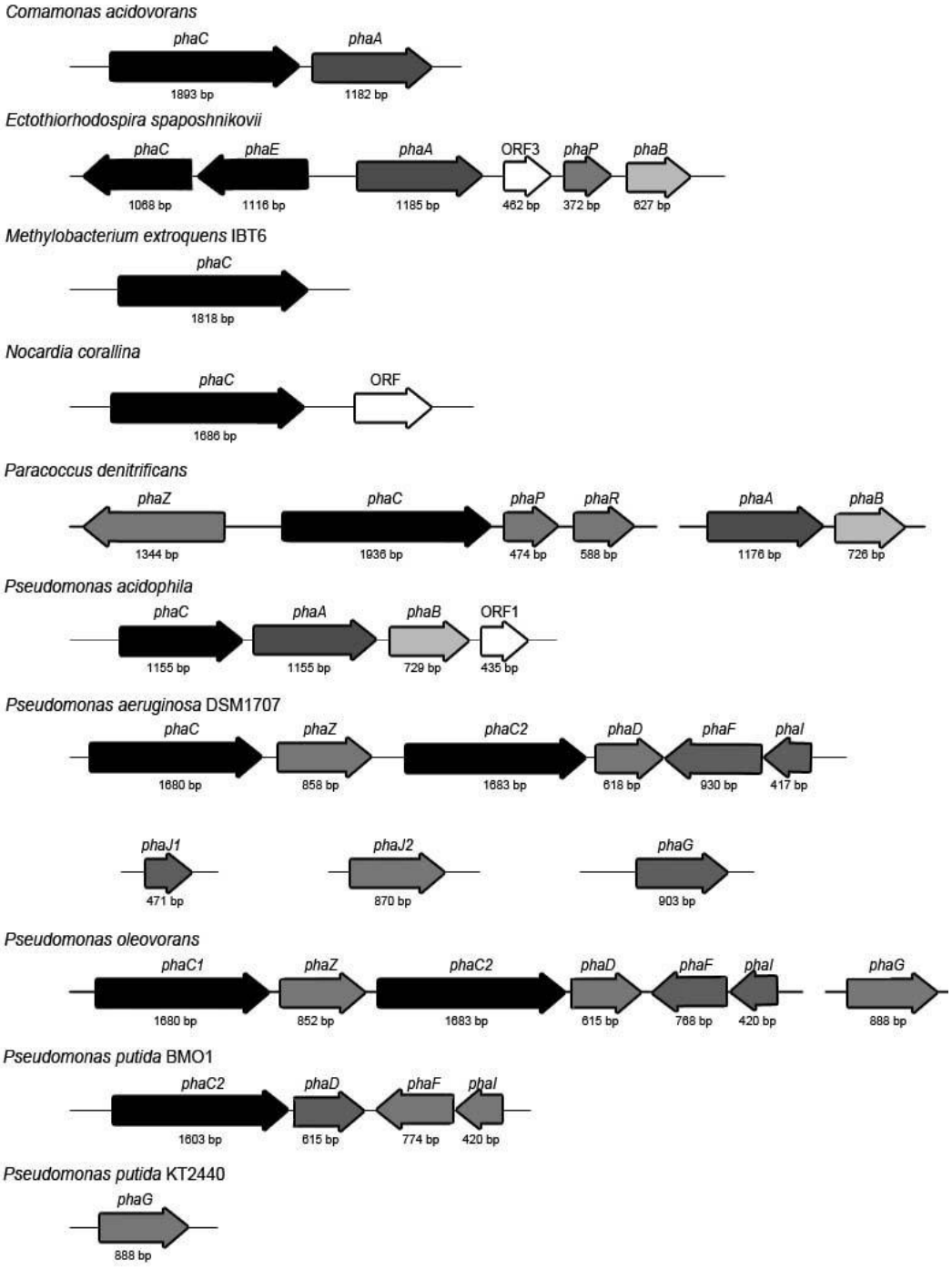

Figure 2.6 Organization of genes involved in PHA synthesis (Continued).

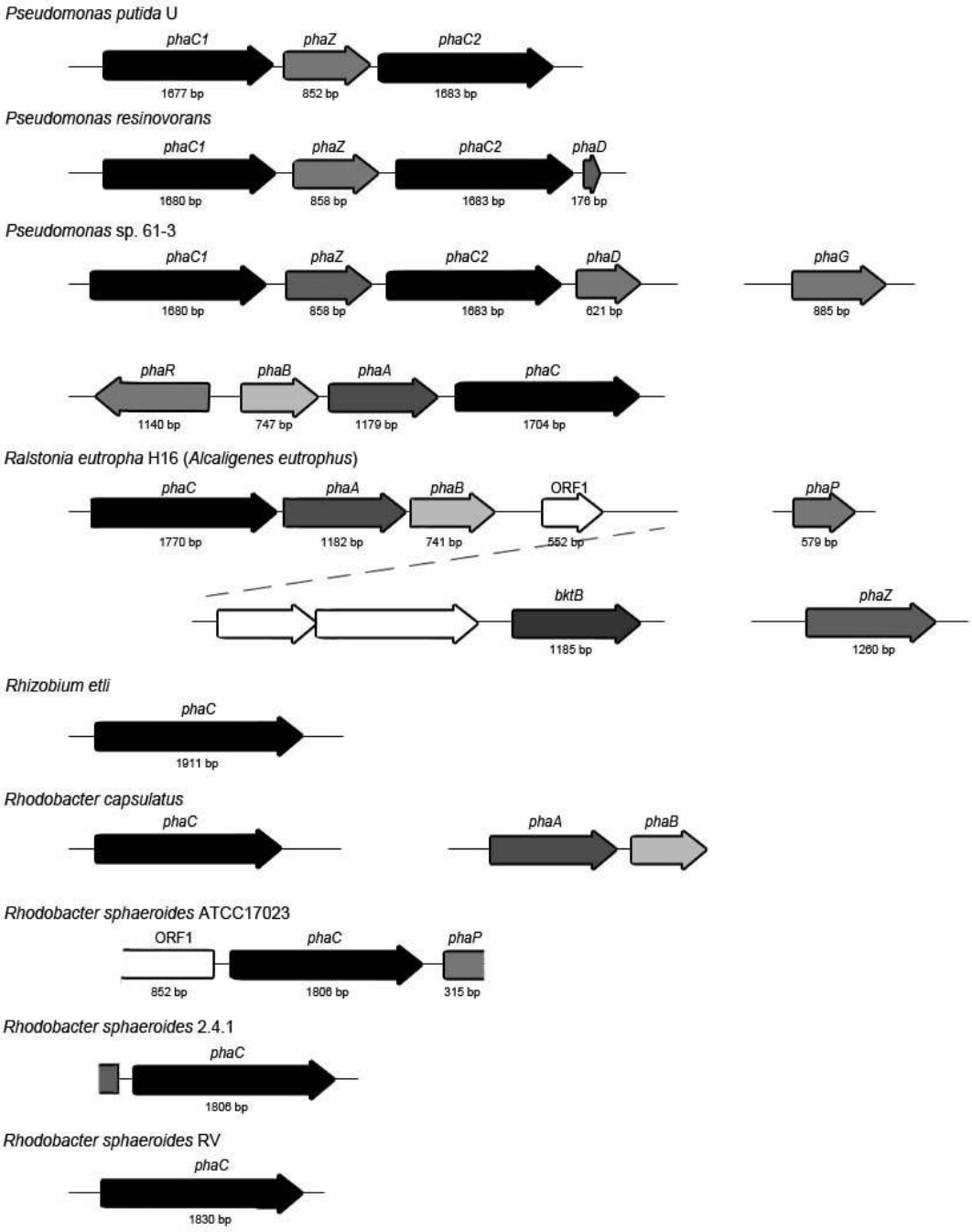

Figure 2.6 Organization of genes involved in PHA synthesis (Continued).

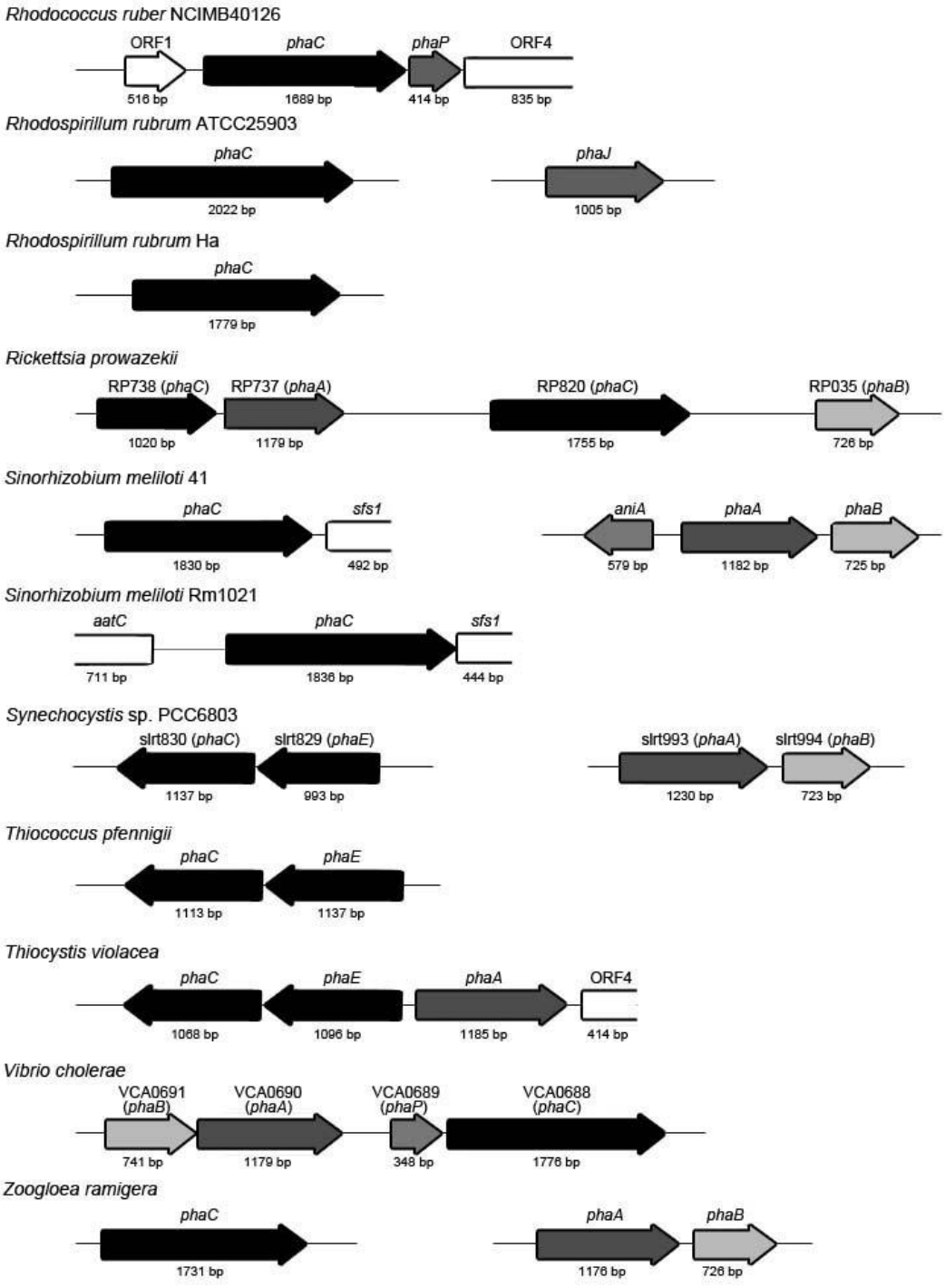

Figure 2.6 Organization of genes involved in PHA synthesis (Continued). The size of genes includes termination codon
(Figure source: Taguchi, *et al*., 2002).

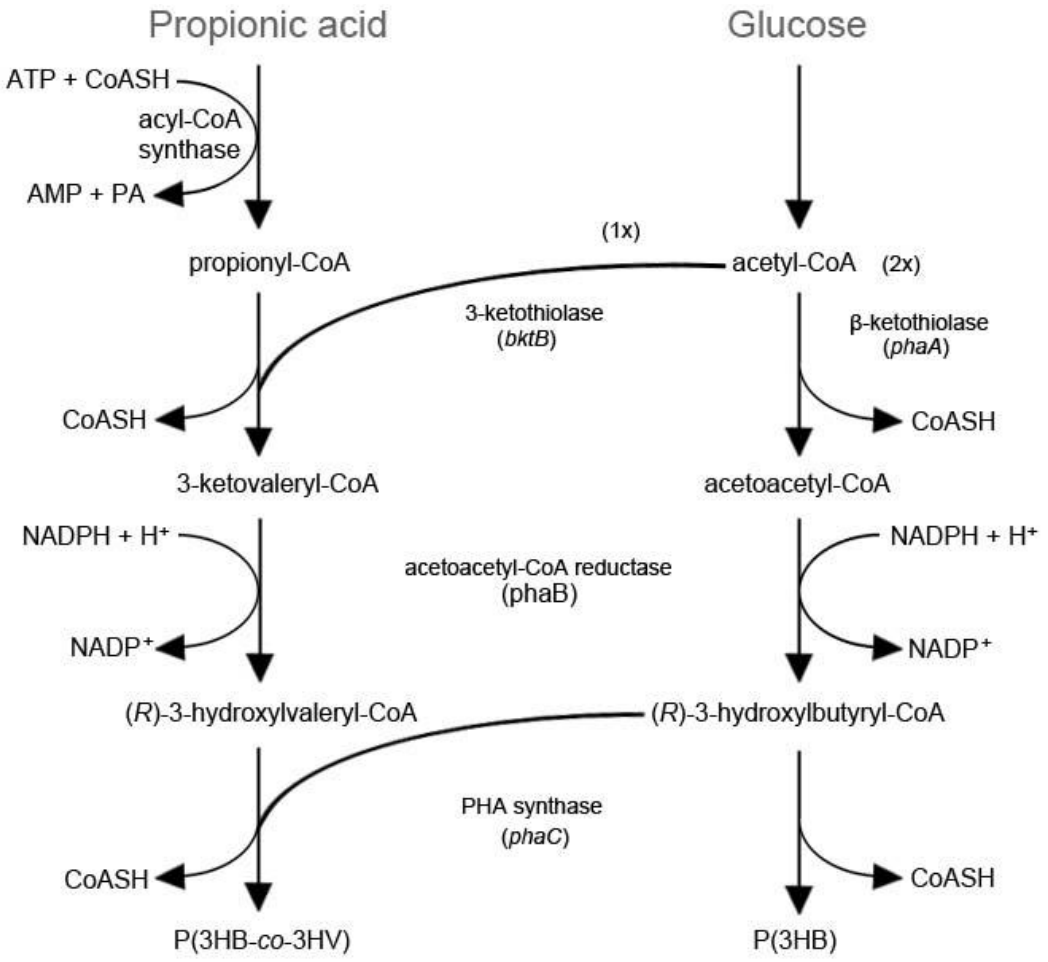

Figure 2.7 P(3HB) and P(3HB-*co*-3HV) biosynthesis pathway in *Cupriavidus necator*. *phaA* and *bktB* encode β-ketothiolase and 3-ketothiolase, enzymes involved in formation of acetoacetyl-CoA and 3-ketovaleryl-CoA, respectively. *phaB* encodes acetoacetyl-CoA reductase, which reduces both acetoacetyl-CoA and 3-ketovaleryl-CoA to form (*R*)-3-hydroxybutyryl-CoA and (*R*)-3-hydroxyvaleryl-CoA, respectively. *phaC* encodes PHA synthase, which is the last enzyme responsible for polymerisation of the monomers (Figure source: Poirier, *et al*., 2002).