IMPROVEMENT OF POLYHYDROXYALKANOATE SYNTHASE FROM *Wautersia eutropha* BY *IN VITRO* EVOLUTION

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by

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LIST OF ABBREVIATIONS

bp	base pair
HB	hydroxybutyryl
kbp	kilobase pair
k_{cat}	enzyme turnover number
K_m	Michaelis-Menten constant
OD	optical density
PCR	polymerase chain reaction
PHA	polyhydroxyalkanoate
PhaC	PHA synthase
PHB	polyhydroxybutyrate
(*R*)-3HB-CoA	(*R*)-3-hydroxybutyryl-CoA
T_m	melting temperature
T_g	glass-transition temperature
v/v	volume/volume
w/v	weight/volume
w/w	weight/weight

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4.0 RESULTS AND DISCUSSION

ABSTRACT

The key enzyme in PHB biosynthesis of the bacterium *Wautersia eutropha* is PHA synthase. In this study, *in vitro* evolution of PHA synthase of *W. eutropha* ($PhaC_{We}$) was performed to obtain "evolved" $PhaC_{We}$ exhibiting improved characteristics. Suppression-type mutagenesis was performed via error-prone PCR to obtain beneficial mutations. The PHA synthase gene ($phaC_{We}$) of the E11 mutant harboring an S80P mutation conferring low PHB accumulation was used as a template. As a result, a G4D mutation conferring high PHB accumulation and molecular weight and *in vivo* level of $PhaC_{We}$ enzyme was obtained. Most of other amino acid substitutions at the G4 position conferred similar phenotypes. Site-directed mutagenesis was also performed at position F420 to investigate the effect of amino acid substitutions on PHB content. This position was chosen as previous F420S substitution resulted in a $PhaC_{We}$ enzyme with enhanced activity (Taguchi *et al*., 2002a). Among all the substitutions, the F420S substitution conferred the highest PHB content. Interestingly, the lag phase of $PhaC_{We}$ enzyme was significantly reduced by this single F420S substitution. This finding strengthened previous postulation reporting the possibility of the F420 substitution in the dimerization of $PhaC_{We}$ enzyme (Taguchi *et al*., 2002a). The G4D and F420S mutations were then combined. The G4D/F420S mutant had high PHB accumulation and *in vivo* concentration of $PhaC_{We}$ enzyme, similar to the G4D mutant. However, the molecular weight of the polymer and the kinetic properties of $PhaC_{We}$ of

G4D/F420S mutant were similar to that of the F420S mutant. These findings suggested the possible dominance of the G4D mutation in determining the PHB content and *in vivo* level of $PhaC_{We}$ enzyme and the F420S mutation in determining the molecular weight of the polymer and the kinetic properties of $PhaC_{We}$ enzyme of the G4D/F420S mutant. Since all the above studies were done using *Escherichia coli* JM109 as the host, the effect of the mutations using native *W. eutropha* $PHB^{-}4$ as the host strain was also investigated. All the recombinants gave similar PHB content when grown on fructose. However, the F420S recombinant gave a slightly higher PHA accumulation than the wild-type when grown on soybean oil. All recombinants had comparable 3HHx mole fractions. Interestingly, the *in vivo* levels of $PhaC_{We}$ enzyme were elevated in most G4 and G4D/F420S recombinants, indicating that the effect of G4 substitution on *in vivo* levels of $PhaC_{We}$ enzyme is not specific to *E. coli* strains only.

PENAMBAHBAIKAN SYNTHASE POLIHIDROKSIALKANOAT DARIPADA *Wautersia eutropha* SECARA EVOLUSI *IN VITRO*

ABSTRAK

Enzim utama dalam biosintesis polihidroksibutirat (PHB) di dalam bakteria *Wautersia eutropha* ialah PHA synthase. Dalam kajian ini, evolusi *in vitro* PHA synthase dari *W. eutropha* ($PhaC_{We}$) telah dilakukan untuk mendapatkan $PhaC_{We}$ yang mempamerkan ciri-ciri yang lebih baik. Mutagenesis jenis supresi secara “error-prone PCR” telah dilakukan untuk mendapatkan mutasi yang berfaedah. Gen PHA synthase (*phaC*$_{We}$) mutan E11 yang mempunyai mutasi S80P yang menyebabkan penghasilan PHB yang rendah telah digunakan sebagai templat. Hasilnya, suatu mutasi G4D yang memberikan penghasilan dan berat molekul PHB serta amaun enzim $PhaC_{We}$ *in vivo* yang tinggi berjaya diperolehi. Kebanyakan asid amino gantian yang lain pada posisi G4 turut mempamerkan fenotip yang sama. Mutagenesis secara tertumpu turut dilakukan pada posisi F420 untuk mengkaji kesan penggantian asid amino pada produksi PHB. Posisi ini telah dipilih berdasarkan kajian terdahulu yang menyatakan mutasi F420S penting dalam meningkatkan aktiviti enzim $PhaC_{We}$ (Taguchi *et al*., 2002a). Di antara kesemua penggantian, penggantian F420S memberikan penghasilan PHB yang tertinggi. Turut menarik perhatian ialah penggantian tunggal ini mengurangkan fasa lag (“lag phase”) enzim $PhaC_{We}$. Penemuan ini mengukuhkan lagi kemungkinan peranan penggantian F420

dalam pendimeran enzim $PhaC_{We}$ (Taguchi *et al*., 2002a). Seterusnya, kedua-dua mutasi G4D dan F420S digabungkan. Mutan G4D/F420S memberikan penghasilan PHB dan amaun $PhaC_{We}$ enzim *in vivo* yang tinggi, sama seperti mutan G4D. Bagaimanapun, berat molekul PHB dan ciri-ciri kinetik $PhaC_{We}$ mutan G4D/F420S menyerupai mutan F420S. Keputusan ini menunjukkan dominasi mutasi G4D dalam menentukan penghasilan PHB dan amaun $PhaC_{We}$ enzim *in vivo* dan mutasi F420S dalam menentukan beral molekul polimer dan ciri-ciri kinetik enzim $PhaC_{We}$ mutan G4D/F420S. Memandangkan kesemua kajian di atas dilakukan menggunakan *Escherichia coli* JM109 sebagai perumah, kajian tentang kesan mutasi-mutasi di atas dengan menggunakan *W. eutropha* $PHB^{-}4$ sebagai perumah turut dilakukan. Kesemua rekombinan memberikan penghasilan PHB yang hampir sama apabila fruktosa digunakan sebagai sumber karbon. Bagaimanapun, rekombinan F420S memberikan penghasilan PHA yang tinggi sedikit daripada jenis liar apabila minyak soya digunakan sebagai sumber karbon. Kesemua rekombinan mempunyai pecahan mol 3HHx yang sama. Satu penemuan yang menarik ialah, amaun enzim $PhaC_{We}$ adalah tinggi dalam kebanyakan rekombinan G4 dan juga G4D/F420S. Ini menunjukkan bahawa kesan mutasi G4 ke atas amaun enzim $PhaC_{We}$ *in vivo* tidaklah terhad pada strain *E. coli* sahaja.

1.0 INTRODUCTION

Polyhydroxyalkanoates (PHA) are polyesters synthesized by various bacteria as intracelullar carbon and energy storage material under excess carbon and limiting nutrient conditions. In the recent decades, PHAs have attracted considerable attention due to similarity in their physical properties to petrochemical-based polyesters. However, unlike conventional polyesters, PHA can be degraded enzymatically by extracellular depolymerase excreted by various bacteria, thus, making these bacterial polyesters more environmentally-acceptable than conventional plastics.

The most well-known type of PHA is poly(3-hydroxybutyrate) (PHB) polymer. PHB is synthesized by various bacteria and the most well-studied PHB producer is *Wautersia eutropha* (formerly known as *Alcaligenes eutrophus* and later *Ralstonia eutropha*). In *W. eutropha*, the key enzyme in PHB biosynthesis is PHA synthase ($PhaC_{We}$). Being the key enzyme, $PhaC_{We}$ has the most influence on PHB accumulation and molecular weight of the polymer produced. Both of these aspects are important in industrial production and commercial application of the bacterial polyester. Modifications of $PhaC_{We}$ are anticipated to lead to the enhancement of either one or both of these aspects, thus taking us one step closer to an economical, efficient large-scale PHB production and/or better application of the polyester.

Modifications to improve $PhaC_{We}$ can be achieved by the introduction of beneficial mutations through directed evolution. Unlike evolution through natural selection, directed evolution enables beneficial mutations to be

generated artificially under induced conditions in a shorter time span. Therefore, directed evolution is considered to be a powerful method to artificially evolve, and hence, improve a given enzyme (Arnold, 1998).

One approach of directed evolution used is *in vitro* evolution (Taguchi and Doi 2004). The *in vitro* evolution approach proved to be a highly successful tool in generating improved PHA synthase enzymes in terms of PHA accumulation, monomer composition and/or molecular weight enhancement (Taguchi *et al*., 2001, 2002a; Kichise *et al*., 2002; Takase *et al*., 2003, 2004; Tsuge *et al*., 2004a, 2004b). One of the successful case-studies using the *in vitro* evolution approach was the improvement of the type II PHA synthase of *Pseudomonas* sp. 61-3 ($PhaC1_{Ps}$) obtained by error-prone PCR mutagenesis, site-directed saturation mutagenesis and combination of beneficial mutations which led to significantly enhanced PHB accumulation in recombinant *Escherichia coli* (Takase *et al*., 2003). Improved type I synthase of *Aeromonas caviae* ($PhaC_{Ac}$) exhibiting enhanced PHA accumulation was achieved through *in vitro* evolution (Kichise *et al*., 2002, Tsuge *et al*., 2004a). However, improvement of type I synthase of *W. eutropha* ($PhaC_{We}$) exhibiting enhanced PHB accumulation has not been widely reported (Tsuge *et al*., 2004b).

In this work, the *in vitro* evolution method was used to obtain evolved $PhaC_{We}$ with improved characteristics. The improved characteristics looked for are those showing:

(1) enhanced PHB accumulation

(2) enhanced PHB properties

(3) enhanced enzyme activity

(4) enhanced $PhaC_{We}$ enzyme production

For this purpose, *in vitro* evolution was carried out in three parts in the present study. The objective for each part was the same, i.e., to obtain evolved $PhaC_{Re}$ enzyme showing one or more of the above enhanced properties. The only difference in each part was the way this objective was achieved. The following are the *in vitro* evolution approaches used for each part:

(1) Part I – suppression-type mutagenesis and site-directed mutagenesis of PHA synthase gene (*phaC*$_{We}$)

(2) Part II – site-directed mutagenesis at position 420 of Pha_{We}

(3) Part III – combination of beneficial mutations obtained from Part I and II

The effect of such approaches on $PhaC_{We}$ was investigated in terms of the changes exhibited in mainly all four of the characteristics mentioned above. The passages below elaborate the historical background and the logic as to why these approaches were used as well as the investigation done on the evolved Pha_{We} enzyme.

The first part (Part I) involved utilizing the *phaC*$_{We}$ gene of the E11 mutant harboring an S80P mutation (in which Ser was substituted with Pro at position 80 of $PhaC_{We}$) as a template to generate beneficial mutations by suppression-type mutagenesis via error-prone PCR. The S80P mutation conferred low PHB accumulation in the E11 mutant (Taguchi *et al*., 2001). Hence, it was hoped that through suppression-type mutagenesis, mutation(s)

which was or were able to suppress this phenotype could be generated. As a result, one mutation which positively enhanced both PHB accumulation and $PhaC_{We}$ enzyme level *in vivo* was identified. Site-directed saturation mutagenesis at this position was performed in order to investigate if other amino acid substitutions exhibit similar or enhanced effects on both PHB accumulation and $PhaC_{We}$ enzyme levels *in vivo*. PHB molecular weight characterization was carried out to investigate the effect of the mutations on PHB molecular weight. In addition, the $PhaC_{We}$ enzyme of the initially identified positive mutant was purified and *in vitro* activity assay was performed to investigate the effect of the mutation on the kinetic properties of the enzyme in (*R*)-3-hydroxybutyryl-CoA ((*R*)-3HB-CoA) polymerization compared to that of the wild-type enzyme.

The second part (Part II) of the study involved site-directed saturation mutagenesis at position 420 of $PhaC_{We}$ enzyme. Previously, an F420S mutation (in which Phe was substituted with Ser at position 420 of $PhaC_{We}$) conferring enhanced $PhaC_{We}$ specific activity was generated by suppression-type mutagenesis (Taguchi *et al*., 2002a). In order to investigate the possibility of other beneficial amino acid substitution(s) at this position, site-directed saturation mutagenesis was carried out at the F420 position. Similarly to Part I above, the effects of the substitutions at F420 on PHB accumulation and *in vivo* levels of $PhaC_{We}$ were investigated. From here, the F420 mutant which gave the highest PHB accumulation was identified and its $PhaC_{We}$ enzyme purified. *In vitro* activity assay of the selected mutant enzyme was performed to investigate the possible differences that might exist between the mutant and wild-type

enzyme in (*R*)-3HB-CoA polymerization.

The third part (Part III) involved combination of two selected beneficial mutations generated from the first and second parts of the present study respectively. Selection of beneficial mutations was done based on the positive phenotype(s) conferred by each of the mutations. The effect of the double mutations on PHB accumulation and molecular weight, and *in vivo* concentration of $PhaC_{We}$ were investigated. In addition, the effect of the double mutations on *in vitro* polymerization of (*R*)-3HB-CoA was also investigated.

Since the above studies were carried out using *Escherichia coli* JM109 strain as the host, similar studies using the native *W. eutropha* strain as the host needed to be addressed. Hence in the last part of this study (Part IV), several mutations generated above were introduced into the native *W. eutropha* $PHB^{-}4$ host strain deficient in PHA biosynthesis (Schlegel *et al*., 1970). The effects of the mutations on PHB and PHA accumulation, monomer composition of the PHA polymer and *in vivo* levels of $PhaC_{We}$ enzyme were investigated.

2.0 LITERATURE SURVEY

2.1 Introduction to PHA

2.1.1 PHA as Storage Material

PHA is a type of polyester synthesized in various bacteria, ranging from many Gram-negative and Gram-positive bacteria, to non-sulfur and sulfur purple bacteria (Sudesh *et al*., 2000). PHA is synthesized under excess carbon and limiting-nutrient conditions (such as shortage of phosphorus, magnesium or nitrogen). The synthesized macromolecules are stored in the cytoplasm in the form of granules (Figure 2.1), serving as carbon and energy storage material.

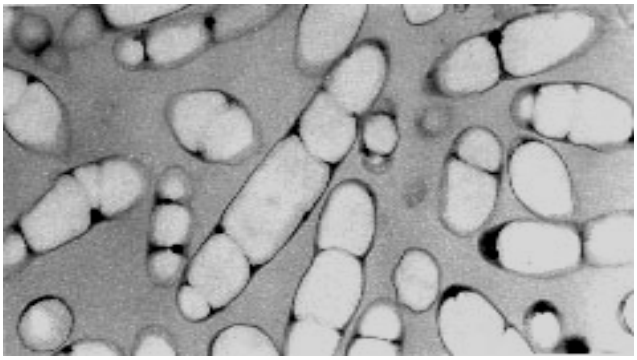


Figure 2.1 PHA granules in bacterial cells.

A vast consortium of microorganisms has evolved the ability to degrade PHA by using intracellular or extracelullar PHA depolymerases (Jendrossek *et al*., 1996). In some PHA producers, such as *Wautersia eutropha* (formerly known as *Alcaligenes eutrophus* and later *Ralstonia eutropha*) and *Zooglea ramigera*, PHA are degraded by intracellular PHA depolymerase (Doi *et al*., 1992 and Saito *et al*., 1992 respectively). On the other hand, other bacteria secrete PHA depolymerase to degrade PHA in the environment (which are released as a

result of cell death of PHA producers). Examples are *Pseudomonas stutzeri* (Mukai *et al.*, 1994) and *Alcaligenes faecalis* (Tanio *et al*., 1982). The ability of microorganisms to degrade PHA by using intracellular or extracellular PHA depolymerases for carbon utilization and energy generation ensures the proper functioning of the microorganism, especially when carbon source is exhausted from the environment. Hence, for these bacteria, the synthesis and/or degradation of PHA under nutrient-limiting conditions is not only an intelligent biological means to ensure the ready availability of intracellular energy and nutrient stores, but also to ensure survival as well.

2.1.2 Types of PHA

The building block of PHA is (*R*)-3-hydroxyalkanoic acid ((*R*)-3HA) monomer unit (Figure 2.2). In these polymers, the carboxyl group (COOH) of one monomer forms an ester bond with the hydroxyl group (OH) of the neighboring monomer (Madison and Huisman, 1999). In all PHA that have been characterized so far, the 3HA monomers are in the *R* configuration due to the stereospecificity of the polymerizing enzyme, PHA synthase (Sudesh *et al*., 2000). Only in one rare case, the *S* configuration of monomers was detected (Haywood *et al*., 1991). The side group (R) of the monomer dictates the overall chain length of the (*R*)-3HA monomer unit.

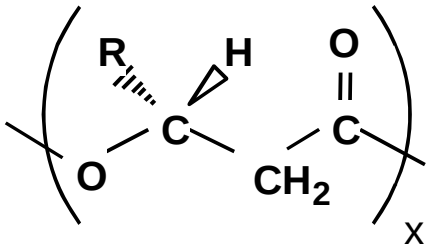


Figure 2.2 Chemical structure of PHA. R refers to side group while x refers to the number of repeating units.

Depending on chain length of the monomer unit, PHA can be classified into three groups (Table 2.1) (Sudesh *et al*., 2000).

Table 2.1 Types of PHA

Type of PHA	Number of C atoms	Example	Representative bacterium
SCL	C3-C5	PHB	*Wautersia eutropha*
MCL	C6-C14	PHO	*Pseudomonas* sp.
SCL-MCL	C3-C14	P(HB-*co*-HO)	*Pseudomonas* sp. 61-3

Short-chain-length PHA (SCL-PHA) comprises of HA monomers with three to five carbon atoms (C3-C5). An example is the polyhydroxybutyrate (PHB) polymer in which 3-hydroxybutyrate (3HB (C4)) is the constituent monomer (Table 2.1). The bacterium *Wautersia eutropha* is a typical example of a PHB producer.

The medium-chain-length PHA (MCL-PHA), on the other hand, comprise of HA monomers with six to fourteen carbon atoms (C6-C14). Examples are PHA consisting of 3-hydroxyoctanoate (3HO (C8)) and

3-hydroxydecanoate (3HD (C10)) as major monomers. Various strains of *Pseudomonas* sp., such as *Pseudomonas oleovorans* and *Pseudomonas putida*, are able to synthesize MCL-PHAs (Huisman *et al*., 1989).

Lastly, the hybrid short-chain-length-medium-chain-length PHA (SCL-MCL-PHA) uniquely consist of both the SCL and MCL HA monomers, with the number of carbon atoms ranging from C3-C14. An example is the random P(HB-*co*-HO) copolymer. Example of a hybrid SCL-MCL-PHA producer is the novel *Pseudomonas* sp. 61-3 bacterium (Matsusaki *et al*., 1998, 2000) (Table 2.1).

2.2 PHB polymer

Among various PHA, PHB is the most widely studied simply because it was the first type of PHA to be discovered, in 1926 by Lemoigne of Pasteur Institute. Several decades later, the discovery that PHB has similar physical and thermal properties with that of polypropylene plastics attracted more attention and interest from scientists and industrialists alike. This is because these properties, plus its biodegradability, make PHB suitable to be used for certain applications. Since then, effort has been made to commercially produce PHB homopolymer (containing solely of 3HB monomer) (Hängii, 1990) or co-polymer (containing 3HB and other 3HA monomers) (Slater *et al*., 1999) with desired characteristics.

2.2.1 Physical and Thermal Properties of PHB

PHB has various molecular weights, depending on various factors such as the type of bacterial strain in which they were produced, culture condition, carbon source used, etc. (Madison and Huisman, 1999). Bacterially produced PHB has sufficiently high molecular weights to have polymer characteristics that are similar to conventional plastics such as polypropylenes (Madison and Huisman, 1999). This is shown in Table 2.2.

Table 2.2 Comparison of PHB homo- and co-polymers with common plastics in properties.

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

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

Table from Sudesh *et al.* (2000).

To start with, the PHB homopolymer films have stiffness (expressed as Young's modulus) (3.5 GPa) and tensile strength (43 MPa) that are close to those of polypropylene (1.7 GPa and 38 MPa respectively). However, the elongation to break for PHB polymer (5%) is markedly lower than that of polypropylene (400%). Although relatively brittle than polypropylene, PHB is relatively stronger and stiffer (Doi, 1990; Holmes, 1998). In addition, PHB homopolymer has a high T_m of 180˚C, similar to that of polypropylene, making PHB polymer behaves as a thermoplastic.

Introduction of other 3HA monomers into the PHB polymer chain greatly influenced the physical properties of the resultant co-polymer (Doi, 1990; Holmes,1998). For instance, co-polymer films of PHB containing 20 mol% of 3-hydroxyvalerate (3HV) (C5) monomer decreased the Young's modulus from 3.5 GPa to 0.8 GPa, tensile strength from 43 MPa to 20 MPa and improved the elongation to break to 50%. This makes the P(3HB-*co*-20 mol% 3HV) co-polymer less stiff and brittle, instead more ductile and easier to mould than PHB homopolymer (Luzier, 1992).

In addition, the introduction of MCL 3HA monomers into PHB chain greatly improved the physical properties of the polymer films (Doi, 1990). For example, P(3HB-*co*-6 mol% 3HA) co-polymer films containing 6 mol% of the respective 3HA monomers (as stated below Table 2.2) have a markedly improved elongation to break of 680%, with markedly reduced Young's modulus (0.2 GPa) and tensile strength (17 MPa) than those of PHB homopolymer. This makes such co-polymers with incorporated MCL 3HA monomer, even at low concentrations, softer, more elastic and flexible than the PHB homopolymer (Matsusaki *et al*., 2000).

The presence of the mentioned monomers (3HV (20 mol%) and 3HA (6 mol%)) in the PHA co-polymer films decreases the T_ms of the co-polymers to 145˚C and 133˚C respectively (Table 2.2). In fact, it is worthy to note that the introduction of these monomers, causes the resultant co-polymers to have physical and thermal properties resembling that of low-density polypropylene (Table 2.2).

Hence, by regulating the monomer composition and content of a given PHB polymer, the physical and thermal properties of the resultant co-polymer can be regulated, influencing the resultant polymer to be either strong and stiff or soft and elastic.

2.2.2 Biological Properties of PHB

Despite the similarity between PHB and petrochemical-based polypropylene, what differentiate PHB with this common plastic are its unique biological properties.

As mentioned above, contrary to common plastics, PHB is biodegradable. Various microorganisms in nature have developed well-evolved mechanisms to degrade PHB via the secretion of intracellular or extracellular PHA depolymerases (Jendrossek *et al*., 1996). The degradation rate depends on various factors such as those related to the environment (temperature, pH, moisture level) and those related to the polymer itself (composition, crystallinity, dimension) (Sudesh *et al*., 2000). Nevertheless, the degradation rate of PHB ranges from a few months (in anaerobic sewage) to years (in seawater) (Jendrossek *et al*., 1996; Mergaert *et al*., 1993, 1994, 1995).

PHB is also a biocompatible material, enabling it to be evaluated for a variety of medial applications such as controlled drug release, surgical sutures, wound dressings, lubricating powders, orthopedic uses (Hocking and Marchessault, 1994) and as a pericardial substitute (Duvernoy *et al*., 1995). The potential of PHB to be used as scaffolds for tissue engineering has also been

evaluated (Hocking and Marchessault, 1994; Williams *et al*., 1999).

Most importantly, PHB can be produced from renewable sources, i.e., from bacteria and plants, making PHB renewable in nature. For example, fermentative production of PHB is based on agricultural products such as sugars (from cane or corn) as carbon and energy sources. These agricultural products used for PHB production are derived from carbon dioxide (CO_2) and water (H_2O). After their conversion into PHB, followed by the degradation of PHB, the end products are again CO_2 and H_2O (Madison and Huisman, 1999) (Figure 2.3). Thus, PHB has gained widespread attention not only because of its biodegradability, which makes this polymer to be more eco-friendly, but also its renewability which can cut our dependence on diminishing fossil fuels.

2.3 Applications of PHB

PHB has been used in various applications, as described in various patents. US patents 4,826,493 and 4,880,592 describe the manufacture of PHB and P(HB-*co*-HV) films and their use as diaper backsheet (Martini *et al.*, 1989a, b). These films can also be used to make laminates with other polymers such as polyvinyl alcohol (Holmes, 1986). In addition, PHB has also been described and used as hot-melt adhesives (Kauffman *et al*., 1992). As mentioned earlier, medical applications of PHB have also been evaluated particularly as scaffolds for tissue engineering (Hocking and Marchessault, 1994; Williams *et al*., 1999) and for orthopedic uses (Hocking and Marchessault, 1994). An interesting future application of PHB will be in the textile industry, whereby effort to produce PHB

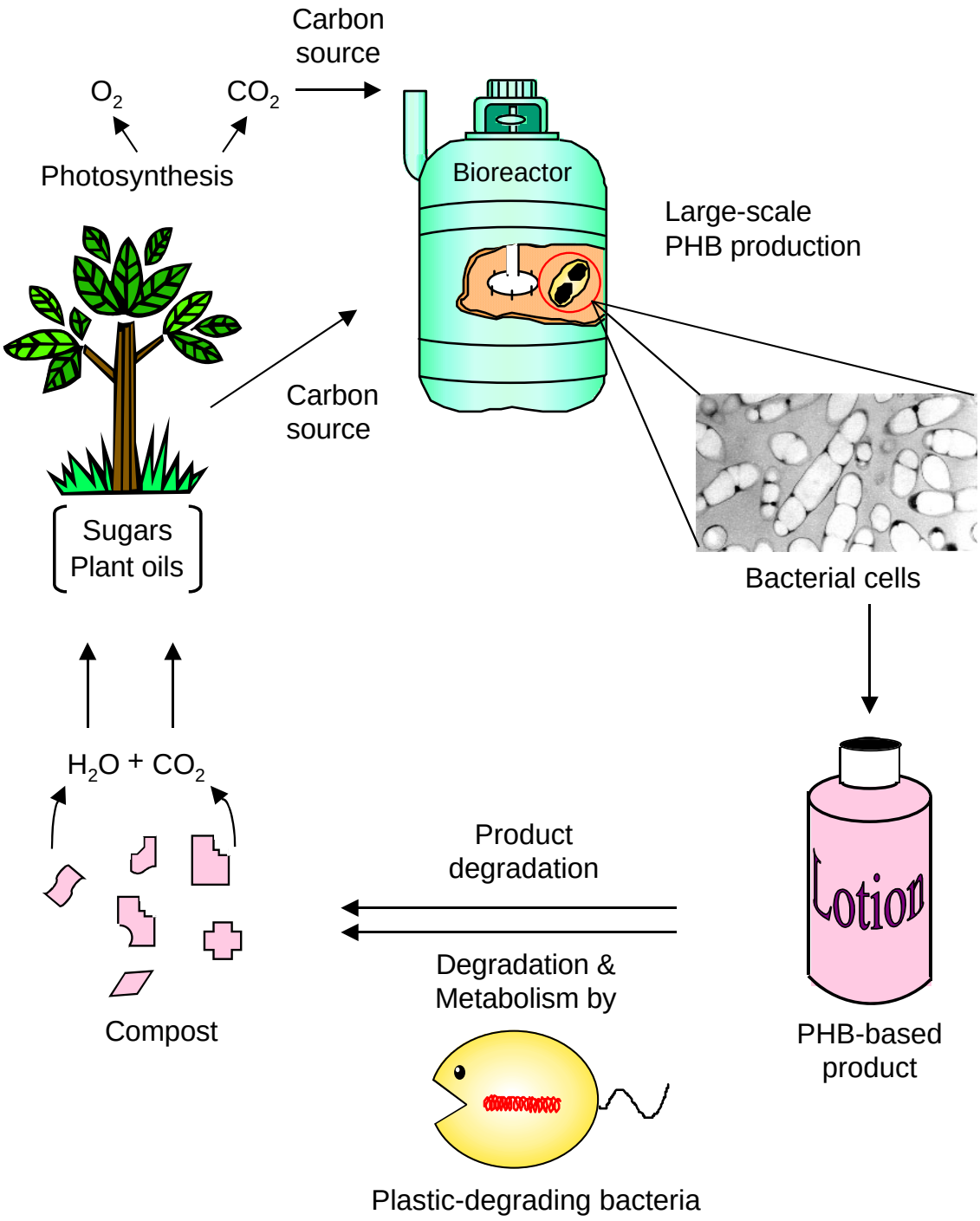


Figure 2.3 The renewability of PHB.

in lumen of cotton fibers to enhance fiber properties for textile applications has been made (Maliyakal and Keller, 1996).

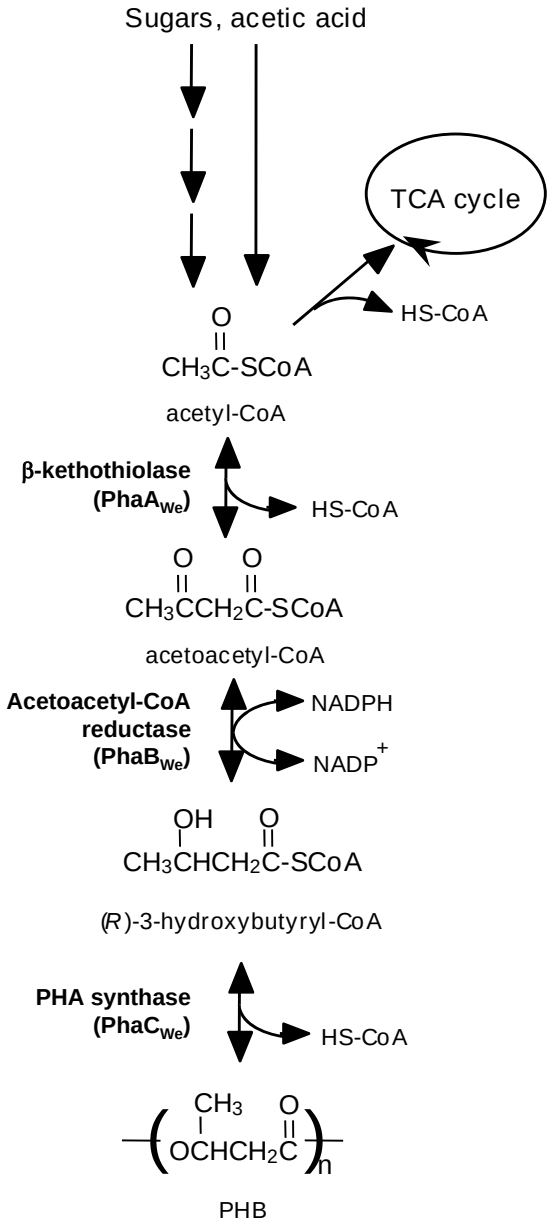
2.4 The PHB Biosynthesis Pathway and Genes in *Wautersia eutropha* – The Model PHB Producer

In *Wautersia eutropha*, the model PHB producer, there are three major enzymes which catalyze PHB biosynthesis, namely, β-ketothiolase ($PhaA_{We}$), acetoacetyl-CoA reductase ($PhaB_{We}$) and PHA synthase ($PhaC_{We}$) (Steinbüchel, 1996) (see Figure 2.4(A)).

First, $PhaA_{We}$ condenses two molecules of acetyl-CoA to form acetoacetyl-CoA, which will then be reduced by $PhaB_{We}$ to form (*R*)-3-hydroxybutyryl-CoA ((*R*)-3HB-CoA). The generated (*R*)-3HB-CoA monomer will then be polymerized by $PhaC_{We}$ to form PHB homopolymer (Figure 2.4(A)). Under this pathway, both $PhaA_{We}$ and $PhaB_{We}$ serve as monomer-supplying enzymes while $PhaC_{We}$ serve as the key enzyme in PHB biosynthesis.

The three major PHB biosynthesis enzymes, $PhaA_{We}$, $PhaB_{We}$ and $PhaC_{We}$, are encoded by the *phaA*$_{We}$, *phaB*$_{We}$ and *phaC*$_{We}$ genes respectively (Figure 2.4(B)). These genes are arranged in a pattern in which *phaC*$_{We}$ is clustered together with *phaA*$_{We}$ and *phaB*$_{We}$, forming the *phaCAB*$_{We}$ operon (Slater *et al*., 1988; Schubert *et al*., 1988; Peoples and Sinskey, 1989) under the control of a single promoter (Schubert *et al*., 1991) (Figure 2.4(B)).

A)



B)

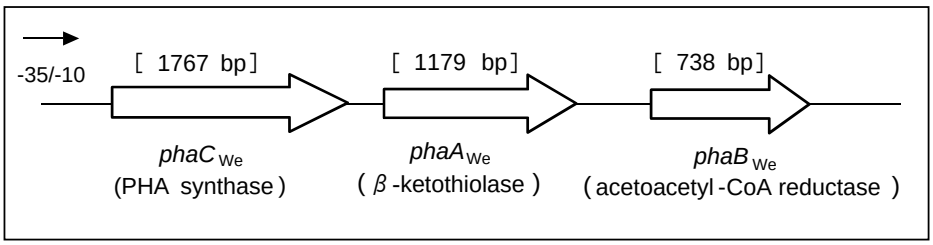


Figure 2.4 (A) PHB biosynthesis pathway and (B) Molecular organizations of genes relevant for PHB biosynthesis in *W. eutropha.*

To date, other *phaC* genes of various bacteria have been cloned, sequenced and their gene arrangements with other PHA biosynthesis related genes were elucidated (Taguchi *et al*., 2002b) (Figure 2.5)

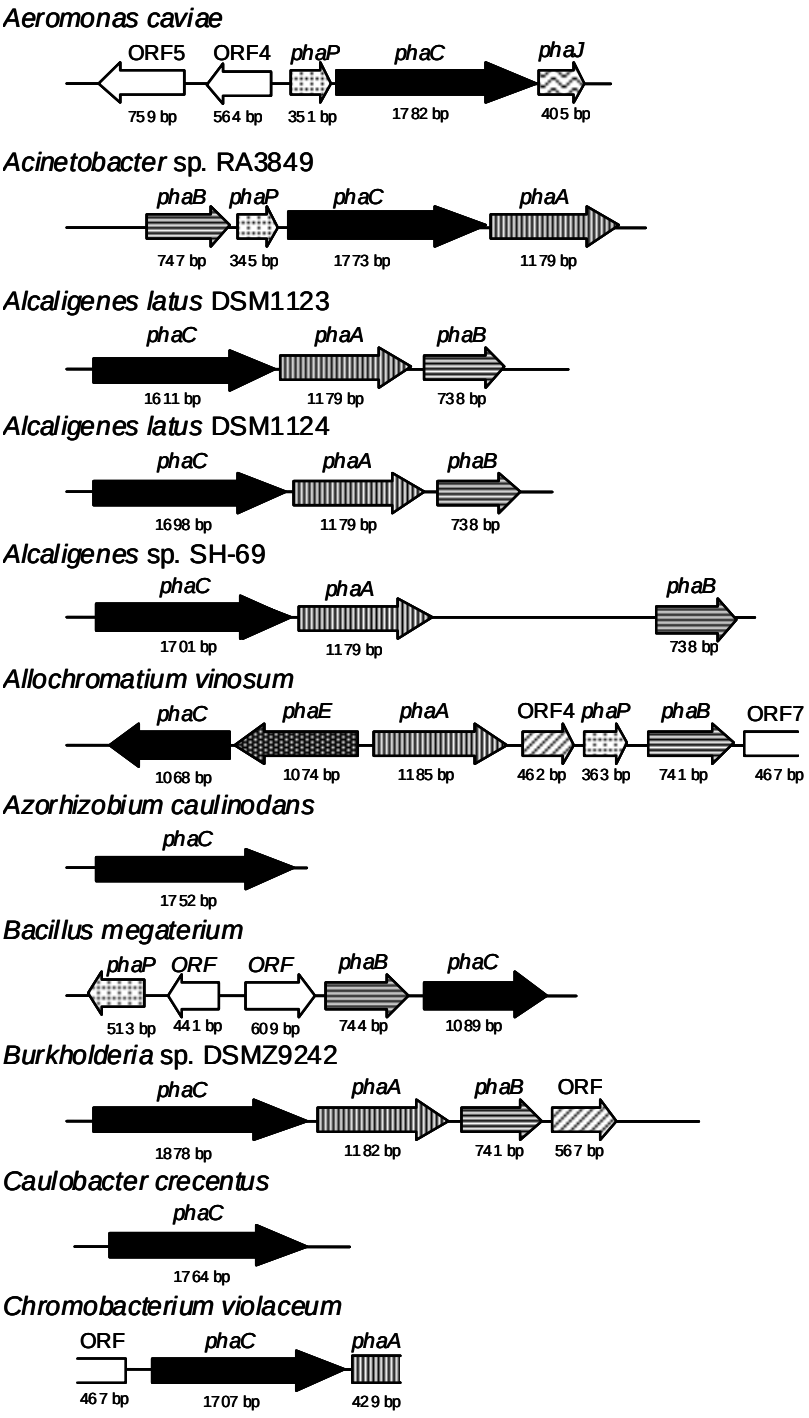


Figure 2.5 Organization of genes involved in PHA synthesis. The size of genes includes termination codon (Figure source: Taguchi *et al*., 2002b).

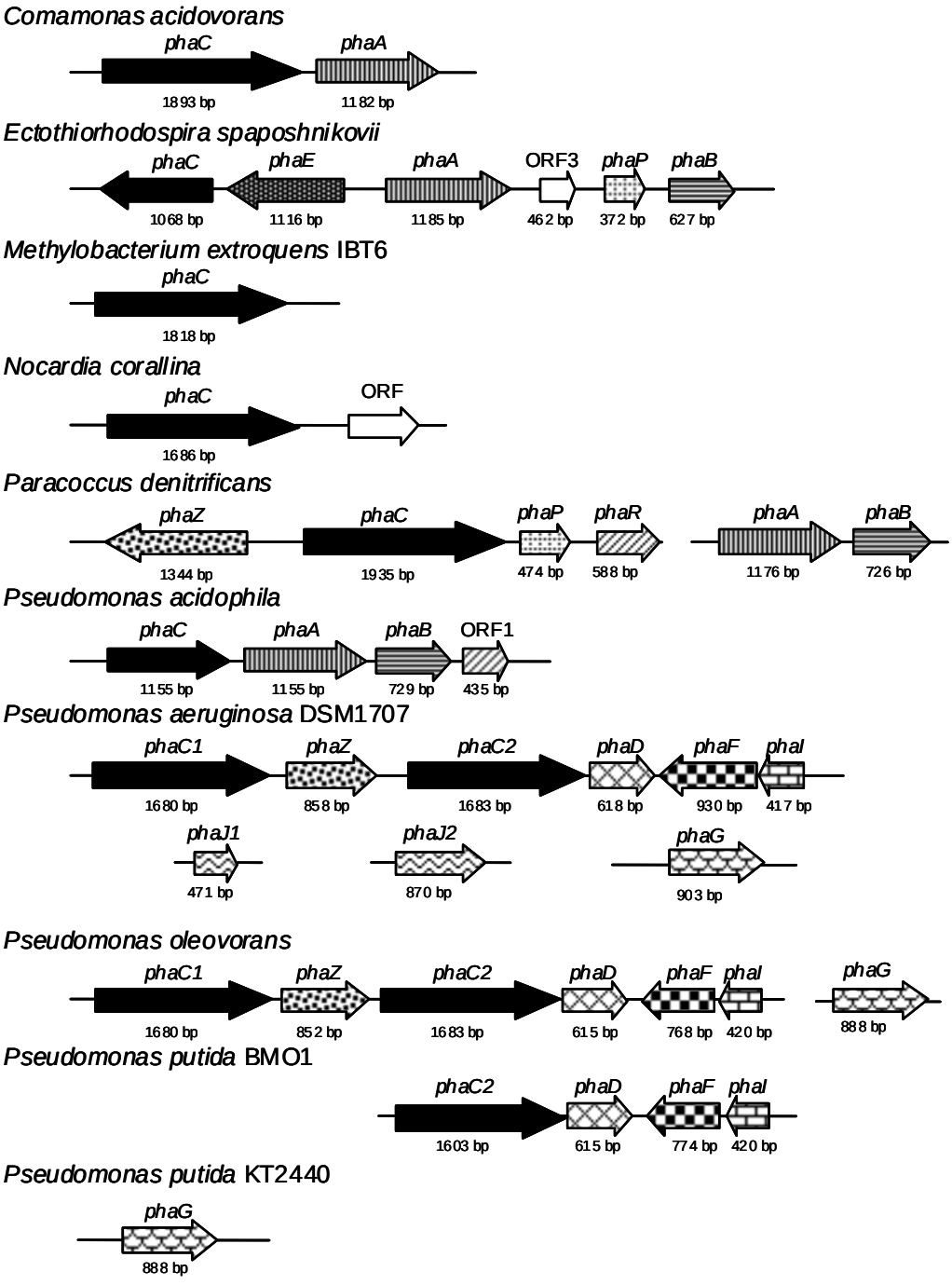


Figure 2.5 (Continued)

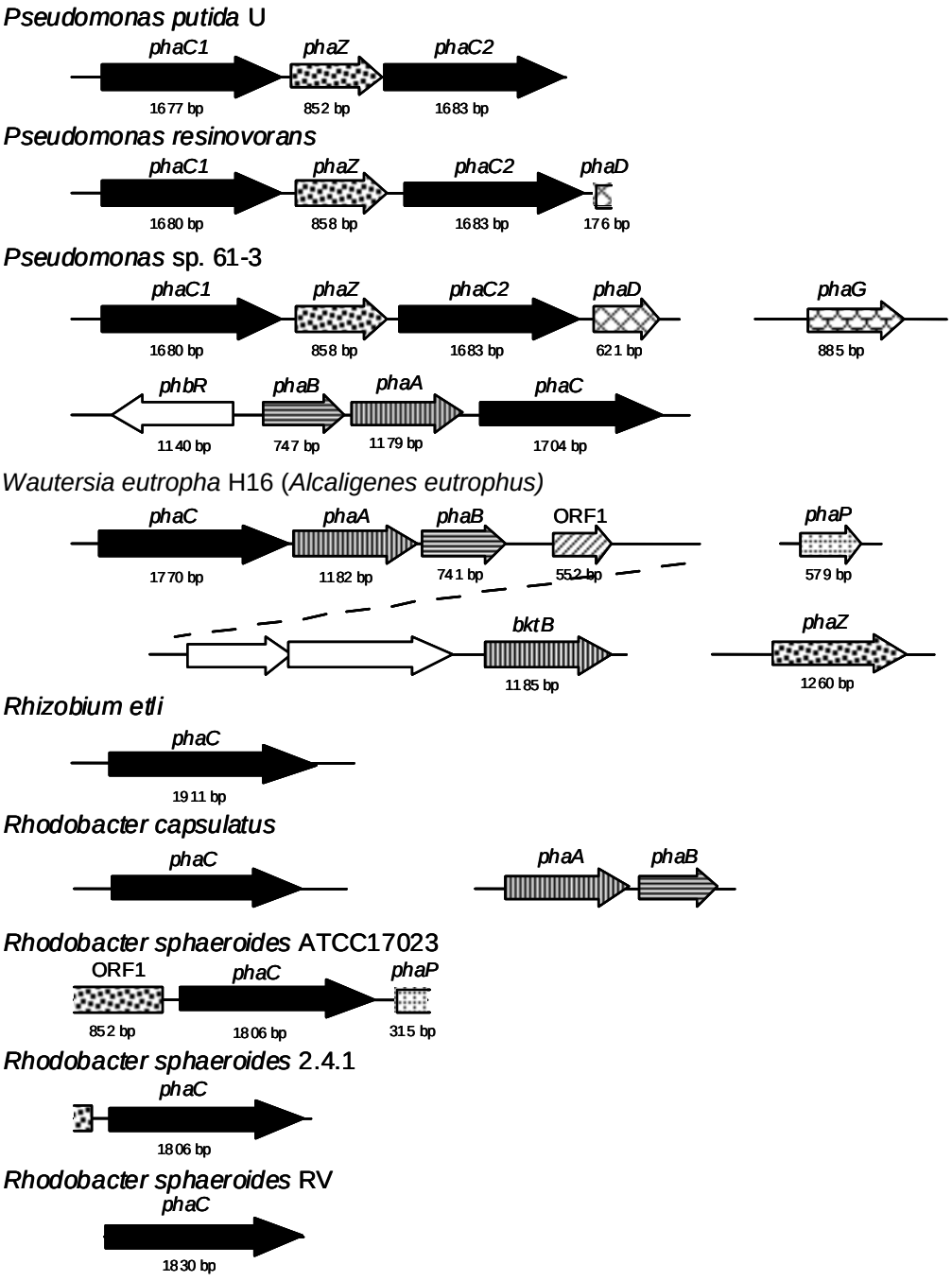


Figure 2.5 (Continued)

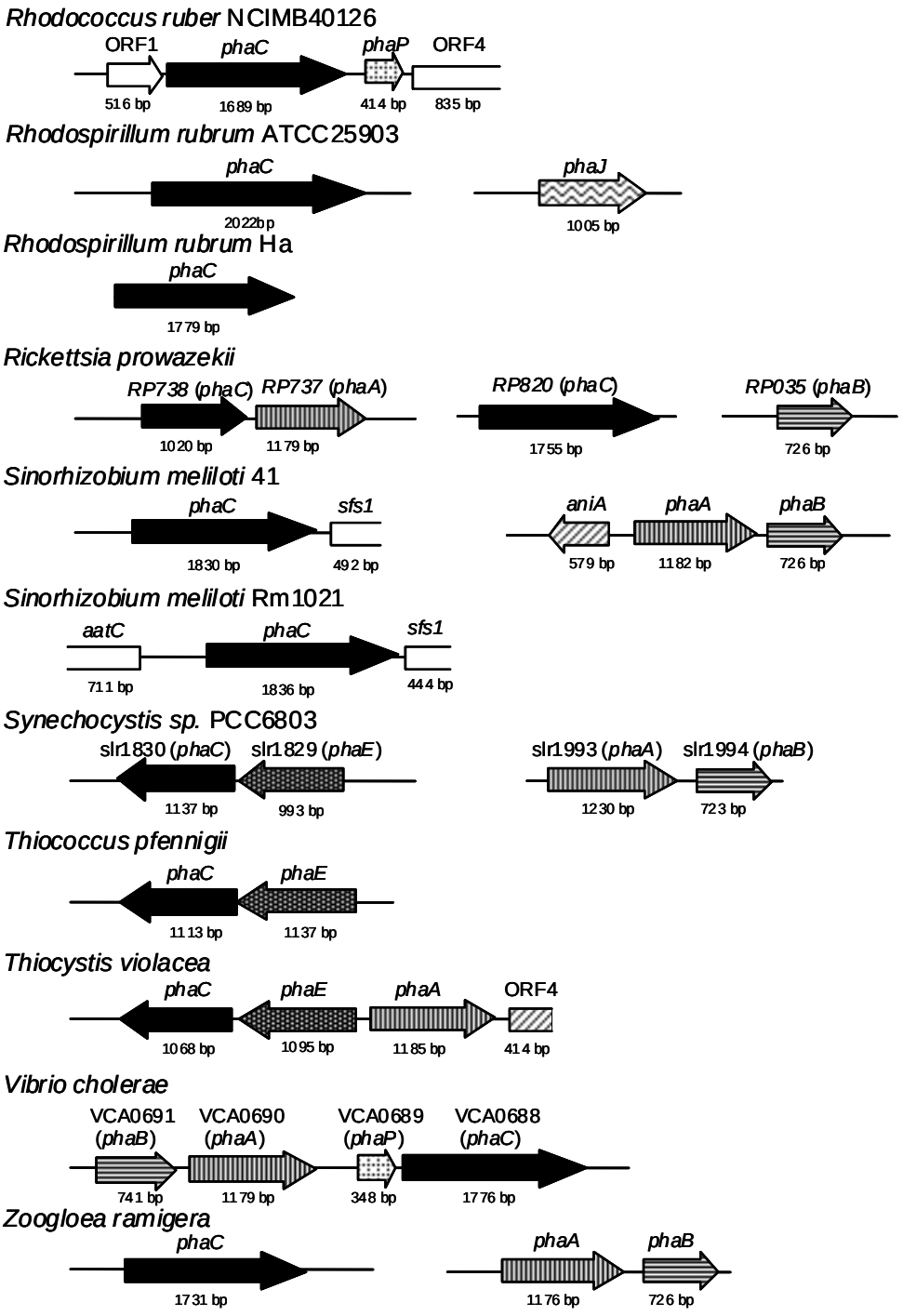


Figure 2.5 (Continued)

2.5 Classes of PHA synthase

Although all PHA synthases catalyze the synthesis of PHAs from (*R*)-3-hydroxyacyl-CoA ((*R*)-3HA-CoA), PHA synthases from different strains exhibit significant differences, such as primary structures (deduced from the nucleotide sequences), subunit compositions and substrate specificities. Based on these aspects, PHA synthases are divided into three major classes (Steinbüchel *et al*., 1992) (Table 2.3).

Table 2.3 Classes of PHA synthases based on their primary structures and substrate specificities

Class of synthase	Gene structure	Preferred substrate	Representative synthase
(I)	*phaC*	scl-HA-CoA	$PhaC_{We}$
(II)	*phaC1* — *phaC2*	mcl-HA-CoA	$PhaC_{Ps}$
(III)	*phaC* — *phaE*	scl-HA-CoA	$PhaC_{Cv}$

Class I PHA synthases, comprise of only one type of subunit (PhaC), with sizes ranging from 61 – 73 kDa. This class of synthases preferentially utilizes SCL-3HA-CoA monomers (C3-C5) to form SCL-PHA polymer. An example is the $PhaC_{We}$ enzyme.

Class II PHA synthases are represented as two PHA synthases comprising of similar type of subunits each (PhaC1 and PhaC2), which are ~ 63 kDa in size. Class II synthases utilize MCL-3HA-CoA monomers (C6-C14) to form MCL-PHA. PHA synthases of most pseudomonads fall into this class of enzymes. An exception is the PHA synthase from *Pseudomonas* sp. 61-3, which can polymerizes both SCL- and MCL-3HA-CoA monomers to form hybrid

SCL-MCL-PHA (Matsusaki *et al*., 1998, 2000).

Class III PHA synthases uniquely possess two different subunits, PhaC (~40 kDa) and PhaE (~40 kDa) (Rehm and Steinbüchel, 1999). The PhaC subunit exhibited 21-28% amino acid sequence similarity to class I and II PHA synthases while the PhaE subunit did not exhibit similarity to any PHA synthases (Rehm and Steinbüchel, 2002). Similar to class I PHA synthases, class III PHA synthases prefer SCL-3HA-CoA monomer (C3-C5). An example is the PHA synthase of *Chromatium vinosum* (formerly known as *Allochromatium vinosum*) (Liebergesell and Steinbüchel, 1992).

2.6 PHA synthase Lipase Box

Alignment of the amino acid sequences of all PHA synthases revealed that the N-terminal regions of the enzymes are highly variable. However, certain stretches in the C-terminal regions showed similarity. One particular stretch of interest found to be conserved in all PHA synthases is the span containing a lipase box (G-X-[S/C]-X-G), in which the essential active site serine (S) of lipase is replaced with a cysteine (C) in PHA synthase (Liebergesell and Steinbuchel, 1992) (Figure 2.6). The significance and the functional implications of the homology between lipase and PHA synthase remained unknown. The possible link between these enzymes was brought up again in a review by Madison and Huisman (1999) in which they suggested that lipase might be a useful model for PHA synthases.

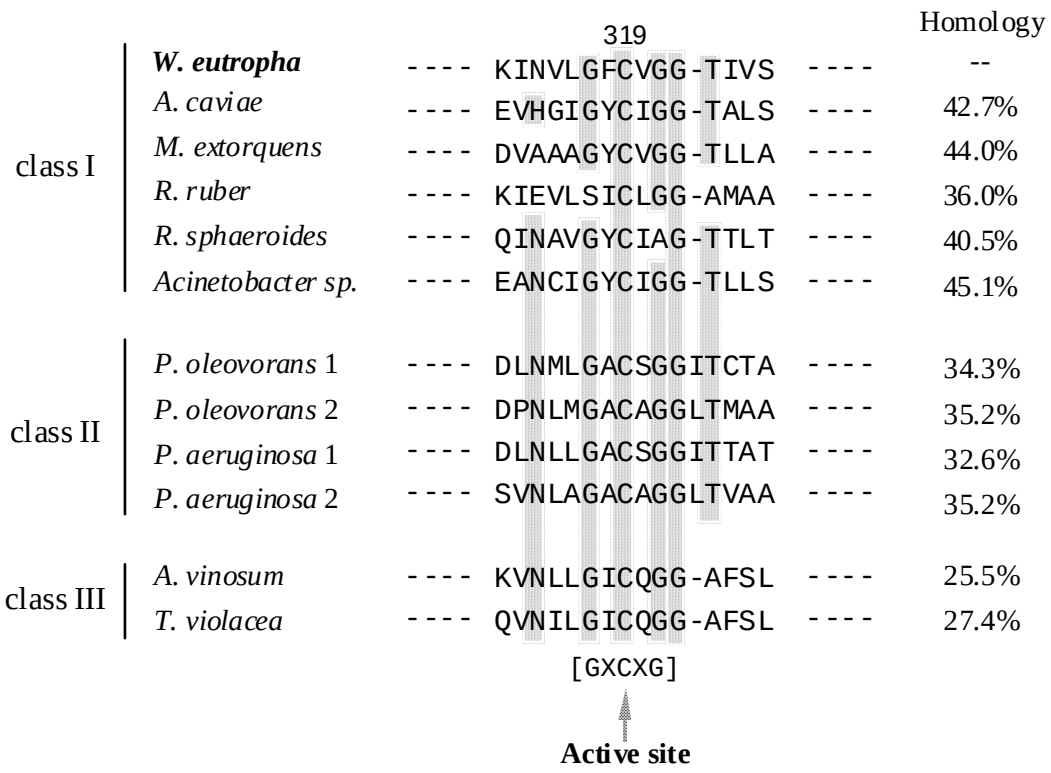


Figure 2.6 Partial alignment of amino acid sequences among various PHA synthases.

A sequence homology search using $PhaC_{Cv}$ protein sequence showed that the C149 residue aligned with the essential Ser in bacterial lipases from *Pseudomonas cepacia*, *Pseudomonas sp. KWI-56* and *Pseudomonas luteola* (Jia *et al*., 2000). Site-directed mutagenesis of *C. vinosum* PhaC ($PhaC_{Cv}$) at C149 (Cys residue at position 149 of $PhaC_{Cv}$) of the lipase box showed that the Cys residue is essential for covalent catalysis (Müh *et al*., 1999). This is similar to the case of C319 residue of $PhaC_{We}$ (Gerngross *et al*., 1994).

Since the crystal structures of *Pseudomonas* lipases have been solved (Schrag *et al*., 1997; Kim *et al*., 1997; Lang *et al*., 1998), threading models of $PhaC_{Cv}$ was performed using the solved structures of lipases in open and close conformations to investigate the possible structure and mechanism of the synthase enzyme (Jia *et al*., 2000). The full-length threading model of $PhaC_{Cv}$ (residues 49-335) revealed that $PhaC_{Cv}$ (and other PHA synthases for that matter) belongs to the protein family possessing an α/β hydrolase fold (α/β hydrolase superfamily) (Jia *et al*., 2000). Interestingly, the α/β hydrolase superfamily also includes prokaryotic lipases (Ollis *et al*., 1992; Schrag and Cygler, 1997).

In many ways, both PHA synthases and lipases share significant similarities. For example, bacterial lipases function as interfacial catalysts by acting at the lipid-water interface of a micellar or emulsified substrate (Desnuelle, 1972). In the case of PHA synthases, as the polymerization process proceeds, the enzymes are attached to the surfaces of insoluble PHA granules. This results in the enzymes carrying out their function at the lipid-water interface,