IMPROVEMENT OF POLYHYDROXYALKANOATE SYNTHASE FROM \textit{Wautersia eutropha} BY IN VITRO EVOLUTION

NORMI MOHD YAHAYA

UNIVERSITI SAINS MALAYSIA

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

by

NORMI MOHD YAHAYA

Thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy

May 2005
ACKNOWLEDGEMENT

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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\textsubscript{We}) was performed to obtain “evolved” PhaC\textsubscript{We} exhibiting improved characteristics. Suppression-type mutagenesis was performed via error-prone PCR to obtain beneficial mutations. The PHA synthase gene (\textit{phaC}\textsubscript{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 \textit{in vivo} level of PhaC\textsubscript{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\textsubscript{We} enzyme with enhanced activity (Taguchi \textit{et al.}, 2002a). Among all the substitutions, the F420S substitution conferred the highest PHB content. Interestingly, the lag phase of PhaC\textsubscript{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\textsubscript{We} enzyme (Taguchi \textit{et al.}, 2002a). The G4D and F420S mutations were then combined. The G4D/F420S mutant had high PHB accumulation and \textit{in vivo} concentration of PhaC\textsubscript{We} enzyme, similar to the G4D mutant. However, the molecular weight of the polymer and the kinetic properties of PhaC\textsubscript{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 \textit{in vivo} level of \( \text{PhaC}_{\text{We}} \) enzyme and the F420S mutation in determining the molecular weight of the polymer and the kinetic properties of \( \text{PhaC}_{\text{We}} \) enzyme of the G4D/F420S mutant. Since all the above studies were done using \textit{Escherichia coli} JM109 as the host, the effect of the mutations using native \textit{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 \textit{in vivo} levels of \( \text{PhaC}_{\text{We}} \) enzyme were elevated in most G4 and G4D/F420S recombinants, indicating that the effect of G4 substitution on \textit{in vivo} levels of \( \text{PhaC}_{\text{We}} \) enzyme is not specific to \textit{E. coli} strains only.
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Wautersia eutropha SECARA EVOLUSI IN VITRO

ABSTRAK

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 intracellular 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<sub>We</sub>). Being the key enzyme, PhaC<sub>We</sub> 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<sub>We</sub> 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<sub>We</sub> 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 (PhaC1Ps) 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* (PhaCAc) 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* (PhaCWe) 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 PhaCWe 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<sub>Fe</sub> 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<sub>Fe</sub> 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<sub>Fe</sub>*)
2. Part II – site-directed mutagenesis at position 420 of Pha<sub>Fe</sub>
3. Part III – combination of beneficial mutations obtained from Part I and II

The effect of such approaches on PhaC<sub>Fe</sub> 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<sub>Fe</sub> enzyme.

The first part (Part I) involved utilizing the *phaC<sub>Fe</sub>* gene of the E11 mutant harboring an S80P mutation (in which Ser was substituted with Pro at position 80 of PhaC<sub>Fe</sub>) 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 \textit{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 \textit{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 \textit{in vitro} activity assay was performed to investigate the effect of the mutation on the kinetic properties of the enzyme in (\textit{R})-3-hydroxybutyryl-CoA ((\textit{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 \textit{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 \textit{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. \textit{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<sub>we</sub> 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. eutrophpha* 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. eutrophpha* 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<sub>we</sub> 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.](image)

A vast consortium of microorganisms has evolved the ability to degrade PHA by using intracellular or extracellular 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 \((R)-3\text{-hydroxyalkanoic acid (}(R)-3\text{HA})\) 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 \((R)-3\text{HA}\) monomer unit.
Depending on chain length of the monomer unit, PHA can be classified into three groups (Table 2.1) (Sudesh et al., 2000).

<table>
<thead>
<tr>
<th>Type of PHA</th>
<th>Number of C atoms</th>
<th>Example</th>
<th>Representative bacterium</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCL</td>
<td>C3-C5</td>
<td>PHB</td>
<td><em>Wautersia eutropha</em></td>
</tr>
<tr>
<td>MCL</td>
<td>C6-C14</td>
<td>PHO</td>
<td><em>Pseudomonas sp.</em></td>
</tr>
<tr>
<td>SCL-MCL</td>
<td>C3-C14</td>
<td>P(HB-co-HO)</td>
<td><em>Pseudomonas sp. 61-3</em></td>
</tr>
</tbody>
</table>

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.

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

* 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 \(T_m\)s 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
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\textsubscript{2}) and water (H\textsubscript{2}O). After their conversion into PHB, followed by the degradation of PHB, the end products are again CO\textsubscript{2} and H\textsubscript{2}O (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 backsheets (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, \( \beta \)-ketothiolase (PhaA<sub>We</sub>), acetoacetyl-CoA reductase (PhaB<sub>We</sub>) and PHA synthase (PhaC<sub>We</sub>) (Steinbüchel, 1996) (see Figure 2.4(A)).

First, PhaA<sub>We</sub> condenses two molecules of acetyl-CoA to form acetoacetyl-CoA, which will then be reduced by PhaB<sub>We</sub> to form (R)-3-hydroxybutyryl-CoA ((R)-3HB-CoA). The generated (R)-3HB-CoA monomer will then be polymerized by PhaC<sub>We</sub> to form PHB homopolymer (Figure 2.4(A)). Under this pathway, both PhaA<sub>We</sub> and PhaB<sub>We</sub> serve as monomer-supplying enzymes while PhaC<sub>We</sub> serve as the key enzyme in PHB biosynthesis.

The three major PHB biosynthesis enzymes, PhaA<sub>We</sub>, PhaB<sub>We</sub> and PhaC<sub>We</sub>, are encoded by the *phaA<sub>We</sub>*, *phaB<sub>We</sub>* and *phaC<sub>We</sub>* genes respectively (Figure 2.4(B)). These genes are arranged in a pattern in which *phaC<sub>We</sub>* is clustered together with *phaA<sub>We</sub>* and *phaB<sub>We</sub>* forming the *phaCAB<sub>We</sub>* 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)).
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).
**Comamonas acidovorans**

\[ \text{phaC} \rightarrow \text{phaA} \]
1183 bp

**Ectothiorhodospira spaposhnikovii**

\[ \text{phaC} \] 1068 bp
\[ \text{phaE} \] 1175 bp
\[ \text{phaA} \] 1156 bp
\[ \text{ORF3} \] 462 bp
\[ \text{phaP} \] 372 bp
\[ \text{phaB} \] 627 bp

**Methylobacterium extroquens IBT6**

\[ \text{phaC} \] 1156 bp

**Nocardia corallina**

\[ \text{phaC} \] 1685 bp

**Paracoccus denitrificans**

\[ \text{phaC} \] 1344 bp
\[ \text{phaZ} \] 1935 bp
\[ \text{phaC} \] 474 bp
\[ \text{phaP} \] 588 bp
\[ \text{phaR} \] 1176 bp
\[ \text{phaB} \] 726 bp

**Pseudomonas acidophila**

\[ \text{phaC} \] 1156 bp
\[ \text{phaA} \] 1156 bp
\[ \text{phaP} \] 435 bp

**Pseudomonas aeruginosa DSM1707**

\[ \text{phaC1} \] 1680 bp
\[ \text{phaZ} \] 888 bp
\[ \text{phaC2} \] 1683 bp
\[ \text{phaD} \] 615 bp
\[ \text{phaF} \] 930 bp
\[ \text{phaI} \] 417 bp

**Pseudomonas oleovorans**

\[ \text{phaC1} \] 1680 bp
\[ \text{phaZ} \] 852 bp
\[ \text{phaC2} \] 1683 bp
\[ \text{phaD} \] 615 bp
\[ \text{phaF} \] 768 bp
\[ \text{phaI} \] 420 bp

**Pseudomonas putida BMO1**

\[ \text{phaC2} \] 1603 bp
\[ \text{phaD} \] 615 bp
\[ \text{phaI} \] 774 bp

**Pseudomonas putida KT2440**

\[ \text{phaG} \] 858 bp

---

**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

<table>
<thead>
<tr>
<th>Class of synthase</th>
<th>Gene structure</th>
<th>Preferred substrate</th>
<th>Representative synthase</th>
</tr>
</thead>
</table>
| (I)               | ![phaC]
|                   | scl-HA-CoA     | PhaC<sub>We</sub>   |
| (II)              | ![phaC1-phaC2]
|                   | mcl-HA-CoA     | PhaC<sub>Ps</sub>    |
| (III)             | ![phaC-phaE]
|                   | scl-HA-CoA     | PhaC<sub>Cv</sub>    |

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<sub>We</sub> 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) (Liebergessell 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 (Liebergessell and Steinbuechel, 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<sub>CV</sub> 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<sub>CV</sub>) at C149 (Cys residue at position 149 of PhaC<sub>CV</sub>) 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<sub>WE</sub> (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<sub>CV</sub> 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<sub>CV</sub> (residues 49-335) revealed that PhaC<sub>CV</sub> (and other PHA synthases for that matter) belongs to the protein family possessing an \(\alpha/\beta\) hydrolase fold (\(\alpha/\beta\) hydrolase superfamily) (Jia *et al.*, 2000). Interestingly, the \(\alpha/\beta\) 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,