

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

NORMI MOHD YAHAYA

UNIVERSITI SAINS MALAYSIA

2005

**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

Jan 2002 marked the first official collaboration between the Institute of Physical and Chemical Research of Japan (RIKEN) with Universiti Sains Malaysia (USM). The luck and opportunity given to pursue my PhD studies in RIKEN under the institute's Joint Graduate School Program took me a step further down the road of science.

Having spent 3 years the Polymer Chemistry Lab in RIKEN, I am very much indebted to my supervisor, Prof. Yoshiharu Doi, for all his advice, supervision, the wonderful scientific experience and environment he and his lab provided me. My deep gratitude also goes to my mentor, Dr. Tomohiro Hiraishi, who had coached and trained me well with dedication and discipline. Thank you to Prof. Seiichi Taguchi, for his suggestions, advice and time. Thank you also to Dr. Kazunori Taguchi, Dr. Ken'ichiro Matsumoto, Dr. Kazuma Takase, Dr. Gan Zhihua, Dr. Christopher T. Nomura, as well as Assoc. Prof. Takeharu Tsuge and Dr. Prihardi Kahar of Tokyo Institute of Technology for their scientific discussions whenever time permitted. A special thanks to Dr. Hideki Abe for his kind supervision, help and discussions regarding polymer chemistry studies.

To the students, Miss Yoko Aoki, Miss Tomoyo Tanaka, Miss Madoka Nagata, Miss Yoko Hirahara and Mr. Takaaki Murata, thanks for the enjoyable moments. Many thanks to Assoc. Prof. Makiko Enoki, Madam Chieko Ogawa and Madam Noriko Tanaka for the many interesting moments and for making my stay very pleasant and enjoyable.

To the USM counterpart, I would like to specially thank my main supervisor Assoc. Prof. Dr. Nazalan Najimudin and my co-supervisor Dr. Sudesh Kumar, for their time, attention and scientific views whenever I needed them.

Lastly, my heartfelt thanks go to my family for their constant support and love.

TABLE OF CONTENTS

ACKNOWLEDGEMENT	i
TABLE OF CONTENTS	iii
LIST OF TABLES	xi
LIST OF FIGURES	xiii
LIST OF ABBREVIATIONS	xix
ABSTRACT	xx
ABSTRAK	xxii
1.0 INTRODUCTION	1
2.0 LITERATURE SURVEY	
2.1 Introduction to PHA	6
2.1.1 PHA as Storage Material	6
2.1.2 Types of PHA	7
2.2 PHB polymer	9
2.2.1 Physical and Thermal Properties of PHB	10
2.2.2 Biological Properties of PHB	12
2.3 Applications of PHB	13
2.4 The PHB Biosynthesis Pathway and Genes in	
<i>Wautersia eutropha</i> – The Model PHB Producer	15
2.5 Classes of PHA synthase	21

2.6	PHA synthase Lipase Box	22
2.7	PHA synthase Catalytic Triad and Dimerization	25
2.8	Mechanism of PHA synthase Polymerization Reaction	26
2.9	Formation and Structure of PHB Granules in Cells	28
2.10	Outline of Present Study	32
3.0	MATERIALS AND METHODS	36
Part I		
3.1	Suppression-type Mutagenesis of <i>phaC_{We}</i> from E11 Mutant by Error-prone PCR	36
3.1.1	Brief Introduction	36
3.1.2	Bacterial Strains	37
3.1.3	Intragenic Suppression-type Mutagenesis by Error-prone PCR	37
3.1.4	Screening of Mutants Leading to Enhanced PHB Accumulation by an <i>In Vivo</i> Assay System	40
3.1.5	PHB Biosynthesis and Determination of PHB Accumulation	42
3.1.6	Determination and Isolation of Secondary Mutations from Selected Suppressor Mutant Generated by Suppression-type Mutagenesis	43
3.1.7	Western Blot Analysis of PhaC _{We}	49

3.1.8	Saturation Mutagenesis of PhaC _{We} at the G4 Position	50
3.1.9	Construction of the pGEM'- <i>phbCAB</i> _{We} G4X Plasmid for Expression in <i>E. coli</i> JM109	52
3.1.10	PHB Polymer Purification	52
3.1.11	Molecular Weight Determination of PHB Homopolymer by Multiangle Laser Light Scattering (MALLS) and Gel Permeation Chromatography (GPC)	54
3.1.12	Thermostability Studies of PhaC _{We}	56
3.1.13	Construction of (His) ₆ -tagged PhaC _{We}	59
3.1.14	Expression of (His) ₆ -tagged PhaC _{We} .	61
3.1.15	Purification of (His) ₆ -tagged PhaC _{We} .	63
3.1.16	<i>In Vitro</i> Enzymatic Assay of Purified PhaC _{We}	64

Part II

3.2	Site-directed Saturation Mutagenesis of PhaC _{We} at the F420 Position	65
3.2.1	Brief Introduction	65
3.2.2	Site-directed Saturation Mutagenesis of PhaC _{We} at the F420 Position	65
3.2.3	Construction of the pGEM'- <i>phaCAB</i> _{We} F420X Plasmid for Expression in <i>E. coli</i> JM109	67

3.2.4	Determination of PHB Accumulation	69
3.2.5	Western Blot Analysis of PhaC _{We}	69
3.2.6	Construction and Purification of (His) ₆ -tagged Mutant PhaC _{We}	69
3.2.7	In Vitro Enzymatic Assay of Purified PhaC _{We}	69

Part III

3.3	Combination of Beneficial Mutations	70
3.3.1	Brief Introduction	70
3.3.2	Construction of the G4X/F420X double mutant	70
3.3.3	Determination of PHB Accumulation	72
3.3.4	Western Blot Analysis of PhaC _{We}	72
3.3.5	Molecular Weight Determination of PHB Homopolymer by Multiangle Laser Light Scattering (MALLS) and Gel Permeation Chromatography (GPC)	72
3.3.6	Thermostability Studies of PhaC _{We}	72
3.3.7	Construction and Purification of (His) ₆ -tagged Mutant PhaC _{We}	73
3.3.8	In Vitro Enzymatic Assay of Purified PhaC _{We}	73

Part IV

3.4 Introduction of Selected Mutations into Native *W. eutropha*

PHB ⁻ 4 Host	73
3.4.1 Brief Introduction	73
3.4.2 Bacterial Strains and Plasmid	74
3.4.3 Construction of the pBBR1CAB _{We} Plasmid for Transconjugational Transfer of Mutations	75
3.4.4 Transconjugational Transfer of Mutations into <i>W. eutropha</i> PHB ⁻ 4	75
3.4.5 PHB and PHA Biosynthesis by <i>W. eutropha</i> PHB ⁻ 4 Transconjugants	78
3.4.6 Determination of PHB and PHA Accumulation and PHA Monomer Composition	78
3.4.7 Western Blot Analysis of PhaC _{We}	80

4.0 RESULTS AND DISCUSSION 81

Part I

4.1 Suppression-type Mutagenesis of *phaC_{We}* from E11

Mutant by Error-prone PCR	81
4.1.1 Isolation of E11-4 Suppressor Mutant Exhibiting Enhanced PHB Accumulation	81
4.1.2 Isolation of Secondary Mutations from the E11-4 Suppressor Mutant – in Search of Beneficial	

	Mutation(s) Leading to Enhanced PHB	
	Accumulation	84
4.1.3	Effect of the G4D Single Mutation on the <i>In Vivo</i>	
	Level of PhaC _{We} Enzyme	85
4.1.4	Was the Effect of G4D Mutation on the <i>In Vivo</i>	
	Level of PhaC _{We} Strain-Dependent?	85
4.1.5	Saturation Mutagenesis of PhaC _{We} at the G4	
	Position – Effect on PHB Content and <i>In Vivo</i>	
	Levels of PhaC _{We}	87
4.1.6	Hypothesis on the Elevation of the <i>In Vivo</i> Level	
	of PhaC _{We} Enzyme Caused by the G4D Mutation	90
4.1.7	Molecular Weight Determination of the PHB	
	Homopolymer Synthesized by G4 Mutants	96
4.1.8	Purification and Kinetic Studies of Wild-type and	
	G4D Mutant PhaC _{We}	99
4.1.9	Conclusion for Part I	102

Part II

4.2	Site-directed Saturation Mutagenesis of PhaC _{We} at the	
	F420 Position	103
4.2.1	PHB content of F420 mutants	103
4.2.2	<i>In Vivo</i> Levels of PhaC _{We} Enzyme of F420 Mutants	104
4.2.3	Enzymatic Reaction of the F420S Mutant Enzyme	106

4.2.4	Conclusion for Part II	109
-------	------------------------	-----

Part III

4.3	Combination of Beneficial Mutations	110
4.3.1	Generation and PHB Content of the G4D/F420S Double Mutant	110
4.3.2	The <i>In Vivo</i> Level of PhaC _{We} Enzyme of the G4D/F420S Double Mutant	112
4.3.3	Hypothesis on the Elevation in the <i>In Vivo</i> Level of PhaC _{We} Enzyme of the G4D/F420S Mutant	112
4.3.4	PHB Molecular Weight Analysis	116
4.3.5	Enzymatic Properties of Wild-type and G4D/F420S Mutant PhaC _{We}	119
4.3.6	Conclusion for Part III	122

Part IV

4.4	Introduction of Selected Mutations into Native <i>W. eutropha</i> PHB ⁻ 4 Host	123
4.4.1	PHB and PHA Contents and Monomer Compositions of <i>W. eutropha</i> PHB ⁻ 4 Transconjugants	123
4.4.2	<i>In Vivo</i> Levels of PhaC _{Re} in <i>W. eutropha</i> PHB ⁻ 4 Transconjugants	127

4.4.3	Conclusion for Part IV	129
5.0	SUMMARY AND FUTURE STUDIES	131
	REFERENCES	136
	VITA	150
	PUBLICATIONS	151

LIST OF TABLES

		Page
2.0 LITERATURE SURVEY		
Table 2.1	Types of PHA.	8
Table 2.2	Comparison of PHB homo- and co-polymers with common plastics in properties.	10
Table 2.3	Classes of PHA synthases based on their primary structures and substrate specificities.	21
3.0 MATERIALS AND METHODS		
Table 3.1	Components of a typical reaction mixture for error-prone PCR.	40
Table 3.2	Components of reaction mixture for <i>MunI</i> restriction site introduction.	47
Table 3.3	Oligonucleotide primers used for site-directed saturation mutagenesis at G4 position.	50
Table 3.4	Reaction mixture for site-directed saturation mutagenesis at G4 position.	51
Table 3.5	Reaction mixture for PHA synthase activity assay.	57
Table 3.6	Components of the reaction mixture for <i>NdeI</i> and <i>BamHI</i> restriction sites introduction.	60

Table 3.7	Oligonucleotide primers used for site-directed saturation mutagenesis at F420 position.	66
-----------	---	----

4.0 RESULTS AND DISCUSSION

Table 4.1	Molecular weight of PHB homopolymers produced in <i>E. coli</i> JM109 recombinants harboring wild-type and various G4 mutant PhaC _{We} from glucose.	97
Table 4.2	Kinetic parameters of wild-type and G4D mutant PhaC _{We} enzymes.	101
Table 4.3	Kinetic parameters of wild-type and F420S mutant PhaC _{We} enzymes.	106
Table 4.4	Molecular weights of PHB homopolymers produced by recombinant <i>E. coli</i> JM109 harboring wild-type and mutants PhaC _{We} .	116
Table 4.5	Kinetic properties of wild-type and mutant PhaC _{We} enzymes.	119

LIST OF FIGURES

	Page
2.0 LITERATURE SURVEY	
Figure 2.1	PHA granules in bacterial cells. 6
Figure 2.2	Chemical structure of PHA. 8
Figure 2.3	The renewability of PHB. 14
Figure 2.4(A)	PHB biosynthesis pathway in <i>W. eutropha</i> . 16
Figure 2.4(B)	Molecular organizations of genes relevant for PHB biosynthesis in <i>W. eutropha</i> . 16
Figure 2.5	Organization of genes involved in PHA synthesis. 17
Figure 2.6	Partial alignment of amino acid sequences among various PHA synthases. 23
Figure 2.7	The proposed lipase-based catalytic mechanism of the <i>C. vinosum</i> class III PHA synthase. 27
Figure 2.8	An illustration of the model explaining PHB granule accumulation and formation in <i>W. eutropha</i> . 29
Figure 2.9	Model for the structure of PHA granule surface. 31

3.0 MATERIALS AND METHODS

Figure 3.1	The pGEM- <i>phaCAB</i> _{We} plasmid of E11 mutant harboring an S80P mutation in its <i>phaC</i> _{We} gene, which is used as a template for suppression-type mutagenesis.	38
Figure 3.2	Strategy for <i>in vitro</i> evolution of PhaC _{We} by error-prone PCR and analysis of mutational effects of PhaC _{We} on PHB accumulation via an <i>in vivo</i> assay system.	39
Figure 3.3	Conversion of PHB to crotonic acid by treatment with hot concentrated sulfuric acid (H ₂ SO ₄).	42
Figure 3.4	Evolutionary lineage of PhaC _{We} through <i>in vitro</i> evolution.	45
Figure 3.5	A simplified figure depicting a three-step procedure of <i>MunI</i> restriction site introduction into the <i>phaC</i> _{We} gene of E11-4 revertant.	46
Figure 3.6	Structure of the pGEM'- <i>phaCAB</i> _{We} plasmid harboring only the wild-type <i>phaC</i> _{We} gene.	50
Figure 3.7	Construction of pGEM'- <i>phaCAB</i> _{We} G4X expression plasmid harboring respective single G4X mutation.	53
Figure 3.8	Principle of the discontinuous assay of PhaC _{We} activity using DTNB.	57

Figure 3.9	pET-15b vector equipped with inducible T7 promoter and (His) ₆ -tag sequence for PhaC _{We} production and purification.	59
Figure 3.10	Construction of pET-15b:: <i>phaC</i> _{We} plasmid for PhaC _{We} purification.	62
Figure 3.11	Structure of the pKAS4-SseI plasmid harboring the wild-type <i>phaC</i> _{We} gene and an attached linker containing the Sse8387I restriction site.	66
Figure 3.12	Construction of the pGEM'- <i>phaCAB</i> _{We} F420X expression plasmid harboring respective single F420X mutation.	68
Figure 3.13	Construction of the pGEM'- <i>phaCAB</i> _{We} G4X/F420X expression plasmid harboring the G4X/F420X double mutation.	71
Figure 3.14	Broad-host-range pBBR1MCS-2 plasmid vector used for transconjugational transfer of mutations into <i>W. eutropha</i> PHB ⁻ 4.	74
Figure 3.15	Construction of the pBBR1CAB _{We} plasmid to transfer mutations into <i>W. eutropha</i> PHB ⁻ 4 via conjugation.	76
Figure 3.16	Conversion of PHA into methyl-esters by methanolysis.	79

4.0 RESULTS AND DISCUSSION

Figure 4.1	Isolation of a promising E11-4 suppressor mutant.	82
------------	---	----

Figure 4.2(A)	PHB accumulated by <i>E. coli</i> JM109 recombinants harboring wild-type and mutant PhaC _{We} enzymes.	83
Figure 4.2(B)	Western blot analysis of wild-type and mutant PhaC _{We} produced in <i>E. coli</i> JM109.	83
Figure 4.2(C)	Amino acid substitutions in PhaC _{We} of E11-4 suppressor mutant generated from error-prone PCR.	83
Figure 4.3	Comparison of the levels of wild-type and G4D mutant PhaC _{We} in <i>E. coli</i> (A) JM109 (B) DH5 α (C) HB101 by Western blot analysis.	86
Figure 4.4(A)	Comparison of dry cell weight of <i>E. coli</i> JM109 recombinants harboring wild-type and individual G4 mutant PhaC _{We} .	88
Figure 4.4(B)	Comparison of PHB content of <i>E. coli</i> JM109 recombinants harboring wild-type and individual G4 mutant PhaC _{We} .	88
Figure 4.4(C)	Comparison of PHB yield of <i>E. coli</i> JM109 recombinants harboring wild-type and individual G4 mutant PhaC _{We} .	88
Figure 4.4(D)	Comparison of residual biomass of <i>E. coli</i> JM109 recombinants harboring wild-type and individual G4 mutant PhaC _{We} .	88
Figure 4.4(E)	Western blot analysis of wild-type and G4 mutant PhaC _{We} produced in <i>E. coli</i> JM109 recombinants.	88

Figure 4.5	Possible effect(s) of G4 substitution on different stages of PhaC _{We} protein production to give high <i>in vivo</i> concentration of the enzyme in bacterial cells.	91
Figure 4.6	Thermostability of G4D PhaC _{We} compared to that of the wild-type.	94
Figure 4.7(A)	SDS-PAGE analysis of purified (His) ₆ -tagged wild-type (WT) and G4D mutant PhaC _{We} .	100
Figure 4.7(B)	Activity assay of purified wild-type and G4D PhaC _{We} .	100
Figure 4.8(A)	PHB accumulated by recombinant <i>E. coli</i> JM109 harboring wild-type and various F420 mutant PhaC _{We} enzymes.	105
Figure 4.8(B)	Western blot analysis of wild-type and F420 mutant PhaC _{We} produced in <i>E. coli</i> JM109.	105
Figure 4.9(A)	SDS-PAGE analysis of purified (His) ₆ -tagged wild-type and F420S PhaC _{We} .	107
Figure 4.9(B)	Profile of <i>in vitro</i> enzymatic reaction catalyzed by purified wild-type and F420S mutant PhaC _{We} at 25°C.	107
Figure 4.10	The adjacent location of F420 residue to that of the W425 residue in PhaC _{We} .	109
Figure 4.11(A)	Dry cell weight of recombinant <i>E. coli</i> JM109 harboring wild-type and various mutant PhaC _{We} .	111
Figure 4.11(B)	PHB content of recombinant <i>E. coli</i> JM109 harboring wild-type and various mutant PhaC _{We} .	111

Figure 4.11(C)	PHB yield of recombinant <i>E. coli</i> JM109 harboring wild-type and various mutant PhaC _{We} .	111
Figure 4.11(D)	Residual biomass of recombinant <i>E. coli</i> JM109 harboring wild-type and various mutant PhaC _{We} .	111
Figure 4.11(E)	Western blot analysis of wild-type and mutant PhaC _{We} produced in recombinant <i>E. coli</i> JM109.	111
Figure 4.12	Thermostability of G4D/F420S PhaC _{We} compared to those of wild-type, G4D and F420S enzymes.	115
Figure 4.13(A)	SDS-PAGE analysis of purified (His) ₆ -tagged wild-type and G4D/F420S PhaC _{We} .	120
Figure 4.13(B)	Profile of <i>in vitro</i> enzymatic reaction catalyzed by purified wild-type and G4D/F420S PhaC _{We} at 25°C.	120
Figure 4.14(A)	PHB content of <i>W. eutropha</i> PHB ⁻ 4 transconjugants.	125
Figure 4.14(B)	PHA content of <i>W. eutropha</i> PHB ⁻ 4 transconjugants.	125
Figure 4.14(C)	3HB mole fraction of PHA polymer isolated from <i>W. eutropha</i> PHB ⁻ 4 transconjugants.	125
Figure 4.14(D)	3HHx mole fraction of PHA polymer isolated from <i>W. eutropha</i> PHB ⁻ 4 transconjugants.	125
Figure 4.14(E)	Western blot analysis of PhaC _{We} produced in <i>W. eutropha</i> PHB ⁻ 4 transconjugants.	125

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
(<i>R</i>)-3HB-CoA	(<i>R</i>)-3-hydroxybutyryl-CoA
T_{m}	melting temperature
T_{g}	glass-transition temperature
v/v	volume/volume
w/v	weight/volume
w/w	weight/weight

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_{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 (PhaC_{1Ps}) 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.

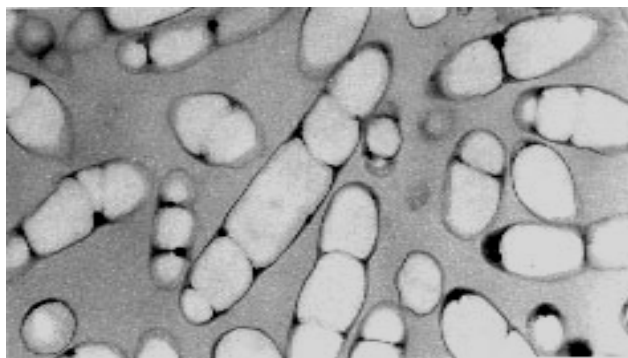


Figure 2.1 PHA granules in bacterial cells.

A vast consortium of microorganisms has evolved the ability to degrade PHA by using intracellular or 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-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.

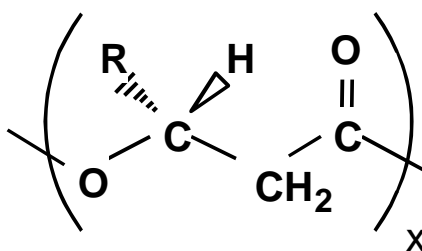


Figure 2.2 Chemical structure of PHA. R refers to side group while x refers to the number of repeating units.

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	<i>Wautersia eutropha</i>
MCL	C6-C14	PHO	<i>Pseudomonas</i> sp.
SCL-MCL	C3-C14	P(HB-co-HO)	<i>Pseudomonas</i> 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_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 medical 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 (CO₂) and water (H₂O). After their conversion into PHB, followed by the degradation of PHB, the end products are again CO₂ and H₂O (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

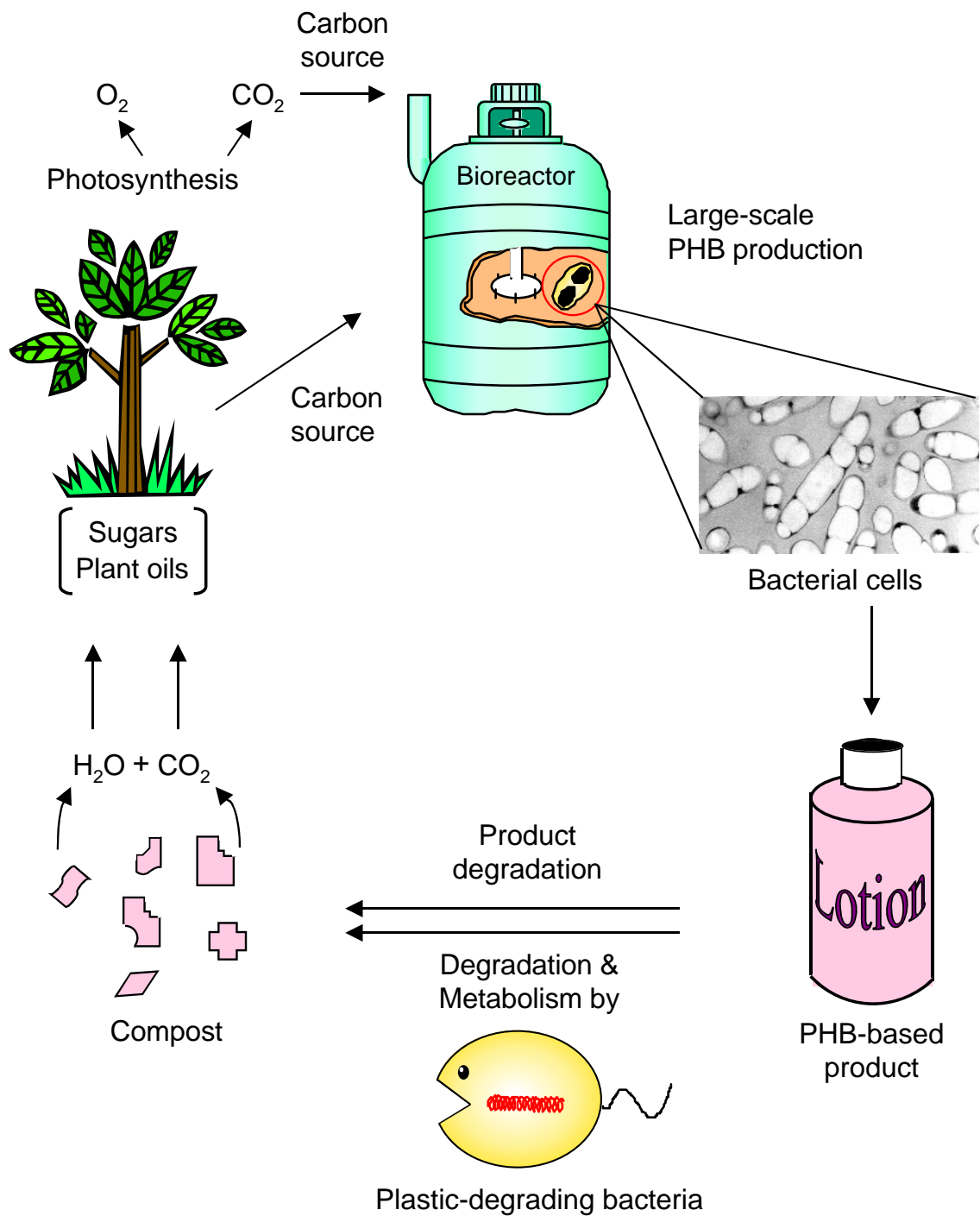


Figure 2.3 The renewability of PHB.

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

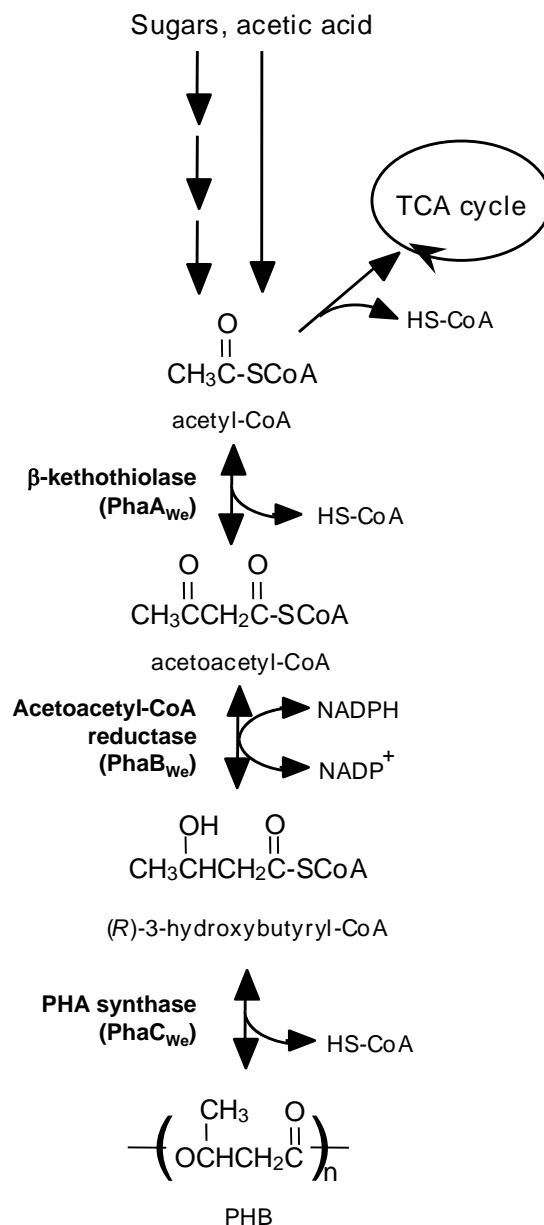
2.4 The PHB Biosynthesis Pathway and Genes in *Wautersia eutropha* – The Model PHB Producer

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)

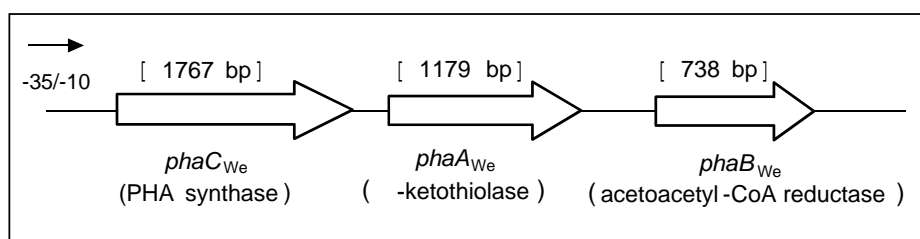


Figure 2.4 (A) PHB biosynthesis pathway and (B) Molecular organizations of genes relevant for PHB biosynthesis in *W. eutropha*.

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)

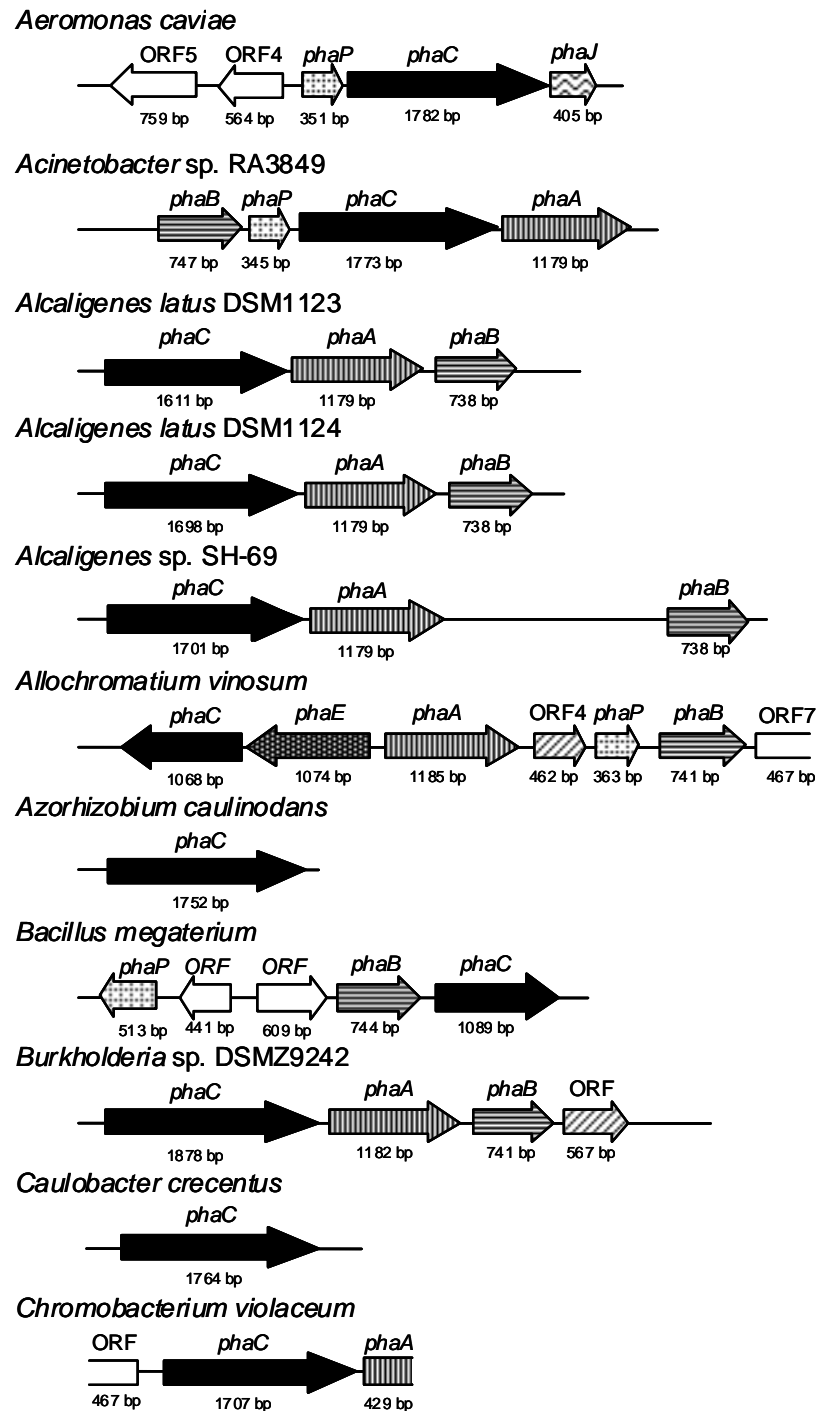


Figure 2.5 Organization of genes involved in PHA synthesis. The size of genes includes termination codon (Figure source: Taguchi *et al.*, 2002b).

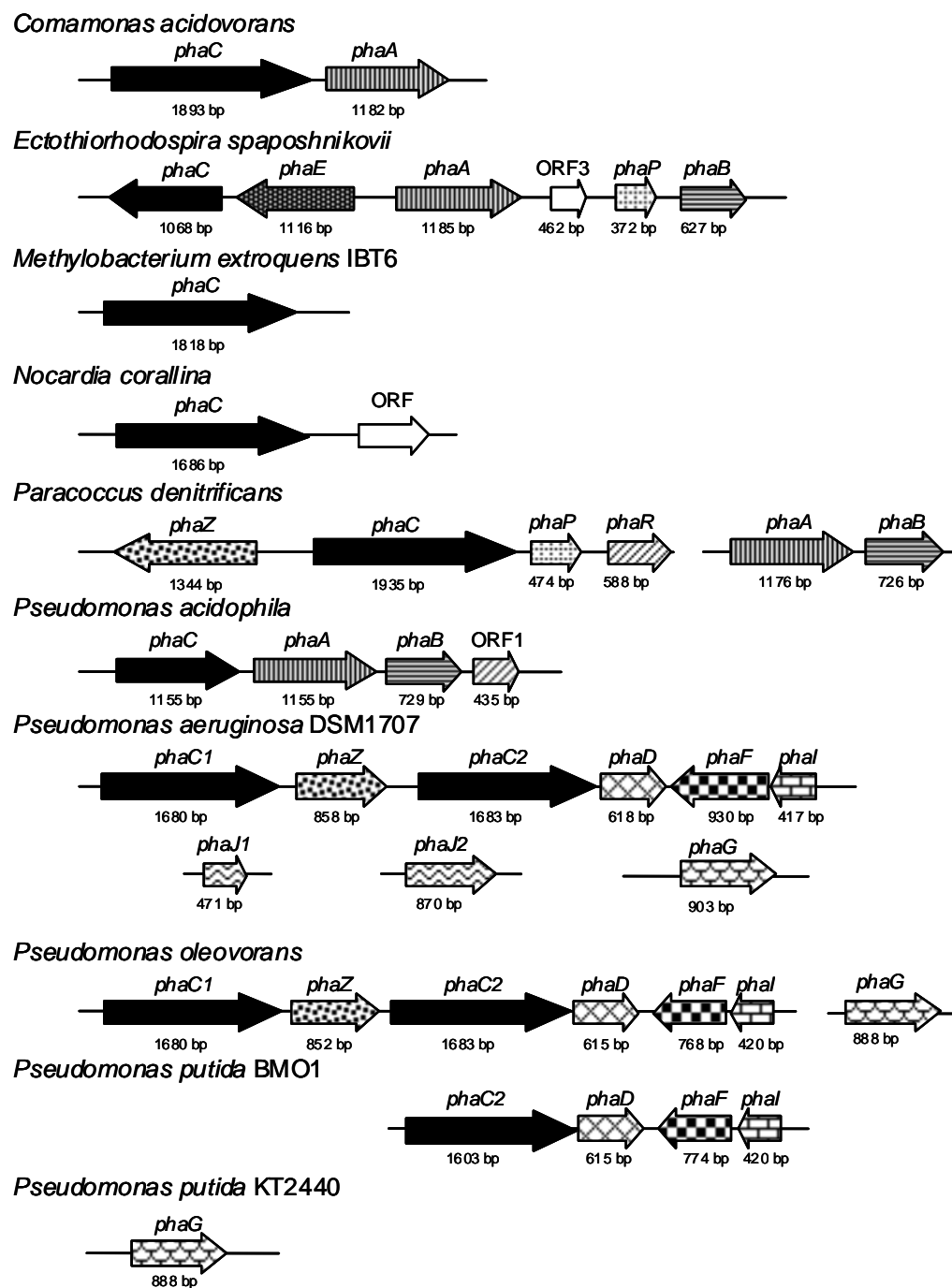


Figure 2.5 (Continued)

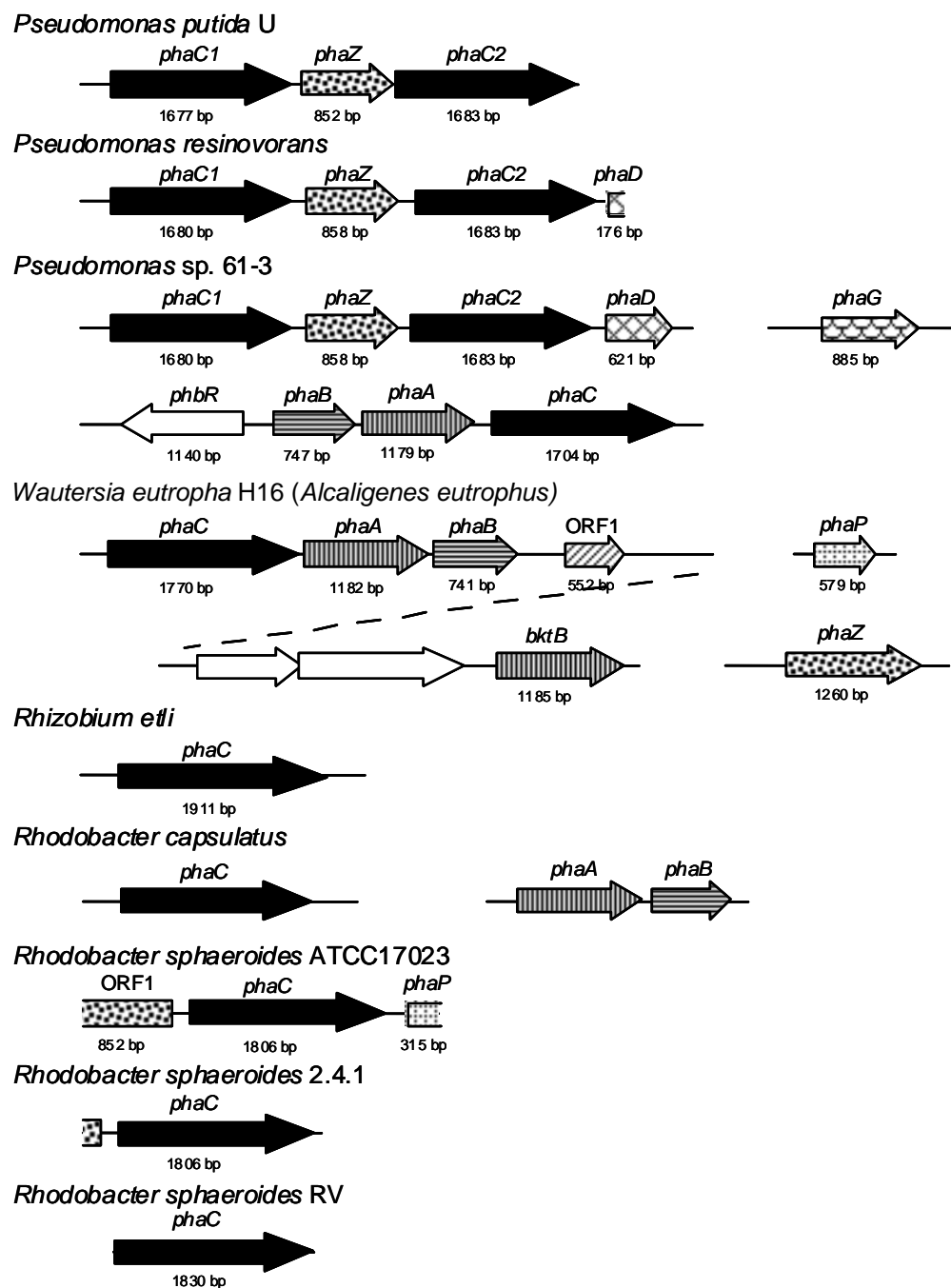


Figure 2.5 (Continued)

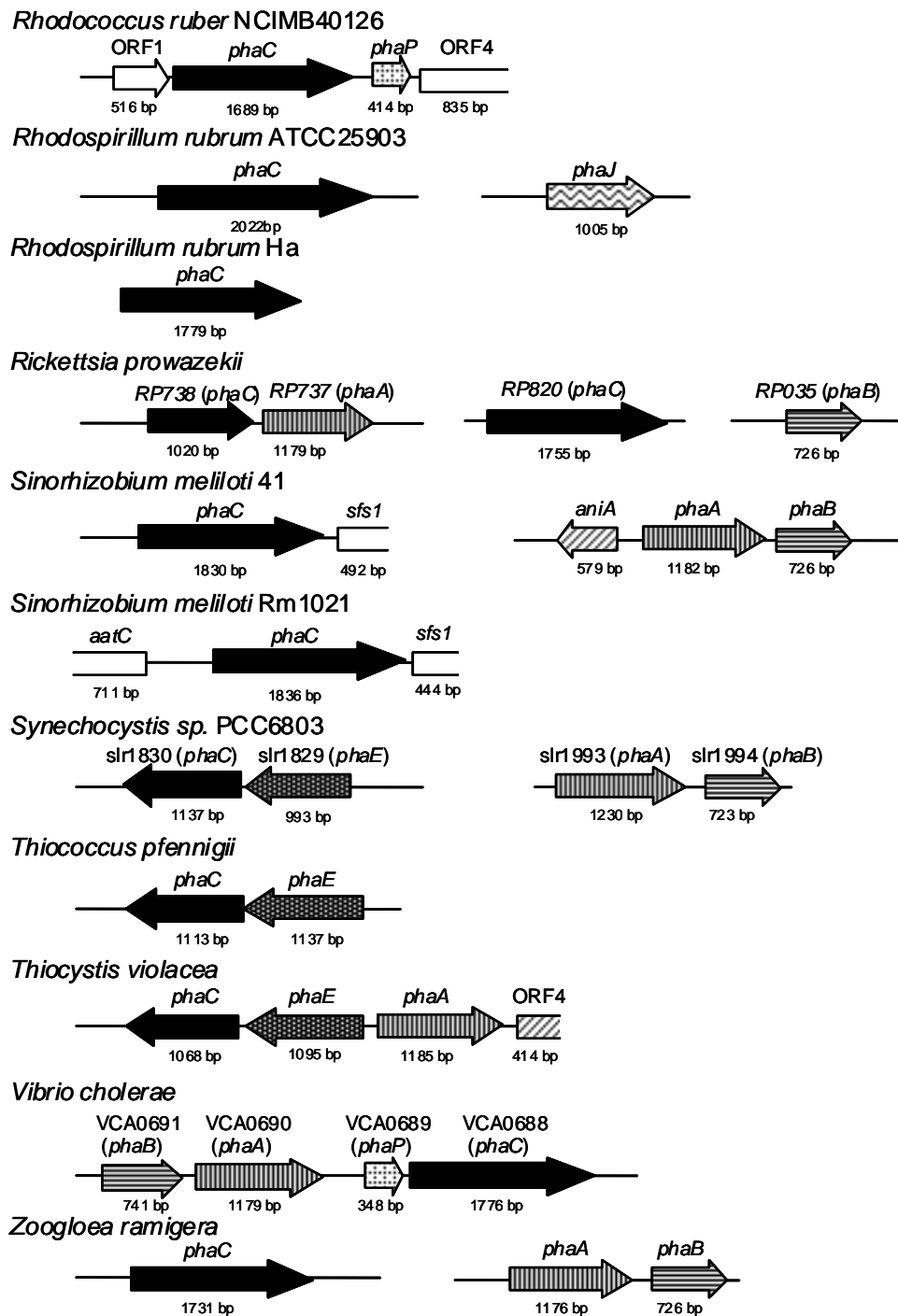
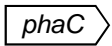
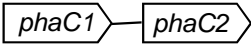



Figure 2.5 (Continued)

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)		scl-HA-CoA	PhaC _{We}
(II)		mcl-HA-CoA	PhaC _{Ps}
(III)		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 Steinbüchel, 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.

			319		Homology
class I	<i>W. eutropha</i>	----	KINVLGFCVGG-TIVS	----	--
	<i>A. caviae</i>	----	EVHGIGYCIGG-TALS	----	42.7%
	<i>M. extorquens</i>	----	DVAAAGYCVGG-TLLA	----	44.0%
	<i>R. ruber</i>	----	KIEVLSICLGG-AMAA	----	36.0%
	<i>R. sphaeroides</i>	----	QINAVGYCIAG-TTLT	----	40.5%
	<i>Acinetobacter sp.</i>	----	EANCIGYCIGG-TLLS	----	45.1%
class II	<i>P. oleovorans</i> 1	----	DLNMLGACSGGITCTA	----	34.3%
	<i>P. oleovorans</i> 2	----	DPNLMGACAGGLTMAA	----	35.2%
	<i>P. aeruginosa</i> 1	----	DLNLLGACSGGITAT	----	32.6%
	<i>P. aeruginosa</i> 2	----	SVNLAGACAGGLTVAA	----	35.2%
class III	<i>A. vinosum</i>	----	KVNLLGICQGG-AFSL	----	25.5%
	<i>T. violacea</i>	----	QVNILGICQGG-AFSL	----	27.4%
			[GXCXG]		
			↑		
			Active site		

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 (Schrage *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; Schrage 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,