CONSTRUCTION OF *phaC* MUTANTS FROM *Pseudomonas* sp. USM4-55

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CONSTRUCTION OF *phaC* MUTANTS FROM *Pseudomonas* sp. USM4-55

by

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PEMBINAAN MUTAN *phaC* DARIPADA *Pseudomonas* sp. USM4-55

oleh

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Tesis yang diserahkan untuk memenuhi
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LIST OF ABBREVIATIONS

3HB	3-hydroxybutyrate
3HD	3-hydroxydecanoate
3HDD	3-hydoxydodecanoate
3HHX	3-hydroxyhexanoate
3HO	3-hydroxyoctanoate
3H5DD	3-hydroxy-cis-5-dodecanoate
3H7TD	3-hydroxy-cis-7-tetradecanoate
bp	Base pair
CME	Caproic methyl ester
CoA	Coenzyme A
DCW	Dry cell weight
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside 5'-triphosphates
EtBr	Ethidium bromide
g	gravity
GFP	Green fluorescent protein
GTE	Glucose tris EDTA
IPTG	Isopropyl β-D-thiogalactopyranoside
Kb	Kilobase pair
km^r	Kanamycin resistant gene
LB	Luria Bertani
MCL-PHA	Medium-chain-length PHA

MCS	Multiple cloning site
OD	Optical density
ONPG	O-nitrophenyl β-galactopyranoside
ORF	Open reading frame
Ori	Origin of replication
PCR	Polymerase chain reaction
PHA	Polyhydroxyalkanoate
P(3HB)	poly(3-hydroxybutyrate)
P(3HB-3HV)	Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)
P(3HB-3HP)	Poly(3-hydroxybutyrate-*co*-3-hydroxypropionate)
P(3HB-4HB)	Poly(3-hydroxybutyrate-*co*-4-hyroxybutyrate)
P(3HO-3HH)	Poly(3-hydroxyoctanoate-*co*-hydroxyhexanoate)
P(4HB)	Poly(4-hydroxybutyrate)
rpm	revolution per minute
RBS	Ribosomal binding site
SCL-PHA	Short-chain-length PHA
sm^r	Streptomycin resistant gene
SDS	Sodium dodecyl sulphate
TAE	Tris-acetate/EDTA buffer
TE	Tris EDTA
Wt/vol	Weight per volume
X-Gal	5-bromo-4-chloro-3-indoyl-β-galactoside

PEMBINAAN MUTAN *phaC* DARIPADA *Pseudomonas* sp. USM4-55

ABSTRAK

Pseudomonas sp. USM4-55 ialah pencilan tempatan yang mampu menghasilkan polihidroksialkanoat (PHA) berantai pendek dan sederhana. Ia mempunyai dua enzim PHA sintase yang menghasilkan PHA berantai sederhana dalam keadaan sumber karbon berlebihan dan sumber nitrogen yang terhad. Kedua enzim dikodkan oleh *phaC1* dan *phaC2*. Untuk mengkaji sumbangan setiap PHA sintase dalam *Pseudomonas* sp. USM4-55, gen pelapor *lacZ* tanpa promoter telah dimasukkan secara berasingan ke dalam *phaC1* dan *phaC2*. Dua vektor penukargantian gen berasaskan plasmid pJRD215 telah dibina. Dua vektor ini, pKEM401 dan pKEM402, mengandungi *phaC1* dan *phaC2* yang diselit oleh kaset *lacZ-km*^{r}. Gen *sacB* yang bertindak sebagai pemilihan bertentangan juga dimasukkan ke dalam kedua-dua plasmid. Kedua-dua plasmid telah dimasukkan ke dalam *Pseudomonas* sp. USM4-55 melalui proses elektroporasi. Untuk *phaC1*, 16 mutan dengan fenotip *lacZ*$^+$, *sm*^{s} and *km*^{r} (dilabel sebagai *Pseudomonas* sp USMLZC1-KH1 hingga KH16) telah dikenalpasti manakala untuk *phaC2,* tiada mutan didapati. Integrasi *phaC1::lacZ-km*^{r} ke dalam *Pseudomonas* sp. USM4-55 melalui rekombinasi homolog telah disahkan melalui amplifikasi PCR pada bahagian huluan dan hiliran binaan *phaC1::lacZ-km*^{r}. Fragmen hulu bersaiz 1160 bp dan fragmen hilir bersaiz 2030 bp telah berjaya diamplifikasikan. Analisis gas kromatografi ke atas *Pseudomonas* sp. USM4-55 dan mutan *phaC1* menggunakan glukosa sebagai sumber karbon mendapati penghasilan polimer oleh jenis liar dan mutan tidak menunjukkan perbezaan yang ketara. Ini mungkin kerana protein PhaC2 boleh menampung ketiadaan aktiviti PhaC1.

CONSTRUCTION OF *phaC* MUTANTS FROM *Pseudomonas* sp. USM4-55

ABSTRACT

Pseudomonas sp. USM4-55 is a local isolate which can produce medium-chain-length (MCL) and short-chain-length (SCL) polyhydroxyalkanoates (PHAs). This bacterium has two PHA synthase isozymes which produce MCL PHA when grown in an excess of carbon source and under nitrogen limitation. They are encoded by the *phaC1* and *phaC2* genes. To investigate the contribution of each PHA synthase, a promoterless *lac*Z reporter gene was separately introduced into *pha*C1 and *pha*C2. Two gene replacement vectors based on plasmid pJRD215 were constructed. Both vectors, pKEM401 and pKEM402, contained either the *pha*C1 or *pha*C2 interrupted by the insertion of a *lacZ-km*^{r} cassette. A *sac*B gene, acting as a counterselection, was also included in the plasmids. Both plasmids were introduced into *Pseudomonas* sp. USM4-55 via electroporation. For *phaC*1, 16 putative mutants (labelled as *Pseudomonas* sp. USMLZC1-KH1 to KH16) were isolated while for *phaC2*, none was obtained. The integration of *phaC1::lacZ-km*^{r} into *Pseudomonas* sp. USM4-55 via homologous recombination was confirmed by PCR amplification on the upstream and downstream regions of *phaC1::lacZ-km*^{r} construct. An upstream 1160 bp DNA fragment and a downstream 2030 bp DNA fragment were successfully amplified. Gas chromatography analysis performed on both parental *Pseudomonas* sp. USM4-55 and *phaC1* mutant strains using glucose as the carbon source did not show any apparent difference in PHA polymer production. This is possibly because the PhaC2 protein could compensate for the absence of PhaC1 activity.

1.0 INTRODUCTION

Polyhydroxyalkanoates (PHAs) are natural polyesters produced by microorganism as a storage compound especially in condition where carbon sources are in excess while an essential nutrient such as nitrogen is limited (Anderson and Dawes, 1990; Steinbuchel *et al*., 1992). These polymers attract a lot of attention because of their many properties and especially their ability to be degraded in natural environment. They can also be produced from renewable substrates such as industrial and agricultural wastes thus increasing the potential of this polymer as a replacement for conventional plastics (Braunegg *et al.,* 1998).

Since the last 20 years, PHAs have been commercially developed and marketed. However the high cost of production compared to the synthetic petroleum-based plastics resulted in the limited usage of this environmental friendly polymer. One way of overcoming the problem is by using cheap substrates such as sugars, corn, cassava or molasses to reduce production cost (Klinke *et al.,* 1999). In order to fully utilize these materials, we need to understand the physiology, genetics and biochemistry of the PHA-producing organism. To accomplish this, various studies involving DNA recombinant and fermentation techniques were done to investigate the structure and organization of the genes involved in PHA biosynthesis.

The bacterium *Pseudomonas* sp. USM4-55 used in this study was isolated from a soil sample taken from Felda Chini in Tasek Chini, Pahang in 1998 by Few Ling Ling (Few, 2001). This isolate was chosen because of its ability to accumulate two groups of polymers, short-chain-length (SCL) and medium-chain-length (MCL) PHA, at the same time. This isolate can utilize carbon sources such as oleic acid (a

component in palm oil) and can accumulate polymers up to 28% of cell dry weight when cultured on C/N=20 medium with glucose as the carbon source (Few, 2001). The *pha* gene cluster of *Pseudomonas* sp. USM4-55 consists of three open reading frame (ORFs) transcribed in the same direction: *phaC1* and *phaC2*, which encode PHA synthases (or PHA polymerases) and the *phaZ*, which codes for a PHA depolymerase (Baharuddin, 2002). Both PHA polymerase genes (*phaC1* and *phaC2*) produce functional proteins. In order to understand the regulation and expression of the *phaC*, we need to assess the effects of different growth conditions on the expression of both *phaC1* and *phaC2* of *Pseudomonas* sp. USM4-55 using a reporter gene.

In this work, we report an attempt to generate mutants of *Pseudomonas* sp. USM4-55 whereby a promoterless *lacZ-km*^{r} cassette was inserted separately in the *phaC1* and *phaC2*. The expression of *phaC1* and *phaC2* could therefore be measured directly by measuring the expression of *lacZ*.

The first part of this study involved the construction of gene replacement vectors having *phaC1* and *phaC2* disrupted by the insertion of a *lacZ-km*^{r} cassette. The *sacB* gene, an effective counterselection marker, was also introduced into these vectors.

The second part involved the integration of the reporter gene into the genome of wild type *Pseudomonas* sp. USM4-55 via homologous recombination. The mutants generated were confirmed using polymerase chain reaction (PCR) on both upstream and downstream regions of *phaC1* and *phaC2*.

The third and final part involved gas chromatography analysis, where both wild type and mutant cells were grown in glucose as carbon source and the types of polymers produced were compared.

1.1 Research objectives

This research was done to fulfill the following objectives:

a) Construction of two gene replacement vectors, pKEM401 and pKEM402 consisting *phaC1* and *phaC2* disrupted by the insertion of *lacZ-km*^{r} cassette.
b) Generation of isogenic *phaC1* and *phaC2* mutants by integration of the *lacZ-km*^{r} cassette into the genome of *Pseudomonas* sp. USM4-55.
c) Comparison of the levels of PHA polymer produced between wild type and mutant strains using glucose as the carbon source.

2.0 LITERATURE REVIEW

2.1 Polyhydroxyalkanoates (PHAs)

Poly(3-hydroxyalkanoates) (PHAs) are high molecular weight macromolecules synthesized as carbon and energy storage compounds by many bacteria including members of the family Halobacteriaceae of the Archaea (Steinbuchel and Fuchtenbusch, 1998). The accumulation of these polymers takes place when the bacteria are grown in excess carbon sources and on nitrogen limited medium (Anderson and Dawes, 1990). The polymers produced depend on the types of carbon sources present and the substrate specificity of the enzymes involved in PHA biosynthesis (Steinbuchel, 1991). Apart from 3-hydroxybutyrate (3HB), many other 3-, 4- and 5-hydroxyalkanoates were identified as components of PHAs (Steinbuchel and Valentin, 1995).

PHAs are accumulated to as much as 90% of the cell dry weight and deposited as water insoluble granules in the cytoplasm (Madison and Huisman, 1999). Bacteria store these as excess nutrients intracellularly for later use when the nutrient supplies are imbalanced. The polymerization of soluble molecules into insoluble molecules is advantageous to bacteria because their osmotic state can be controlled and leakage of these compounds towards the outside of the cells is prevented. Therefore these nutrient stores are available at low maintenance cost and do not affect the cell's general fitness (Madison and Huisman, 1999).

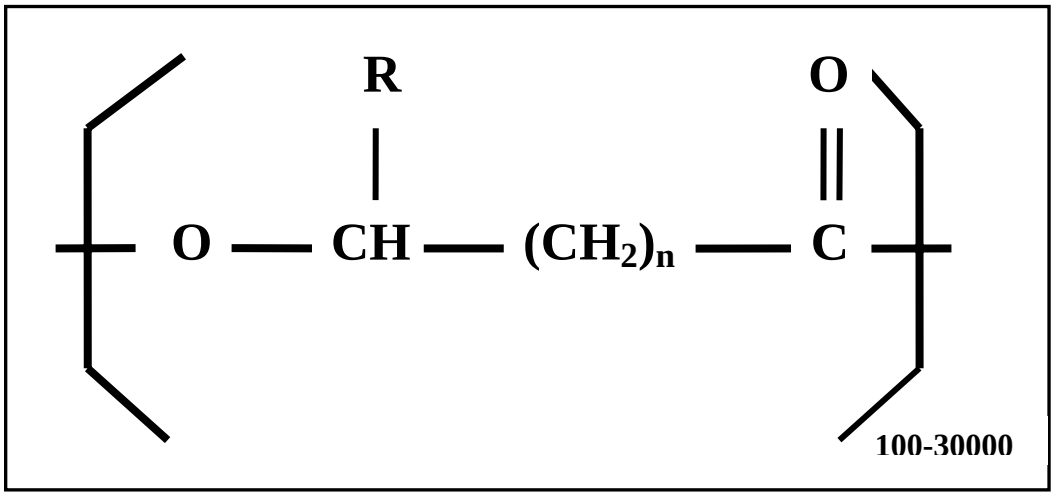
PHAs comprised of R(-)-3-hydroxyalkanoic acid monomers of 3 to 14 carbon atoms with a variety of saturated or unsaturated and straight or branched side chain containing aliphatic or aromatic group (Doi *et al*., 1992; De Smet *et al*., 1983). Therefore it is possible to produce different types of biodegradable polymers

with a wide range of properties. PHAs have properties similar to polyefins and can be degraded rapidly under unfavourable conditions. They can substitute petroleum-based polymers in many applications (Fuller and Lenz, 1990).

Various PHAs with linear head to tail polyester that are made up of 3-hydroxyalkanoate (3HA) were found naturally. Their general structure is shown in Figure 2.1. The identity of a monomer unit is determined by the side chain, R. The most common PHA is poly-3-hydroxybutyrate [P(3HB)], containing repeating units of (R)-3HB (Madison and Huisman, 1999; Sudesh *et al*., 2000).

2.2 Discovery of PHAs

Discovery of PHAs other than P(3HB) has marked a new era in biopolymer research. These thermoplastic polymers are degradable and can be produced from cheap and renewable carbon sources thus drawing great interest since their discovery. Meyer (1903) first observed PHA as a lipid-like inclusion that was soluble in chloroform in *Azotobacter chroococcum* early last century (Adapted from Sudesh *et al*., 2000). Later in 1926, Lemoigne found a similar inclusion in *Bacillus megaterium* and it was later identified as P(3HB) by Wallen and Rohwedder in 1974 (Anderson and Dawes, 1990). In 1950s, scientists discovered that P(3HB) in microorganisms function as intracellular carbon and energy sources. However, in the early 1970s, the P(3HB) unit was thought to be the only hydroxyalkanoate (HA) monomer that formed the basic building block for this biodegradable polymer until the discovery of other HAs (Wallen and Rohwedder, 1974; Sudesh *et al*., 2000).



n=1	R=	Hydrogen	poly(3-hydroxypropionate)
		Methyl	poly(3-hydroxybutyrate)
		Ethyl	poly(3-hydroxyvalerate)
		Propyl	poly(3-hydroxyhexanoate)
		Pentyl	poly(3-hydroxyoctanoate)
		Nonyl	poly(3-hydroxydecanoate)
n=2	R=	Hydrogen	poly(4-hydroxybutyrate)
n=3	R=	Hydrogen	poly(5-hydroxyvalerate)

Figure 2.1: The general structure of polyhydroxyalkanoates (PHA) (Adapted from Ojumu *et al*., 2004).

Following that discovery, over a hundred of other PHAs constituents have been found in prokaryotes isolated from diverse places including soil, domestic sewage plants and estuarine sediments (Steinbuchel and Valentin, 1995).

2.3 Production of PHAs

PHAs are synthesized and accumulated intracellularly under unfavourable growth condition such as nitrogen limitation and in excess supply of carbon source (Anderson and Dawes, 1990). Bacterial PHAs can be classified into two groups according to the number of carbon atoms constituting monomer units. SCL-PHAs consisted of 3-5 carbon atom and MCL-PHAs consisted of 6-14 carbon atoms. There have been several reports about PHAs consisted of both SCL and MCL monomer units (SCL-MCL-PHA) (Kato *et al*., 1996; Matsusaki *et al*., 1998; Matsumoto *et al*., 2001).

Several PHA producing bacteria accumulate large amounts of PHAs during cultivation. This includes bacteria such as *Cupriavidus necator* (formerly known as *Wautersia eutropha*), *Alcaligenes lactus*, *Chromobacterium violaceum*, *Pseudomonas* strain K, *Azotobacter vinelandii*, *Pseudomonas oleovorans* and genetically engineered bacteria such as recombinant strain of *Escherichia coli*. These microorganisms produce various PHAs in high amount by utilizing several substrates such as glucose, sucrose, molasses or fatty acids (Steinbuchel and Fuchtenbusch, 1998).

C. necator mutants can accumulate up to 80% (wt/wt) P(3HB) with glucose as the sole carbon source (Holmes, 1985). Fluorescent pseudomonads were shown to accumulate PHAs consisting of MCL-PHA 3-hydroxyacids, but not

P(3HB). Timm and Steinbuchel (1990) showed that many strains of *Pseudomonas aeruginosa* and other *Pseudomonas* species are capable of accumulating substantial amounts of PHAs containing 3-hydroxydecanoate with gluconate as the sole carbon source. According to Huisman *et al*. (1989), the ability to accumulate these PHAs may be of taxonomic value.

2.4 Physical properties of PHAs

PHAs can be found in the cell cytoplasm of prokaryotes as small insoluble inclusions usually 0.2 μm to 0.5 μm in diameter (Sudesh *et al*., 2000). The molecular mass of PHAs depends on the producer and growth conditions but is generally between 50,000 to 1,000,000 Dalton (Madison and Huisman, 1999). The use of phase contrast light microscopy enables the PHA granules to be seen clearly due to their high refractivity (Sudesh *et al*., 2000). Byrom (1994) reported that about 8-13 granules per cell were observed in *C. necator.* Observation of PHA within these microorganisms can be done by staining with Sudan black or Nile blue A (Anderson and Dawes, 1990).

P(3HB) can be found in a fluid, amorphous state inside the cell. However, they become a highly crystalline, stiff but brittle material after extraction from the cell, and therefore, is not stress resistant (Doi, 1995). Its high melting temperature (177°C), which is near the decomposition temperature, also limits the ability to work with this polymer (Madison and Huisman, 1999). MCL-PHAs have lower crystallinity and higher elasticity than P(3HB) but their tensile strength is low while their elongation to breaking point is high (Doi, 1995).

Few studies on *Pseudomonas* strain showed the ability of these microorganisms to produce both SCL-PHA and MCL-PHA monomers with mechanical properties similar to low density polyethylene (LDPE) (Liebergesell *et al*., 1993; Kato *et al.,* 1996; Chen *et al*., 2001). They have hard crystalline to elastic properties, depending on the percentage of different monomers incorporated into the copolymer (Table 2.1). These superior properties of SCL-MCL PHA will enhance industrial applications of PHA (Doi and Abe, 1990).

2.5 Application of PHAs

The unique features of PHAs and the flexibility of PHA biosynthesis have given them a wide range of applications industrially. Materials produced from PHAs have physical properties ranging from stiff and brittle plastic to rubbery polymers (Ojumu *et al.,* 2004). These biopolymers can also be used in the manufacture of consumer packaging items such as bags, bottles, pen and golf tees. It can also be applied to paper or cardboard to form a water resistant layer (Reddy *et al*., 2003). Other than that, they can also be used as conventional commodity plastics in disposable items such as razors, utensils, diaper back sheet, feminine hygiene products, cosmetic containers and cups (Reddy *et al*., 2003). PHAs have also been used as a material for non-woven fabrics (Hocking *et al*., 1994).

In the medical and agricultural fields, P(HB-HV) can be used as packaging materials for slow release of drugs, herbicides, insecticides and hormones. PHAs can also be used in bone plates, surgical sutures, blood vessel replacements and as osteosynthetic materials to stimulate bone growth due to their piezoelectric properties (Reddy *et al*., 2003).

Table 2.1: Properties of PHA, polypropylene and LDPE (adapted from Park *et al*., 2001)

Type of polymers[a]	T_g(°C)[b]	T_m(°C)[c]	Tensile strength (MPa)	Crystallinity (%)	Elongation to break (%)
P(3HB)	4	177	40	60	5
P(3HB-*co*-10mol%3HV)	6	162	36	69	10
P(3HB-*co*-20mol%3HV	-1	145	32	53	-
P(3HB-*co*-10mol%3HHx)	-1	127	21	34	400
P(3HB-*co*-15mol%3HHx)	0	115	23	26	760
P(3HB-*co*-17mol%3HHx)	-2	120	20	22	850
P(3HB-*co*-6mol%3HA)	-8	133-146	17	-	680
Commercial plastic (film)					
PP	-30	130-161	29.3	40	400
LDPE	-30	120	15.2	-	620

[a] P(3HB) is poly(3-hydroxybutyate), P(3HB-*co*-10mol%3HV) is poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) containing 10% 3HV, P(3HB-*co*-20mol%3HV) is poly(3-hydroxybutyrate-co-3-hydroxyvalerate) containing 20% 3HV, P(3HB-*co*-10mol%3HHx) is poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) containing 10% 3HHx, P(3HB-*co*-15mol%3HHx) is poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) containing 15% 3HHx, P(3HB-*co*-17mol%3HHx) is poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) containing 17% 3HHx, P(3HB-*co*-6mol%3HHx) is poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate containing 6% 3HHx, PP is polypropylene and LDPE is low density polyethylene.
[b] T_g is glass transition temperature.
[c] T_m is melting temperature.

Its extremely slow biodegradation and high hydrolytic stability in sterile tissues however, has restricted the application of P(3HB) in medical and pharmaceutical areas (Wang and Bakken, 1998; Steinbuchel and Fuchtenbusch, 1998).

Apart from being potential plastic materials, PHAs can be used as stereo regular compounds for the synthesis of optically active compounds (Senior and Dawes, 1973). PHA can also be hydrolyzed chemically and the monomers can be transformed to useful molecules such as β-hydroxyacids, 2-alkenoic acids, β-hydroxyalkanols, β-acyllactones, β-amino acids and β-hydroxyacid esters (William and Peoples, 1996). PHAs can be used in toners and developers thus replacing petrochemical polymers (Madison and Huisman, 1999). PHAs also have potential as dairy cream substitutes or flavour delivery agents in the food industry (Madison and Huisman, 1999). They are also being considered as sources for the synthesis of enantiomerically pure chemicals and as raw materials for the production of paints (Muller and Seebach, 1993). Plant derived PHAs can be depolymerised after certain processing which includes esterification and can be used in the production of bulk chemicals (Brandl *et al*., 1988).

2.6 Biological degradation

One of the unique features that differentiate PHAs from petroleum based plastics is their ability to degrade in natural environment caused by the enzymatic activities of microorganisms. PHAs are degraded when exposed to soil, compost, sea water and lake water over a period of time. This ability to be degraded has been evaluated by monitoring their properties such as dimension, molecular weight and mechanical strength (Poirier *et al*., 1995; Sudesh *et al*., 2000).

The rate of polymer biodegradation depends on a number of factors including surface area, microbial activity of the disposal environment, pH, temperature, moisture and the pressure of other nutrient materials (Lee, 1996). The nature of monomer units, polymer composition and crystallinity also affect the rate of degradation. Copolymers with P(3HB) monomer degraded more rapidly compared to either P(3HB) alone or P(3HB-*co*-HV) copolymers (Reddy *et al.,* 2003). Electron microscopy showed that degradation takes place at the crystal's surface by enzymatic hydrolysis or surface erosion (Sudesh *et al*., 2000).

PHAs are degraded into water soluble oligomers and monomers by the action of extracellular PHA-degrading enzymes secreted by bacteria and fungi in soil, sludge and seawater (Sudesh *et al*., 2000). In an aerobic condition, carbon dioxide and water are the end products of PHA degradation, while methane is also produced in an anaerobic environment. Degradation occurs most rapidly in anaerobic sewage and slowest in seawater (Lee, 1996). P(3HB-*co*-HV) was completely degraded after 6, 75 and 350 weeks in anaerobic sewage, soil and seawater, respectively (Lee, 1996).

2.7 Biosynthesis of PHAs

2.7.1 Biosynthesis of P(3HB)

C. necator is the most well known producer of SCL-PHA (Poirier *et al*., 1995). The P(3HB) biosynthetic pathway is catalyzed by three different enzymes through three enzymatic reactions as shown in Figure 2.2**.** The first reaction is the condensation of two acetyl coenzyme A (acetyl-CoA) molecules into acetoacetyl-CoA by β-ketoacyl-CoA thiolase (encoded by *phbA*). This is followed by the

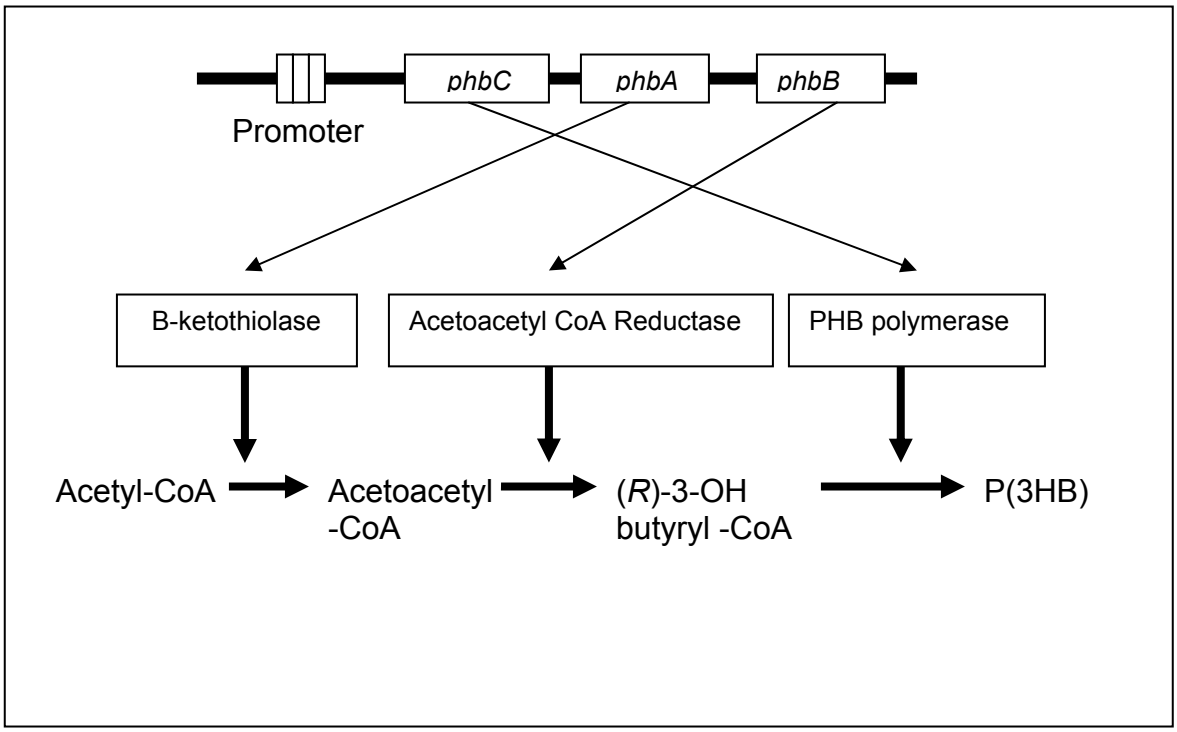


Figure 2.2: P(3HB) biosynthesis pathway and genes arrangement of *C. necator*. P(3HB) is synthesized by the successive action of β-ketoacyl-CoA thiolase (*phbA*), acetoacetyl-CoA reductase (*phbB*) and P(3HB) polymerase (*phbC*) in a three step pathway. The genes of the *phbCAB* operon encode the three enzymes. The promoter (P) upstream of *phbC* transcribes the complete operon (*phbCAB*) (adapted from Madison and Huisman, 1999).

reduction of acetoacetyl-CoA to (*R)*-3-hydroxybutyryl-CoA by an NADPH-dependent acetoacetyl-CoA dehydrogenase (encoded by *phbB*). Lastly, the (*R*)-3-hydroxybutyryl-CoA monomers are polymerized into P(3HB) by P(3HB) polymerase (encoded by *phbC*) (Madison and Huisman, 1999).

2.7.2 Biosynthesis of MCL-PHAs

The MCL-PHA biosynthesis pathway needs an additional enzyme to channel down 3-hydroxyacyl coenzyme A thioesters, the substrates of the PHA synthases, from the central pathway. Three different pathways (as shown in Figure 2.3) were found to be involved in the synthesis of the 3-hydroxyalkanoate precursors from studies on *Pseudomonas putida* KT2442 (Madison and Huisman, 1999; Huijberts *et al*., 1995).

2.7.2.1 Chain elongation reaction

The chain elongation reaction, in which acetyl-CoA molecules are condensed to 3-hydroxyacyl-CoA, takes place during growth on hexanoate. In this reaction, acyl-CoA is added to acetyl-CoA to form ketoacyl-CoA. Ketoacyl-CoA was then converted to (*R*)-3-OH-acyl-CoA by the reaction of ketoacyl-CoA reductase (Hoffmann and Rehm, 2004).

2.7.2.2 β-oxidation pathway

When fatty acids are utilized as carbon source, β-oxidation is the main pathway. In this pathway, degradation of fatty acid resulted in the removal of C2 units as acetyl-CoA. The intermediates consist of acyl-CoA, enoyl-CoA, (*S*)-3-OH-

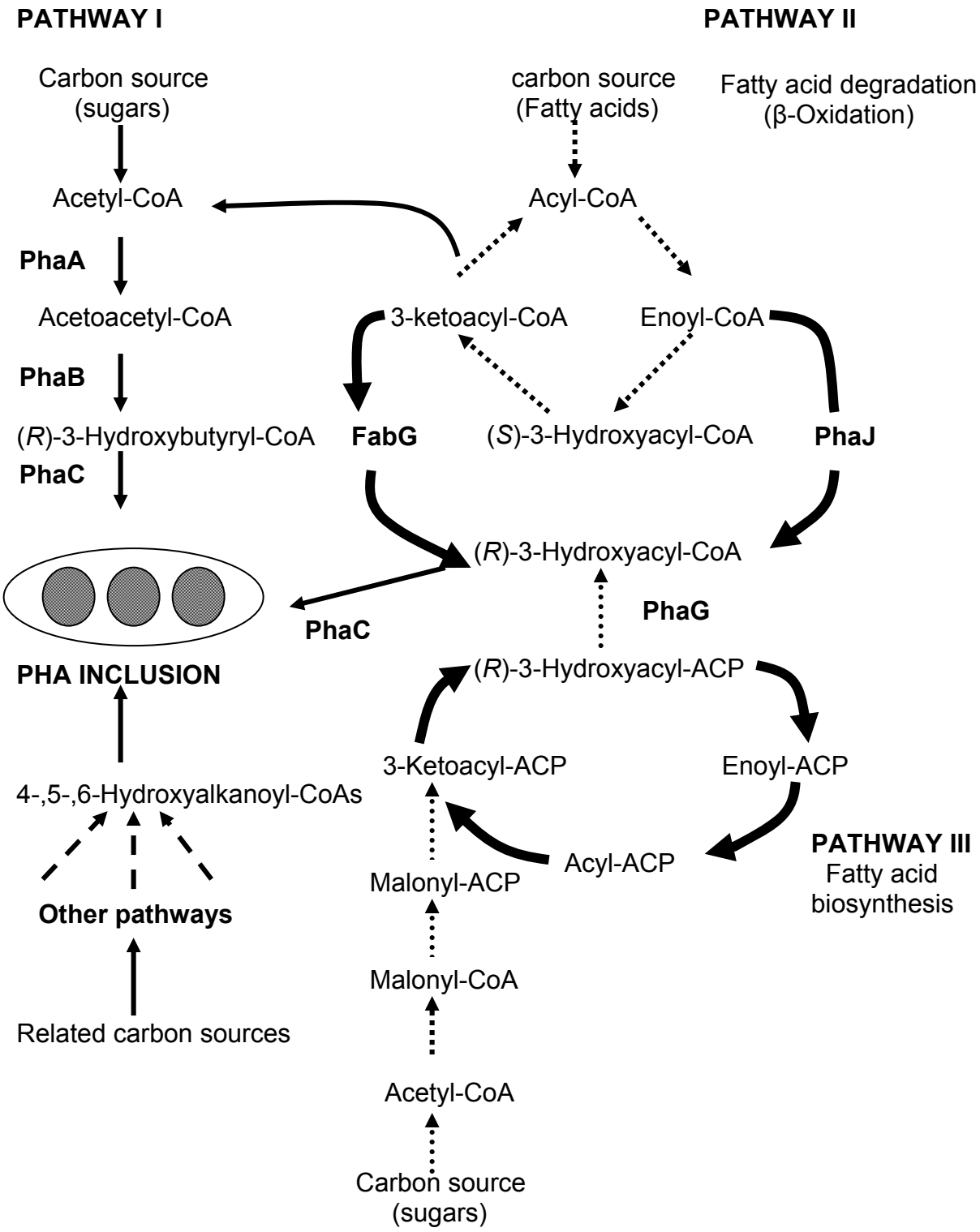


Figure 2.3: Major metabolic pathways that supply hydroxyalkanoate monomers for PHA biosynthesis. PhaA, β-ketothiolase; PhaB, NADPH-dependent acetoacetyl-CoA reductase; PhaC, PHA synthase; PhaG, 3-hydroxyacyl-ACP-CoA transferase; PhaJ (*R*)-enoyl-CoA hydratase; FabG, 3-ketoacyl-CoA reductase (adapted from Sudesh *et al*., 2000).

acyl-CoA and 3-ketoacyl-CoA. For the synthesis of the (*R*)-3-OH-acyl-CoA monomer, additional biosynthesis by enoyl-CoA hydratase, 3-OH-acyl-CoA epimerase and 3-ketoacyl-CoA reductase were required. PHAs were formed after polymerization of (*R*)-3-OH-acyl-CoA by PHA polymerase (Poirier, 2002).

2.7.2.3 Fatty acid *de novo* synthesis

Fatty acid *de novo* biosynthesis is the main route during growth on simple carbon sources such as gluconate, acetate and ethanol. Monomers for PHA are derived from this pathway as (R)-3-OH-acyl-ACP intermediates and are converted to (R)-3-OH-acyl-CoA through an (R)-3-hydroxyacyl-(ACP to CoA) transferase encoded by the *phaG* (Rehm *et al*., 1998).

It has been shown that both the β-oxidation and *de novo* fatty acid biosynthesis routes can function simultaneously in the synthesis of PHA (Huijbert *et al.,* 1995). *P. putida* and *P. aeruginosa* utilize the fatty acid *de novo* synthesis pathway and produced copolyesters with 3-hydroxydecanoic acid as their main constituent and some other MCL-PHA as minor constituent from glucose or gluconic acid (Haywood *et al*., 1990).

2.8 Genes involved in PHA biosynthesis

Numerous genes involved in the formation and degradation of PHAs have been cloned and characterized from various microorganisms (Madison and Huisman, 1999). Studies showed that nature has evolved different pathways for PHA formation, each suited to the environment of the PHA-producing microorganism (Madison and Huisman, 1999). The diversity of the P(3HB)

biosynthetic pathways shows the distance of the divergence of the *pha* loci. The separation and gene organization of *pha* (genes encoding enzymes for MCL-PHA) and *phb* (genes encoding enzymes for SCL-PHA) differ from species to species (Reddy *et al*., 2003).

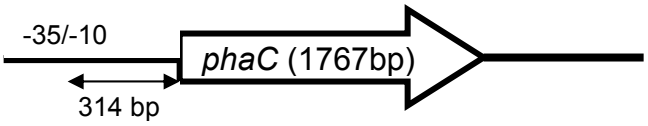
Genes coding for proteins involved in the biosynthesis of PHA are known as *phaA* (β-ketothiolase), *phaB* (acetoacetyl-CoA reductase), *phaC* (PHA synthase), *phaG* (3-hydroxyacyl-acyl carrier protein-coenzyme A tranferase) and *phaJ* (enoyl-CoA hydratase). The genes required for the degradation are referred in reverse alphabetical order such as *phaZ* for PHA depolymerase. PHA depolymerase is needed to mobilize PHA granules by releasing carbon and energy when the limited nutrient is restored (Rehm and Steinbuchel, 1999).

Pseudomonas strains contain two types of PHA synthase known as PhaC1 and PhaC2 that slightly differ in substrate specificities and monomer composition of the accumulated PHAs (Matsusaki *et al*., 1998). Both genes are encoded on the same open reading frame (ORF) together with *phaZ* (PHA depolymerase) and *phaD* (putative transcriptional regulator) (Huisman *et al*., 1991; Klinke *et. al*, 2000).

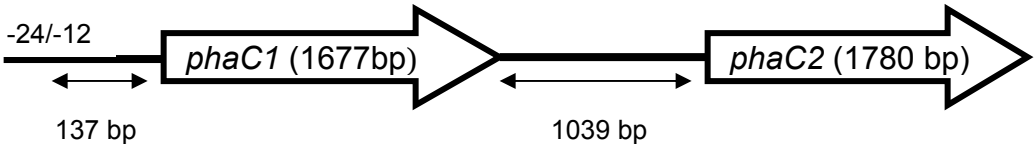
2.8.1 Classification of PHA synthases (*phaC*) genes

The key enzyme that determines the type of PHA synthesized was identified as PHA synthase and it is encoded by the gene *phaC.* This enzyme can be divided into three different classes based on the number of subunits that constitute the active PHA synthase protein, their primary amino acid sequences and also *in vivo* substrate specificities as shown in Figure 2.4 (Rehm and Steinbuchel, 1999).

Type I: Represented by the PHA synthase of *C. necator*



Type II: Represented by the PHA synthase of *P. oleovorans*



Type III: Represented by the PHA synthase of *C. vinosum*

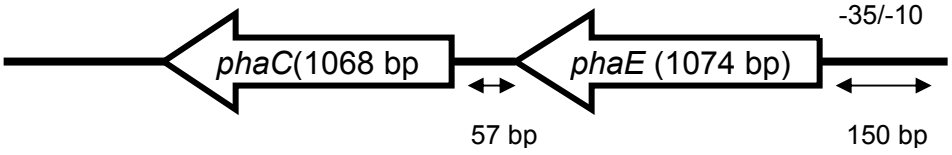


Figure 2.4: Classification of PHA synthases (adapted from Sudesh *et al*., 2000).

Type I and type II PHA synthases comprise enzymes consisting of only one type of subunit (PhaC) with molecular weight (M_W) between 61 and 68 kDa. Type I PHA synthases utilize coenzyme A thioesters of SCL-PHAs, containing 3 to 5 carbon atoms. The SCL PHA synthase in *C. necator* is an example of the type I enzyme. Type II PHA synthase as exemplified by *P. aeruginosa* utilize coenzyme A thioester comprising 6 to 14 carbon atoms. Type III PHA synthases as exemplified by *C. vinosum*, comprise enzymes with two different subunits (Rehm and Steinbuchel, 1999). The first subunit, PhaC (approximately 40kDa), shows 21% to 28% amino acid sequence similarity to type I and type II PHA synthases. The second subunit, PhaE (about 40kDa) showed no resemblance to PHA synthase. These PHA synthases prefer coenzyme A thioester of SCL-PHA (Rehm and Steinbuchel, 1999). Studies showed that both of these subunits are equally important for this group of PHA synthases to function actively (Steinbuchel *et al.*, 1992).

Rehm and Steinbuchel (1999) discovered that type I PHA synthases synthesize bigger PHAs (500 000 to several millions Dalton). Type II PHA synthases on the other hand synthesize smaller PHAs with molecular weights ranging from 50 000 to 500 000 Dalton while the size of PHAs synthesized by type III PHA synthase are in between type I and type II (Rehm and Steinbuchel, 1999).

2.8.2 Regulation of PHA synthase operon

In *Pseudomonas* species, the PHA biosynthesis gene locus comprised of two PHA synthase genes, *phaC1* and *phaC2,* which are separated by the *phaZ* (encoding PHA depolymerase), followed by *phaD* (encoding putative transcriptional

regulator, *phaF* (negative regulator) and *phaI* (granule associated protein) (Hoffman and Rehm, 2004). For *P. oleovorans*, sequencing data showed the presence of three complete open reading frames: ORF1, *phaI* and *phaF* (Prieto *et al*., 1999). A homologous *pha* gene cluster showing a similar gene organization was also found in *P. aeruginosa* and *P. putida* (Prieto *et al*., 1999). Previous studies on *P. aeruginosa* and *P. oleovorans* found a sequence similar to sigma 54 (RpoN) and 70 (SigA) of *E. coli* consensus promoter, upstream of *phaC1* while a SigA *E. coli* consensus promoter was found upstream of *phaC2* (Huisman *et al*., 1991). Identification of RpoN dependent consensus promoter sequence upstream of *phaC1* suggested that it was involved in the regulatory network of polyhydroxyalkanoate metabolism in these bacteria (Timm and Steinbuchel, 1992).

From the studies on *P. aeruginosa,* it was found that other than nitrogen limitation, the *phaC1* promoter region activation depends on the type of carbon source present in the medium. The *phaC1* promoter was less active when citric acid or glucose is used as carbon source but more active in the presence of octanoic acid (Prieto *et al*., 1999). Prieto *et al*. (1999) also reported that in *P. oleovorans* disruption of *phaF* resulted in the increase expression of *phaC1*, thus suggesting that this gene acted as a negative regulator for *phaC1* expression. Under PHA accumulating conditions, PhaF was bound to PHA granules and less PhaF is available for repression, which resulted in enhanced transcription of *phaC1* and *phaI* (Prieto *et al*., 1999).

In *P. aeruginosa*, PHA accumulation was nitrogen-dependent and RpoN-dependent when gluconate or octanoate was used as carbon source whereas in *P. putida* PHA accumulation was RpoN-independent when octanoate was used as

carbon source. However, when cultivated on gluconate, *P. putida* showed a stronger nitrogen-dependency (Hoffmann and Rehm, 2004). These observations showed that in *P. putida*, the gluconate pathway was controlled by RpoN subunit of RNA polymerase while fatty acid pathway was RpoN independent (Timm and Steinbuchel, 1992).

2.9 Reporter genes

Reporter genes are usually used to measure the rates of transcription of certain genes in prokaryotic and eukaryotic systems. They are used in transcriptional fusions to elucidate the transcriptional activity of a promoter under various environmental or physiological conditions. The products of the genes fusion are readily measured, and mutations affecting their expression can be identified (Simon and Schumann, 1987). Some of the reporter genes (as shown in Table 2.2) such as *lacZ*, *luxAB* and *gusA* are widely used in prokaryotic system while others such as *inaZ* or *gfp*, are not as common (Lindgren *et al*., 1989).

The most popular reporter system is *lacZ* of *E. coli*, encoding β-galactosidase. Fusion of the *lacZ* structural gene to the promoter region of other gene or operon of interest is a useful tool for promoter's identification and for the regulation of gene expression studies (Silhavy and Beckwith, 1985). There are more advantages to using the *lacZ* as compared to other reporter genes. Its product, β-galactosidase (β-gal) can be measured easily and accurately using an assay for β-galactosidase activity as first described by Miller (1972). There are also various indicators such as 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-Gal) that can be diffused into the medium agar to generate colour changes. Apart from that,

Table 2.2: Reporter genes (adapted from Kohler *et al*., 2000).

Reporter protein	Reporter gene	origin	Potential substrate	Detection method
Bacterial luciferase	*lux*	Luminescent bacteria	Long chain aldehyde (C9-C14)	Luminescence
Insect luciferase	*luc*	Fireflies, click beetles	Luciferin	Luminescence
β-galactosidase	*lacZ*	*E. coli*	Galactopyranosides	Colorimetric, electrochemical, fluorescence, chemiluminescence
Greenfluorescent protein	*gfp*	*A. victoria*	No substrate	Fluorescence
Alkaline phosphatase	*phoA*	various	Phosphorylated organics	Colorimetric, Chemiluminescence
β-glucuronidase	*gusA, gurA*	*E. coli*	β-glucuronides	Colorimetric, Fluorescence, luminescence
β-lactamase	*bla*	*E. coli*	lactamides	Colorimetric

the enzyme has a high turnover rate and generates strong signals by using fluorescence, electrochemical or chemiluminescent substrates (Kohler *et al.*, 2000).

Another type of reporter gene is the green fluorescent protein gene (*gfp*) isolated from the jellyfish *Aequorea victoria* (Chalfie *et al*., 1994). This gene produces a protein in this animal which fluoresces due to an energy transfer from Ca^{2+}-activated photoprotein aequorin. The GFP protein is highly stable and can be expressed in both prokaryotic and eukaryotic systems without the need of a substrate or cofactor (Kain and Kitts, 1997).

Among the reporter genes, the expression of β-glucuronidase (*gusA)* and β-galactosidase (*lacZ)* can be easily measured and monitored on plates using a chromogenic substrate making them the most convenient reporter systems (Jefferson *et al*., 1986). The activity of *gusA* and *lacZ* are easily and rapidly quantified on bacterial extracts. However the small size of *gusA* makes it easier to use compared to *lacZ* (Platteeuw *et al*., 1994).

2.10 Counterselection using *sacB*

Gene replacement through homologous recombination is a powerful mechanism by which DNA fragments can be inserted, deleted or altered at specific sites in the genome. The use of suicide systems offers a good counterselection method whereby microorganism harbouring the counterselection gene will die under certain growth condition (Stibitz, 1994). Among the popular counterselectable markers are genes that confer sucrose, streptomycin or fusaric acid sensitivity (Reyrat *et al*., 1998).

The *sacB* gene from *Bacillus subtilis,* coding for levansucrase, is a 50 kDa secretory enzyme which is induced by sucrose and is widely used as a counterselectable marker (Gay *et al.,* 1985). The expression of *sacB* is harmless to the natural hosts, Gram positive bacteria. However the presence of 5% sucrose will result in the death of Gram negative bacteria harboring the *sacB*. Induction by sucrose will result in the inhibition of cell growth and within 1 hour, the cell will lyse (Gay *et al.,* 1983). The levansucrase enzyme catalyzes a fructosylation reaction that hydrolyzes sucrose, releasing glucose and fructosylating an acceptor molecules (Gay *et al*., 1983). It has been proposed that the accumulation of the product of this reaction, levans (high molecular weight fructose polymers synthesized by the levansucrase), in the periplasm is toxic to the Gram negative bacteria (Gay *et al*., 1983). The *sacB* counterselection system has been successfully used in several Gram-negative bacteria (Schweizer, 1992; Quandt and Hynes, 1993; Blomfield *et al.*, 1991).