MOLECULAR CHARACTERIZATION OF POLYHYDROXYALKANOATE (PHA) SYNTHESIZING GENE (S) FROM STREPTOMYCES AUREOFACIENS NRRL 2209

BY
LATA H. MAHISHI

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MOLECULAR CHARACTERIZATION OF POLYHYDROXYALKANOATE (PHA) SYNTHESIZING GENE (S) FROM *STREPTOMYCES AUREOFACIENS* NRRL 2209

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BY

LATA H. MAHISHI

PLANT TISSUE CULTURE DIVISION
NATIONAL CHEMICAL LABORATORY
PUNE – 411008
INDIA

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DEDICATED TO MY FAMILY

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CERTIFICATE

This is to certify that the work incorporated in the thesis entitled "**Molecular characterization of polyhydroxyalkanoate (PHA) synthesizing gene (s) from *Streptomyces aureofaciens* NRRL 2209**" submitted by Lata H. Mahishi was carried out under my supervision at the Plant Tissue Culture Division, National Chemical Laboratory, Pune. Material obtained from other sources has been duly acknowledged in the thesis.

Dr. S. K. Rawal
(Research Guide)

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ABBREVIATIONS

BSA	Bovine serum albumin
bp	Base pairs
CTAB	Cetyltrimethylammonium Bromide
EDTA	Ethylene diamine tetra acetic acid disodium salt
h	hours
IPTG	Isopropyl β-D-thiogalactoside
Kb	Kilobases
µCi	Microcurie
MCS	Multiple cloning sites
min	Minutes
PEG	Polyethylene glycol
PHB/P(3HB)	Poly(3-hydroxybutyrate)
PHBV/P(3HB-3HV)	Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)
SDS	Sodium dodecyl sulphate (sodium lauryl sulphate)
s	Seconds
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

ABSTRACT

ABSTRACT

Globally plastic waste accumulates in the environment at the rate of about 30 million tonnes per year. Problems associated with global environment and solid waste management have generated interest in the development of novel plastics. These, while retaining the desired properties of conventional synthetic plastics, must also be biodegradable. Among the various biodegradable plastics available, there is growing interest in the group of polymers known as polyhydroxyalkanoates (PHAs). PHAs are polyesters of various hydroxyalkanoate monomers which accumulate as granular inclusions in the cytoplasm of various bacterial cells. Of all the PHAs, poly(3-hydroxybutyrate) (PHB) has attracted considerable interest as a candidate for biodegradable and biocompatible plastics. PHAs have immense applications as packaging films, disposable items, bone replacements; blood vessel replacements, scaffold material in tissue engineering of heart valves etc.

PHB biosynthetic pathway involves sequential action of three genes: *phaA* encoding for β - ketothiolase, *phaB* encoding for NADPH dependent acetoacetyl CoA-reductase and *phaC* encoding for PHA synthase.

Streptomyces species are Gram-positive soil bacteria. *Streptomyces* DNA is remarkable for its high G+C content of more than 70%, probably the highest of all eubacteria. They display a complex life cycle, which culminates in spore formation and involves the production of a large number of secondary metabolites such as enzyme inhibitors, herbicides and over 70% of naturally occurring antibiotics. These characteristics make this genus an attractive research subject from both academic and application point of view.

In our laboratory, at the National Chemical Laboratory, Pune, India, research on PHB synthesis by *Streptomyces aureofaciens* NRRL 2209 was initiated. *Streptomyces aureofaciens* partial *Sau*3A I genomic library was constructed in pGEM-3Z plasmid vector. The main features of the present thesis are:

1. Isolation and cloning of the PHA synthesizing genes from *Streptomyces aureofaciens* NRRL 2209
2. *Sequencing and sequence analysis of a ~5.0 kb genomic DNA fragment of* Streptomyces aureofaciens *harboring the PHB biosynthetic genes*

3. PHB synthesizing genes from Streptomyces aureofaciens *NRRL 2209: Heterologous expression in* Escherichia coli

1. Isolation and cloning of the PHA synthesizing genes from *Streptomyces aureofaciens* NRRL 2209

Cultural studies with Streptomyces aureofaciens NRRL 2209 for accumulation of PHB

S. aureofaciens cells grown in Kannan – Rehacek medium were stained with Nile blue-A and observed under fluorescence microscope. The cells showed characteristic orange fluorescence indicating intracellular accumulation of PHA granules. Freeze-dried cells of *S. aureofaciens* when analyzed by gas chromatography revealed the accumulated PHA to be poly(3-hydroxybutyrate). Maximum accumulation of 2.4% PHB of cell dry weight was achieved in 16h of culture.

Isolation of PHB synthesizing genes from S. aureofaciens NRRL 2209

*Streptomyces aureofaciens Sau*3A I partial genomic library in *Escherichia coli* was screened for the presence of *phaC* (PHA synthase) gene by colony hybridization using 3' region of the $phaC_{Re}$ gene of *Ralstonia eutropha* as the hybridization probe. Plasmid DNA was isolated from 12 positive clones and was further subjected to Southern hybridization using the 3'region of the $phaC_{Re}$ gene of *Ralstonia eutropha.* Based on signal intensity three clones were selected and the *Escherichia coli* cells harboring putative $phaC_{Sa}$ clones were checked for PHA accumulation by fluorescence microscopy and gas chromatography. Recombinant *E. coli* cells harboring pSa240 clone showed characteristic orange fluorescence when stained with Nile blue-A. GC analysis revealed a peak corresponding to the propyl ester of standard PHB. Thus, Plasmid pSa240 which contains an approximate 5.0 kb *Sau*3A I genomic DNA fragment from *S. aureofaciens* NRRL 2209, apparently carries all the necessary genetic information to order and direct poly(3-hydroxybutyrate) synthesis in recombinant *Escerichia coli* harboring it.

2. Sequencing and sequence analysis of a ~5.0 kb genomic DNA fragment of *Streptomyces aureofaciens* harboring the PHB biosynthetic genes

pSa240 plasmid DNA was restriction digested with various restriction endonucleases singly and in combinations. A restriction endonuclease map of the insert was generated. To get the complete nucleotide sequence of the ~ 5.0 kb DNA insert nine

overlapping subclones were generated. Attempts were made to sequence these. But due to high G+C content of *S. aureofaciens*, nine subclones proved insufficient to get complete sequence data. Therefore Tn5 transposon insertion system was used to generate frequent and overlapping DNA sequences. These were sequenced to derive a sequence of 4826 bp. This sequence has 74.6% G+C content. The restriction endonuclease map of this sequence matches the one generated earlier. The sequence was analyzed using Frameplot method for predicting protein coding region of bacterial DNA with high G+C content. Six ORFs with GTG as the start codon and four ORFs with ATG as start codon were obtained. None of these showed sequence similarity with any of the reported sequences at amino acid level. ORF 3, however, did show presence of a lipase box motif found in all the polyhydroxyalkanoate synthase (*phaC*) genes reported tilldate. ORF 3 also has two histidine residues present as Gly^{522}-His^{523} and Asp^{600}-His^{601} dyads, probably forming a part of the catalytic triad, needed for synthase function. This ORF has all the attributes of a putative $phaC_{Sa}$ gene. ORF 5 shows the presence of NADP binding site at its 5' end indicating it to be putative NADPH dependent acetoacetyl-CoA reductase ($phaB_{Sa}$) gene. Putative promoter regions, ribosome binding sites and transcription termination site have been identified for ORF 3 and ORF 5. ORF 4 shows significant homology with the hypothetical regulatory protein of *Streptomyces coelicolor*. No ORF was found to which β - ketothiolase function could be assigned.

β-ketothiolase and NADPH dependent acetoacetyl Co-A reductase activities were determined in crude cellular extracts of untransformed and recombinant *E. coli.* β-ketothiolase levels were similar in untransformed and recombinant *E. coli* while NADPH dependant acetoacetyl-CoA reductase activity was detected only in the recombinant *E. coli.*

Transposon insertion clones with one or more of the ORFs disrupted were analyzed for PHB accumulation and NADPH dependent acetoacetyl-CoA reductase. Based on results obtained, it was concluded that ORF 3 (or 8) and ORF 5 codes for PHA synthase and NADPH dependent acetoacetyl-CoA reductase respectively. The pSa240 DNA fragment from *S. aureofaciens* probably does not carry the *phaA* gene and the host *E. coli* provides this function. In the eventuality of the *E. coli* catabolic β-ketothiolase not participating in PHB synthesis, intermediates of fatty acid β-oxidation or fatty acid synthesis might be channelized to PHB biosynthesis in recombinant *E. coli.*

3. PHB synthesizing genes from *Streptomyces aureofaciens* NRRL 2209: Heterologous expression in *Escherichia coli*

The well-defined physiological environment, biochemistry and genetics of *Escherichia coli* make it a perfect host for heterologous expression of foreign proteins.

PHB accumulation in recombinant *E. coli* harboring pSa240 clone from *S. aureofaciens* was studied in complex medium supplemented with different 'C' sources (such as glycerol, glucose, molasses, palm oil, sucrose and ethanol) and nitrogen sources (as yeast extract, peptone and corn steep liquor). Glycerol and yeast extract-peptone were found to support maximum PHB accumulation in recombinant *E. coli*. PHB accumulation in recombinant *E. coli* was studied in defined media supplemented with different amino acids. The results were analyzed using 2^3 factorial analysis.

PHB was extracted from the recombinant *E. coli* using chloroform, sodium hypochlorite dispersion followed by non-solvent precipitation. The PHB was further characterized by, (i) NMR spectroscopy, (ii) Gel permeation chromatography and (iii) Scanning electron microscopy.

The identity of PHB was confirmed by NMR analysis of polymer extracted from recombinant pha_{Sa}^{+} *E. coli*. Gel permeation chromatography studies of polymer extracted from recombinant pha_{Sa}^{+} *E. coli* revealed weight average molecular weight (Mw), number average molecular weight (Mn) and polydispersity index (PI) of the polymer to be 2.85×10^5, 1.065×10^5 and 2.64 respectively. The PHB granules extracted from recombinant pha_{Sa}^{+} *E. coli* were spherical in shape as observed by SEM. The size distribution of PHB granules ranged from 0.11 to 0.35 μm with the mean value of 0.23 ± 0.06 μm.

CHAPTER 1

GENERAL INTRODUCTION

As a rule, synthetic polymers are relatively inexpensive materials developed for durability, rigidity, permeability and transparency. These conveniences enhance the quality and comfort of life in modern society. The intrinsic qualities of durability and resistance to degradation have over the last two decades been increasingly regarded as a source of environmental and waste management problem emanating from plastic materials (Poirier *et al*. 1995). The fact that a major portion of domestic waste comprises of non-biodegradable plastics, a great deal of interest has developed in recycling plastics and in producing plastic materials that can be safely and easily disposed off in the environment. One option is to produce truly biodegradable polymers, which may be used in the same applications as the existing synthetic polymers. These materials, however, must be processible, impervious to water and retain their integrity during normal use but readily degrade in a biologically rich environment. Several different types of degradations occur in the environment. These include biodegradation, photodegradation, oxidation, and hydrolysis. However, a fully biodegradable polymer is defined as a polymer that is enzymatically and completely converted by living organisms, usually microorganisms, to carbon dioxide, water and humic material (in case of anaerobic biodegradation, to carbon dioxide, methane and humic material). Biodegradable materials under development include polylactides, polyglycolic acids, polyhydroxyalkanoates (PHAs), aliphatic polyesters, polysaccharides and their co-polymers and/or blends (Steinbüchel, 1991).

1.1 Polyhydroxyalkanoates (PHAs)

PHAs are polyesters of various hydroxyalkanoate monomers (Fig. 1.1). The first PHA, the homopolymer poly(3-hydroxybutyrate) (PHB) was discovered by Maurice Lemoigne in 1925 (Jackson and Srienc 1994). Approximately 150 different hydroxyalkanoic acids are at present known as constituents of these bacterial storage polyesters (Steinbüchel and Valentin 1995). PHAs synthesized by many Gram positive and Gram negative bacteria as storage compounds are deposited as insoluble inclusions in the cytoplasm (Steinbüchel 1991). These water insoluble PHAs exhibit rather high molecular weights, thermoplastic and/or elastomeric features and some other interesting physical and material properties. The main hindrance in use of PHAs as replacement for existing petroleum based plastics is their higher cost of production. Inputs in the areas such as isolation of new PHA accumulating wild type strains, isolations of genes directing PHA synthesis, molecular characterization of PHA synthesizing genes, *in*

vivo/in vitro metabolic engineering for PHA production would aid in efficient and cost effective production of PHAs.

1.2 Properties of PHAs

PHAs are a family of optically active biological polyesters, containing (*R*)-3HA monomer units (Anderson and Dawes 1990). The 3-hydroxyalkanoic acids are all in the *R* configuration due to the stereospecificity of the polymerizing enzyme, PHA synthase. In one rare case a small portion of the *S* monomers were also detected (Haywood *et al.* 1991). At the C-3 or β position, an alkyl group, which can vary from methyl to tridecyl is positioned. This alkyl chain can be saturated, aromatic, unsaturated, halogenated, epoxidized or with branched monomers (Abe *et al.* 1990, Doi and Abe 1990, Fritzsche *et al.* 1990a b c, Kim *et al.* 1991, Kim *et al.* 1992b, Choi and Yoon 1994, Hazer *et al.* 1994, Curley *et al.* 1996, Song and Yoon 1996, Garcia *et al.* 1999, Arkin *et al.* 2000). Along with the variation in the alkyl substituent, the position of the hydroxyl group is variable, and 4-, 5- and 6- hydroxyacids have been incorporated. Side chains can be modified further to get different PHAs (de Koning *et al.* 1994, Gagnon *et al.* 1994a, b). Such a vast array of variations forms the basis for diversity of the PHA polymer family. PHAs are insoluble in water and exhibit a rather high degree of polymerization ranging from 10^5 to almost 10^7 Da. They are nontoxic and biocompatible.

PHAs are classified as ssc – PHAs, msc – PHAs and lsc – PHAs based on short, medium and long chain length, respectively, of hydroxyalkanoic acid monomers (Steinbüchel *et al.* 1992). ssc – PHAs are composed of C_3 to C_5 3-hydroxy/4-hydroxy fatty acids. Poly(3-hydroxybutyrate) (PHB), the first of the PHAs to be studied extensively falls in this group of PHAs. msc – PHAs are composed of C_6 to C_{16} 3-hydroxy fatty acids. This group includes polymers such as poly(3-hydroxyhexanoate) P(3HHx), poly(3-hydroxyheptanoate) P(3HHp), poly(3-hydroxyoctanoate) P(3HO), poly(3-hydroxydodecanoate) (3HDD), etc. The term lsc – PHAs is reserved for the PHAs composed of more than C_{16} 3-hydroxy fatty acids. These classes are also referred to as scl-, mcl- and lcl-PHAs.

PHB, the most well known member of the PHAs, is an isotactic polymer. It exists in a fluid amorphous state within the cell (Ellar *et al.* 1968, Dunlop and Robards 1973, Barnard and Sanders 1989, Doi 1990, Lauzier *et al.* 1992). However, freeze-drying/organic solvent treatment during its extraction irreversibly converts it to a

crystalline state (Hahn *et al.* 1995). In this crystalline state PHB is stiff but brittle material. The glass transition temperature and melting temperature for PHB are 4°C and 180°C respectively (Sudesh *et al.* 2000). The specific density is 1.25 g /cm^3 (Ramsay *et al.* 1993). It is moisture resistant, shows piezoelectric effect and above all is truly and completely biodegradable (Barham *et al.* 1984, Doi 1990, Steinbüchel 1991).

The properties of some PHAs and synthetic polymers are shown in Table 1.1 for comparison (Lee 1996a, Poirier *et al.* 1995, Chen *et al.* 2000, Sudesh *et al.* 2000).

1.3 Uses and applications of PHAs

PHAs are natural thermoplastic polyesters, which can be replacements for petrochemical polymers currently in use for packaging and coating applications. They can be used for consumer packaging items such as bottles, cosmetic containers, pens and golf-tees. They have been used as hot-melt adhesives (Madison and Huisman 1999). These can be used for manufacture of disposable items such as razors, utensils, diapers or different personal hygiene products (Lee 1996a). PHAs have been processed into fibers, which then were used to construct materials such as nonwoven fabrics (Steel and Norton-Berry 1986). They can be used in the manufacture of latex paints (Steinbüchel 2001). PHAs also promise to be a new source of small molecules, some of which have potential applications as biodegradable solvents. These include β-hydroxy acids, 2-alkenoic acids, β-hydroxyalkanols, β-acyllactones, β-amino acids, and β-hydroxyacid esters (Williams and Peoples 1996). Synthesis of PHAs in plants can be used as a new tool to study the quality and relative quantity of the carbon flow through beta-oxidation as well as to analyze the degradation pathway of unusual fatty acids (Mittendorf *et al.* 1999). Transgenic *Arabidopsis* expressing the *pha* synthase from *Pseudomonas aeruginosa* in peroxisomes has been used to analyze how fatty acids having a double bond at an even numbered carbon are degraded (Allenbach and Poirier 2000).

PHAs being biodegradable and biocompatible have applications in medical therapeutics. PHAs can be used to fabricate three-dimensional, porous, biodegradable heart valve scaffold (Sodian *et al.* 2000a, b, c), in bone fracture fixation (Galego *et al.* 2000), in manufacture of surgical pins, sutures, staples, swabs, fixation rods and cardiovascular stents (Scholz 2000). PHAs can be used as carriers for long term slow release of drugs, insecticides, herbicides and fertilizers and in wound dressing (Lee 1996a). The biomedical field is in need of tissue adhesives and tissue sealants,

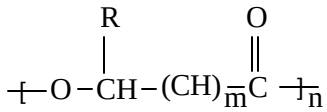

Fig 1.1 Chemical structure of PHAs. m = 1,2,3, yet m = 1 is common, 'n' can range from 100 to several thousands. 'R' is variable. When m = 1, R = CH_3, the monomer structure is 3-hydroxybutyrate. While m = 1, R = C_3H_7, it is a 3-hydroxyhexanoate monomer.

Table 1.1 Properties of some PHAs and synthetic polymers (Lee 1996a, Poirier *et al.* 1995, Chen *et al.* 2000, Sudesh *et al.* 2000)

Polymer	**Melting Temperature °C**	**Glass-transition temperature °C**	**Young's modulus GPA**	**Elongation to break %**	**Tensile strength MPa**
P(3HB)	180	4	3.5	5	40
P(3HB-co-3HV)*	145	-1	-	-	32
P(4HB)	53	- 48	149	1000	104
PHA_{mcl}	45-54	-25 to – 40	-	-	-
Polypropylene	176	- 10	1.7	400	34.5
Polystyrene	240	100	3.1	-	50

* P(HB-HV) copolymer containing 20 mol% C5 and 80 mol% C4 monomers

soft tissue fillers to replace collagen,and tissue adhesion preventives for the treatment of severe burns to mention just a few of the current challenges. PHAs and modified PHAs will be considered in the future as candidates for an increasing variety of biomedical challenges.

1.4 Physiology of PHA metabolism

Bacteria that are used for the production of PHAs can be divided into two groups based on the culture conditions required for PHA synthesis. The first group that includes *Ralstonia eutropha*, methylotrophs, and pseudomonads, require the limitation of an essential nutrient element in the presence of an excess carbon source for efficient synthesis of PHAs. The second group which includes *Alcaligenes latus*, *Azotobacter vinelandii* and recombinant *Escherichia coli*, does not require nutrient limitation for PHA synthesis and can accumulate PHA during growth (Wang and Lee 1997). However, even in *Alcaligenes latus* PHB synthesis is enhanced by nitrogen limitation suggesting that this growth during PHA accumulation is not an efficient process.

PHAs are stored as intracellular cytoplasmic inclusions. Intracellular deposition of metabolites as a reduced polymer provides the advantage that they become unavailable as a carbon source for the competing organisms, and that they are osmotically inert and do not therefore affect the osmotic pressure of the cells (Steinbüchel 1991). The presence of PHB in a cell frequently, but not universally, retards the degradation of cellular components such as RNA and proteins during nutrient starvation. PHB enhances survival of some, but not all, of the bacteria and serves as a carbon and energy source for spore formation in *Bacillus* sp. (Anderson and Dawes 1990). Similarly, PHB acts as a carbon and energy source for the encystment of azotobacters.

Senior and Dawes (1971) proposed that PHAs serve as a sink for reducing power and could therefore be regarded as a redox regulator within the cell. It was found that for the members of the Azotobacteriaceae, the reductive step of PHA synthesis appeared to serve as an electron sink for the reducing power, which accumulated when electron flow through the electron transfer chain was affected as a consequence of oxygen limitation (Senior and Dawes 1973).

In *Ralstonia eutropha* and *Rhodospirillum rubrum* the PHA biosynthesis includes pyridine nucleotide dependent reduction of acetoacetyl-CoA. Thus, PHB is a sink for reducing equivalents and the can be considered to be a fermentation product. By contrast, in *Pseudomonas oleovorans* and *P. aeruginosa*, oxygen deficiency exerts a negative effect on the accumulation of PHA, because in these organisms either reducing equivalents are formed or energy in the form of ATP is consumed (Steinbüchel 1991).

In *Rhizobium* and *Bradyrhizobium japonicum*, at the bacteroid stage, the nitrogen fixation apparatus competes with PHB formation for reducing equivalents (Povolo *et al.* 1994). *Rhizobium* apparently evolved mechanisms to maintain a

functional TCA cycle under anaerobic or microaerobic conditions. In the bacteroids, the TCA cycle may be limited by the redox state of NADH/NAD^+ at the 2-ketoglurate-dehydrogenase complex, and a number of pathways may be involved in bypassing this block. These pathways include PHB synthesis, glutamate synthesis, glycogen synthesis, GABA shunt and glutamine cycling (Poole and Allaway 2000).

The lipidic polymer PHB found in the plasma membranes of *Escherichia coli* complexed to calcium polyphosphate reportedly functions as calcium channels and are probably evolutionary antecedents of protein Ca^{2+} channels (Reusch and Sadoff 1988, Reusch *et al.* 1995). The *Streptomyces lividans* KcsA potassium channel was found to contain poly-(R)-3-hydroxybutyrate. KcsA protein creates an environment such that discrimination between K(+) and Na(+) is accomplished by adjusting the ligand geometry in cation binding cavities formed by PHB and polyP (Reusch 1999). PHB and polyP have also been found in human erythrocyte Ca^{2+}-ATPase pump, indicating that these probably are supramolecular structures in which proteins, polyP and PHB cooperate in forming well-regulated and specific cation transfer systems (Reusch 2000). PHB has also been found to be a constituent of the membranes and the cytoplasms of genetically competent *Azotobacter vinelandii, Bacillus subtilis, Haemophilus influenzae* and *Escherichia coli*, and is thought to play an important role in transmembrane transport of DNA (Reusch and Sadoff 1983, Reusch *et al.* 1986).

It is increasingly convincing that PHA, especially PHB, is not just an inert storage polymer confined to certain bacteria, but is instead a ubiquitous, interactive, solvating biopolymer involved in important physiological functions. This low molecular weight non-storage PHA referred to as cP(3HB) (c-complexed) is associated to other macromolecules and is widely distributed in biological cells, being found in representative organisms of nearly all phyla (Reusch 1995, Huang and Reusch 1996).

1.5 PHA biosynthesis

Nature has evolved several different pathways for PHA formation, each optimized for the ecological niche of the PHA producing microorganisms. These include:

PHA biosynthesis represented by *Ralstonia eutropha*,

PHA synthesis with an enoyl-CoA hydratase,

Methylmalonyl-CoA pathway for P(3HB-3HV) synthesis from sugars,

msc-PHAs biosynthesis from fatty acids represented by the pseudomonads,

msc-PHAs biosynthesis from carbohydrates represented by the pseudomonads

1.5.1 PHA biosynthesis represented by *Ralstonia eutropha*

The PHA-biosynthetic pathway in *Ralstonia eutropha* is most extensively studied. In this bacterium, PHB is synthesized from acetyl-CoA by sequential action of three enzymes (Fig. 1.2). Biosynthetic ketothiolase (EC 2.3.1.9) catalyses the formation of a carbon-carbon bond by a biological Claisen condensation of two acetyl-CoA moieties (Masamune *et al.* 1989a, b). NADPH dependent acetoacetyl-CoA reductase (EC 1.1.1.36) catalyses the stereoselective reduction of acetoacetyl-CoA formed in the first reaction to R-(-)-3-hydroxybutyryl CoA. The third reaction of this pathway is catalyzed by the enzyme PHA synthase that catalyzes the polymerization of R-(-)-3-hydroxybutyryl-CoA to form PHB. The EC number is yet to be assigned to PHA synthase (Steinbüchel and Schlegel 1991, Belova *et al.* 1997).

The nature of the polyester depends on the specific bacterium, the biosynthetic enzyme, the genetic background, the substrate(s) and the growth conditions. Various carbon sources can be utilized by *R. eutropha* for growth and/or PHA production. Addition of propionic acid or valeric acid to the growth medium containing glucose leads to the production of a random copolymer composed of 3-hydroxybutyrate and 3-hydroxyvalerate (Fig. 1.2). Successful utilization of lactic acid and plant oils for PHB production has also been reported (Linko and Vaheri 1993, Fukui and Doi 1998). Under autotrophic conditions *R. eutropha* can also utilize carbon dioxide for the production of P(3HB) (Taga *et al.* 1997). *R. eutropha* is also capable of accumulating PHA from specialized carbon sources such as 4-hydroxybutyric acid, γ-butyrolactone and 1,4 butanediol which give rise to the incorporation of 4HB monomers along with 3HB (Sudesh *et al.* 2000).

Two β-ketothiolases are reported from *R. eutropha*, which together accept C4 to at least C10 β-ketoacyl-CoAs (Haywood *et al.* 1988a). The NADPH dependent acetoacetyl-CoA reductase, however, has been shown to be active with C4 to C6 β-ketoacyl-CoAs to generate D (-)-3-3hydroxyacyl-CoAs (Haywood *et al.* 1988b). In *R. eutropha*, the PHAs formed generally contains monomers having only 3-5 carbon atoms. This has led to the conclusion that in *R. eutropha*, the PHA synthase enzyme is

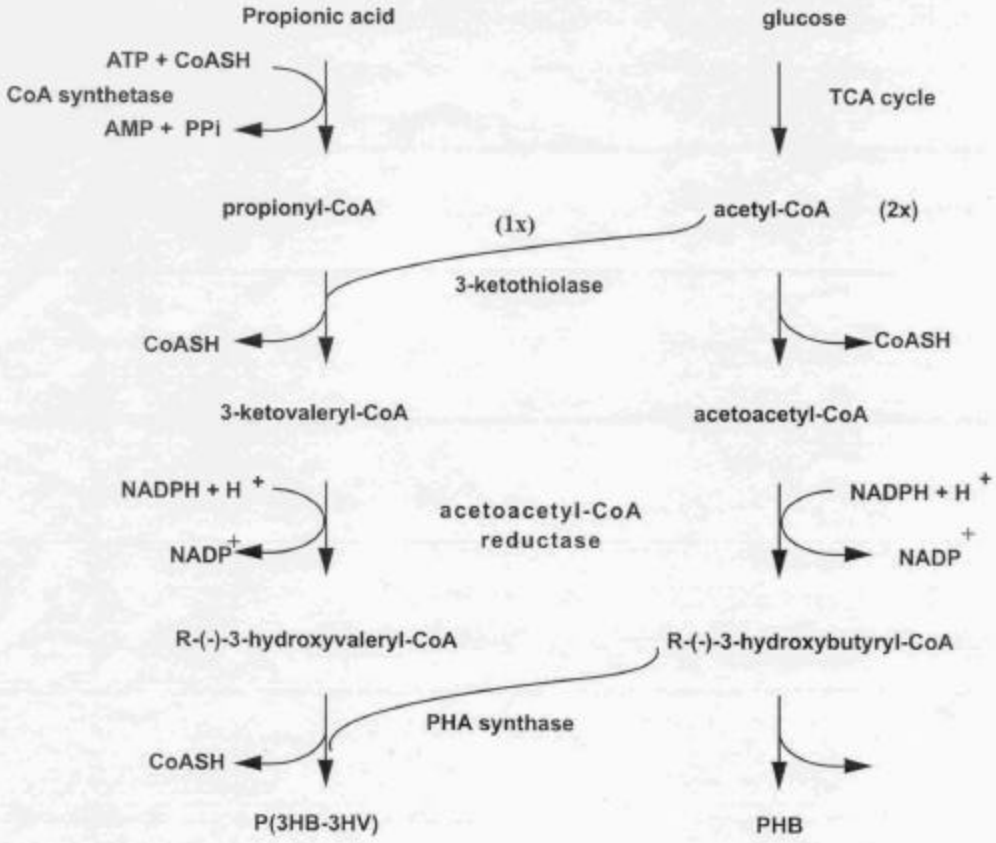

Fig. 1.2 PHA biosynthesis represented by *Ralstonia eutropha* (Poirier and Nawrath 1998)

only active towards ssc-HA. However, the position of the oxidized carbon in the monomer doesn't seem to be a crucial factor, which explains the incorporation of 4-HA besides the more common 3-HA (Doi *et al.* 1990, Steinbüchel *et al.* 1994). Recent findings, however, indicate that the polymerizing enzyme of *R. eutropha* may actually have a broader range of substrate specificity. This was evident when the PHA synthase gene of *R. eutropha* was expressed in a heterologous environment which can provide for a wider range of HA monomers. At present it has been shown that *R. eutropha* PHA synthase can incorporate 3HO and 3HDD units (Antonio *et al.* 2000, Clemente *et al.* 2000).

1.5.2 PHA synthesis with an enoyl-CoA hydratase

In *Rhodospirillum rubrum*, Moskowitz and Merrick (1969) proposed a pathway that included two hydratases, one specific for the *R* enantiomer and the other specific for the *S* enantiomer (Fig. 1.3). In *Aeromonas caviae* the PHA biosynthetic pathway proceeds from enoyl-CoA derivatives of the fatty acid oxidation pathway (Fig. 1.4). PhaJ converts crotonyl-CoA, pentenoyl-CoA and hexenoyl-CoA to PHA precursors but it does not convert octenoyl-CoA (Fukui and Doi 1997, Fukui *et al.* 1998). *Methylobacterium rhodesenium* again, uses two hydratases for PHB synthesis (Mothes and Babel 1995). Along with two hydratases, this bacterium has two constitutive acetoacetyl-CoA reductases, one NADH dependent and the other NADPH dependent (Mothes and Babel 1994). These four enzymes work in combination under different conditions to synthesize 3-hydroxybutyryl-CoA needed for PHB formation.

1.5.3 Methylmalonyl-CoA pathway for P(3HB-3HV) synthesis from sugars

In *Rhodococcus ruber* and *Nocardia corallina*, during P(3HB-3HV) synthesis monomers are derived from both the fatty acid degradation pathway and the traditional *R. eutropha* PHB biosynthetic pathway. In this pathway referred to as methylmalonyl-CoA pathway, succinyl-CoA is converted to methylmalonyl-CoA, which is decarboxylated to propionyl-CoA that is incorporated in P(3HB-3HV) (Fig. 1.5) (Williams *et al.* 1994, Valentin and Dennis 1996).

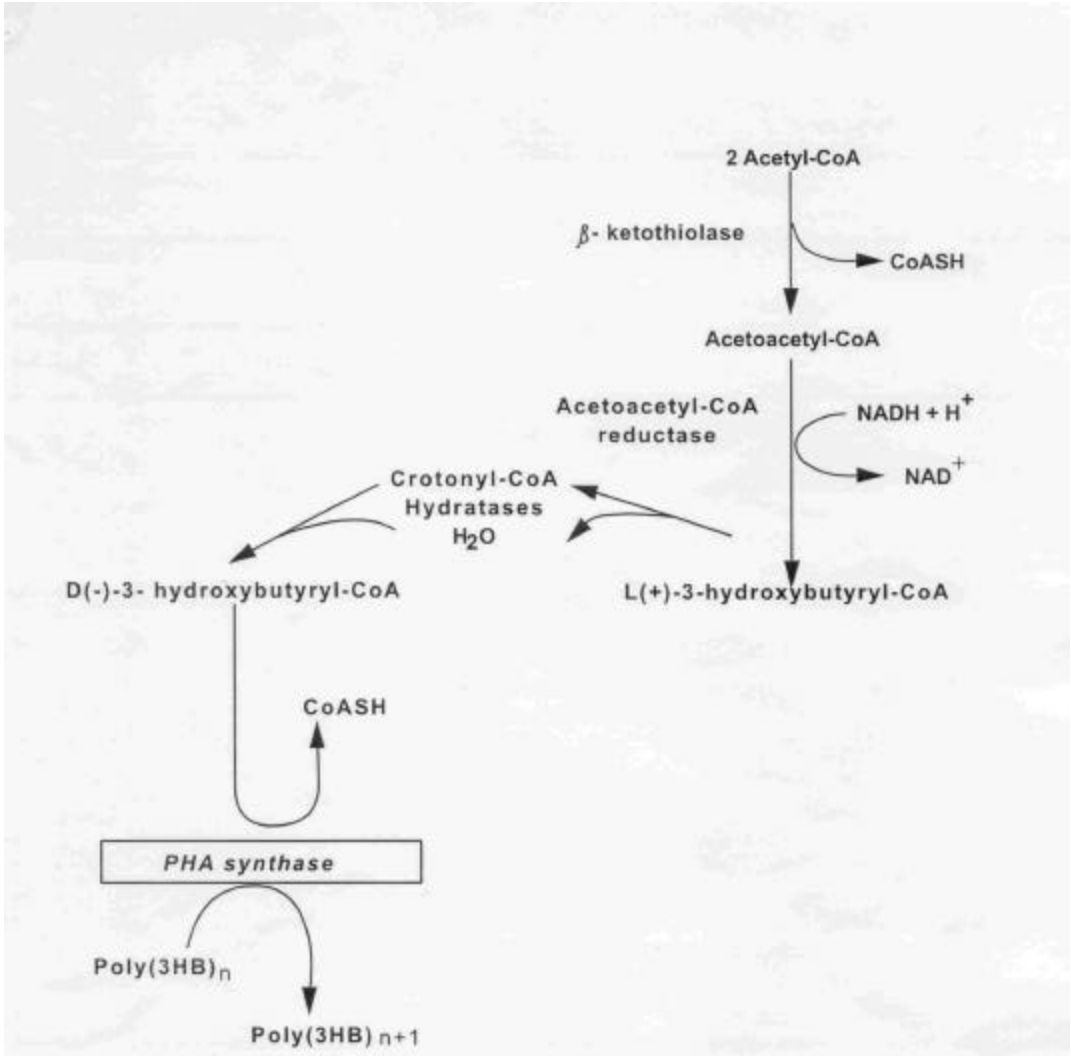

Fig. 1.3 *Rhodospirillum rubrum* PHA-biosynthetic pathway. (Steinbüchel 1991)

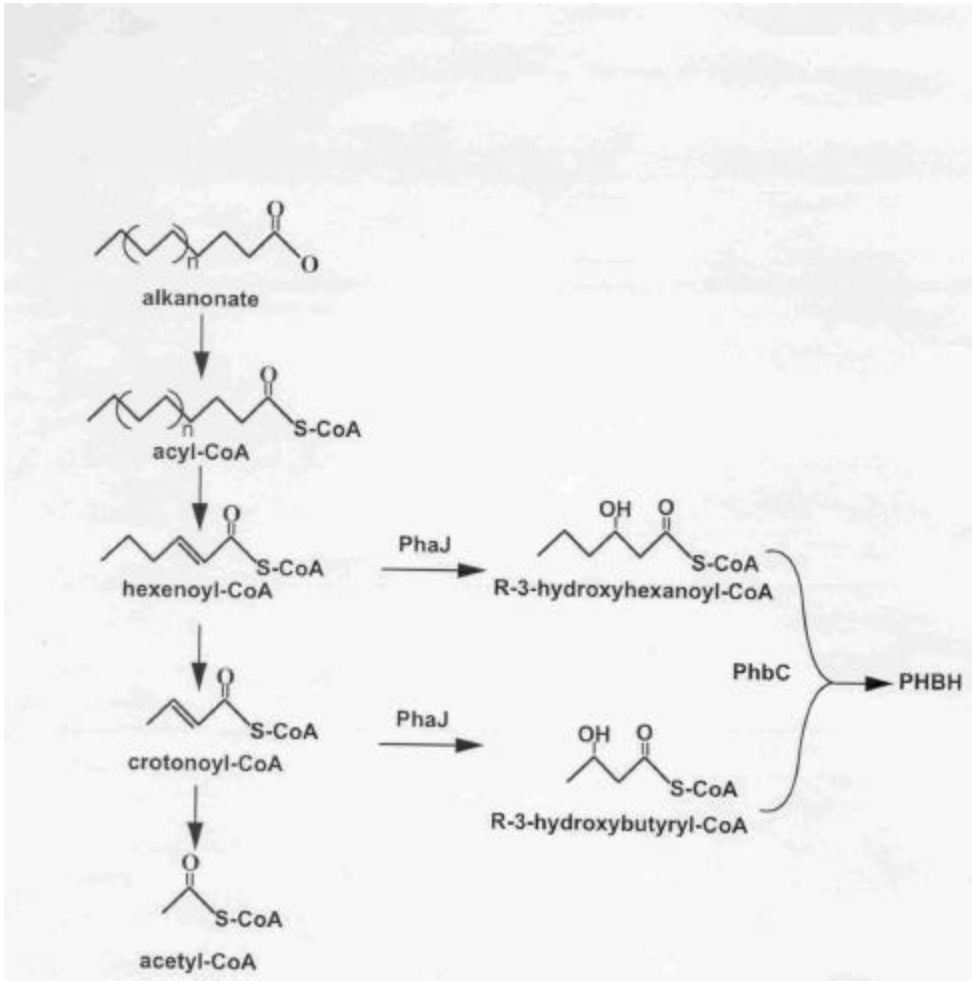

Fig. 1.4 PHA biosynthesis with an enoyl -CoA hydratase in *Aeromonas caviae* (Madison and Huisman 1999)

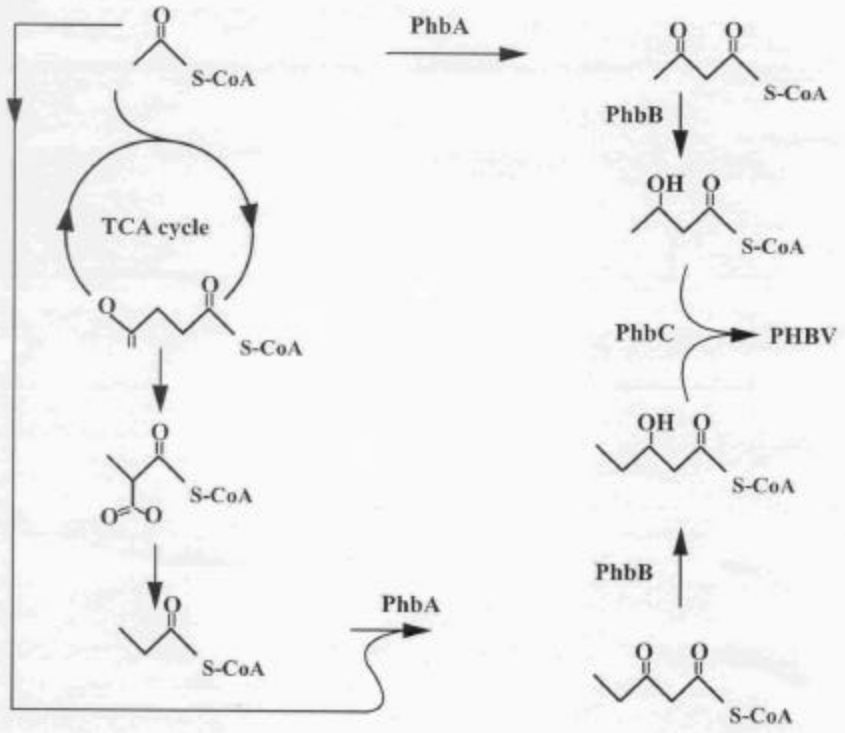

Fig. 1.5 Methylmalonyl-CoA pathway for P(3HB-3HV) synthesis from sugars (Williams *et al.* 1994, Valentin and Dennis 1996)

1.5.4 msc-PHAs biosynthesis from fatty acids represented by the pseudomonads

msc-PHAs were first discovered in *Pseudomonas oleovorans* grown on octane (DeSmet *et al.* 1983). The inclusions formed were determined to be made of a copolyester consisting of 89% (*R*)-3-hydroxyoctanoate and 11% (*R)*-3-hydroxyhexanoate (Lageveen *et al.* 1988). PHAs formed by pseudomonads of the rRNA homology group I were directly related to the structure of the alkane, alkene, or fatty acid carbon source (Lagaveen *et al.* 1988, Brandl *et al.* 1988, Huisman *et al.* 1989). The composition of the polymers depends on the length of the carbon backbone of the substrate used. For C-even substrates, only C-even monomers were found, the smallest was 3HHx. For C-odd substrates only C-odd monomers were found, with 3HHp being the smallest monomer.

In pseudomonads, the intermediates of fatty acid oxidation are used as precursors for PHA biosynthesis (Fig. 1.6). In this pathway, fatty acids are degraded by the removal of C_2 units as acetyl-CoA. The acyl-CoA thus formed is oxidized to 3-ketoacyl-CoA via 3-hydroxyacyl-CoA intermediates (Lageveen *et al.* 1988). The substrate specificity of this msc-PHA synthase ranges from C6 to C14 (*R*)-3-hydroxyalkanoyl-CoAs, with preference for the C8, C9 and C10 monomers (Huisman *et al.* 1989). However, the intermediate of β-oxidation pathway is (*S*)-hydroxyacyl-CoA. For PHA biosynthesis, (*S*) form needs to be converted to (*R)*-hydroxyacyl-CoA. Whether this PHA precursor is the product of a reaction catalyzed by a hydratase, by the epimerase activity of the β-oxidation complex, or by a specific 3-ketoacyl-CoA reductase is unknown.

1.5.5 msc-PHAs biosynthesis from carbohydrates represented by the pseudomonads

Most of the rRNA homology group I pseudomonads except *P. oleovorans*, can also synthesize PHA containing msc/mcl monomers from unrelated carbon sources such as carbohydrates: gluconate, fructose, acetate, glycerol and lactate. PHA accumulated contains 3-hydroxydecanoate as the predominant monomer (Anderson and Dawes 1990). Psudomonads that have shown to possess this pathway are *P. aeruginosa, P. aureofaciens, P. citronellolis, P. mendocina, P. putida, P. chlororaphis, P. marginalis* and *Pseudomonas* sp. strain DSM 1650 (Haywood *et al.* 1990, Timm and Steinbüchel 1990, Steinbüchel 1991). In this pathway, the 3-hydroxyacyl monomers are derived

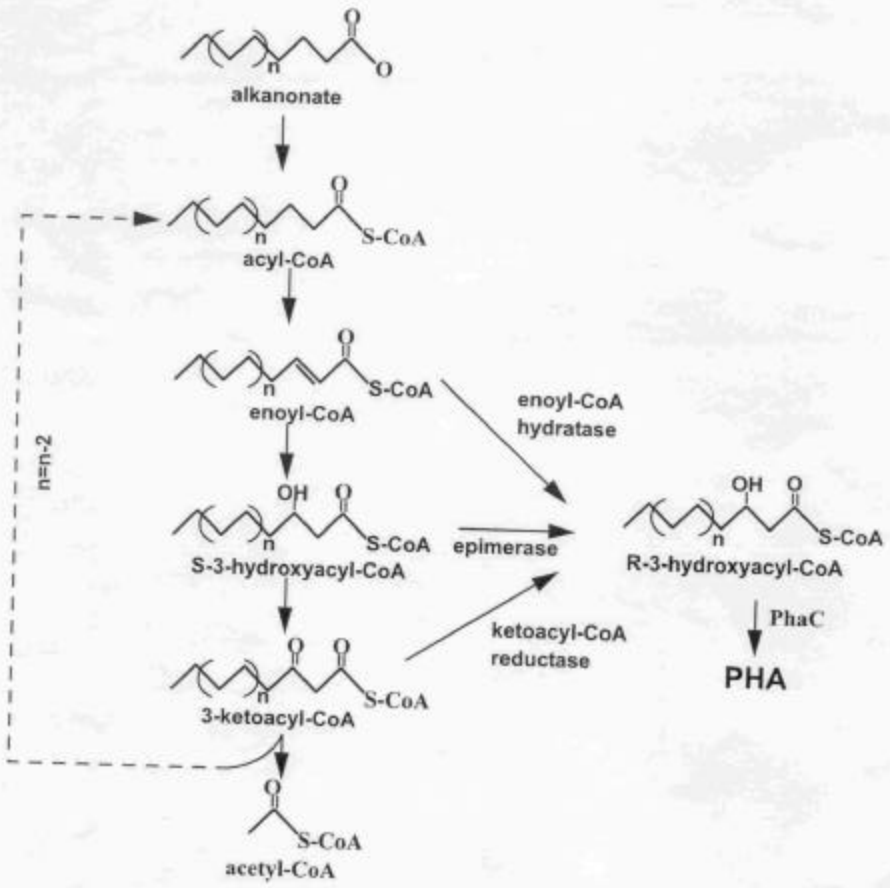

Fig. 1.6 msc-PHAs biosynthesis from fatty acids represented by the pseudomonads (Lageveen *et al.* 1988)

from the *de novo* fatty acid biosynthesis pathway (Fig. 1.7) (Huijberts *et al.* 1992). The intermediates of *de novo* fatty acid biosynthesis pathway are in the form of (*R)*-3-hydroxyacyl-ACP that need to be converted to (*R*)-3-hydroxyacy-CoA form for incorporation in PHA. The 3-hydroxyacyl-CoA-ACP transferase encoded by phaG gene, has been shown to be capable of channeling the intermediates of the *de novo* fatty acid biosynthesis pathway to PHA biosynthesis (Rehm *et al.* 1998).

1.6 Molecular organization of PHA-biosynthetic genes

Given the diversity of P(3HB) biosynthetic pathway discussed above, it is not surprising that the *pha* loci have diverged considerably. Based on the molecular data available till-date, seven types of arrangements have been observed for PHA synthesizing genes (Fig. 1.8) (Madison and Huisman 1999, McCool and Cannon 2001).

Genes encoding for proteins involved in the biosynthesis of PHA are referred in alphabetical order as *phaA* (β-ketothiolase), *phaB* (acetoacetyl-CoA reductase), *phaC* (PHA synthase), *phaG* (3-hydroxyacyl-acyl-carrier protein-coenzyme A transferase), *phaJ* (enoyl-CoA hydratase) etc., whereas the genes required for the degradation are referred in reverse alphabetical order such as *phaZ* for PHA depolymerases, *phaY, phaX, phaW,* etc. The genes for phasins are referred to as *phaP*. The gene product i.e protein is indicated with first letter in upper case e.g β-ketothiolase is written as PhaA. The origin of a gene or a protein is indicated by the first letter of the genus and the species and added as a subscript (Rehm and Steinbüchel 1999).

In *Acinetobacter* sp., *Burkholderia* sp., *Alcaligenes latus*, *Pseudomonas acidophila* and *Ralstonia eutropha*, the *phaC, phaA* and *phaB* genes are arranged in tandem on the chromosome although not necessarily in the same order (Schembri *et al.* 1995, Peoples and Sinskey 1989a, Peoples and Sinskey 1989b, Umeda *et al.* 1998, Madison and Huisman 1999, Rodrigues *et al.* 2000). However, in *Paracoccus denitrificans*, *Rhizobium meliloti* and *Zoogloea ramigera* the *phaAB* and *phaC* loci are unlinked (Peoples and Sinskey 1989c, Tombolini *et al.* 1995, Yabutani *et al.* 1995, Lee *et al.* 1996, Ueda *et al.* 1996).

PHA polymerase in *Allochromatium vinosum* (formerly called *Chromatium vinosum*), *Thiocystis violacea*, *Thiocapsa pfennigii* and *Synechocystis* is unique in that it is a two -subunit enzyme encoded by the *phaE* and *phaC* genes (Liebergesell and

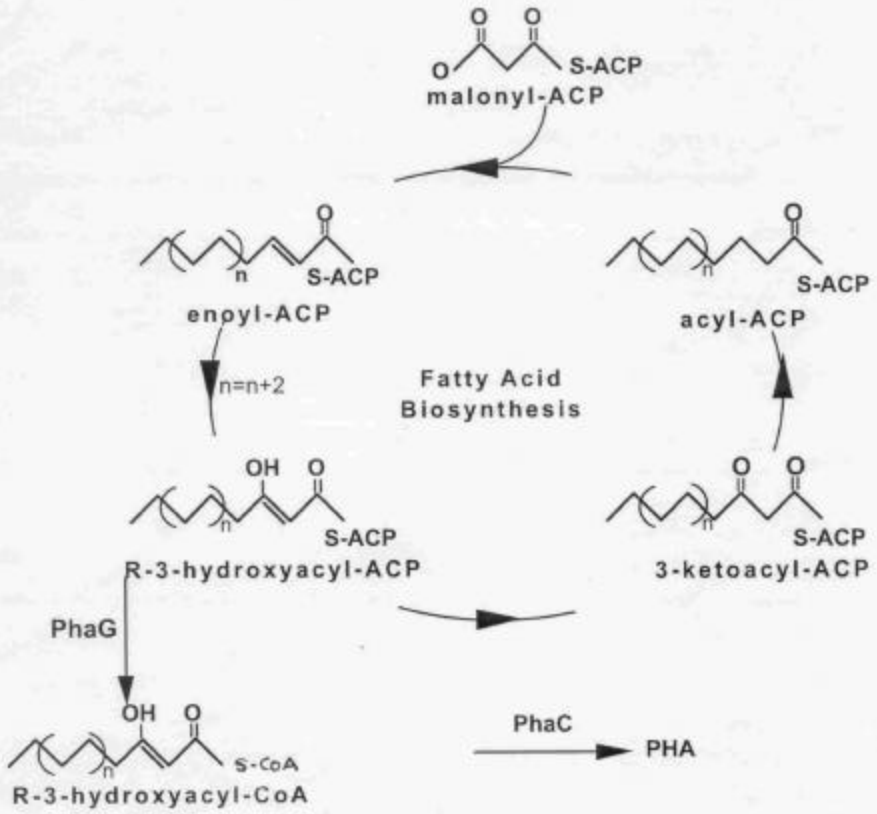

Fig. 1.7 msc-PHAs biosynthesis from carbohydrates represented by the pseudomonads (Rehm *et al.* 1998)

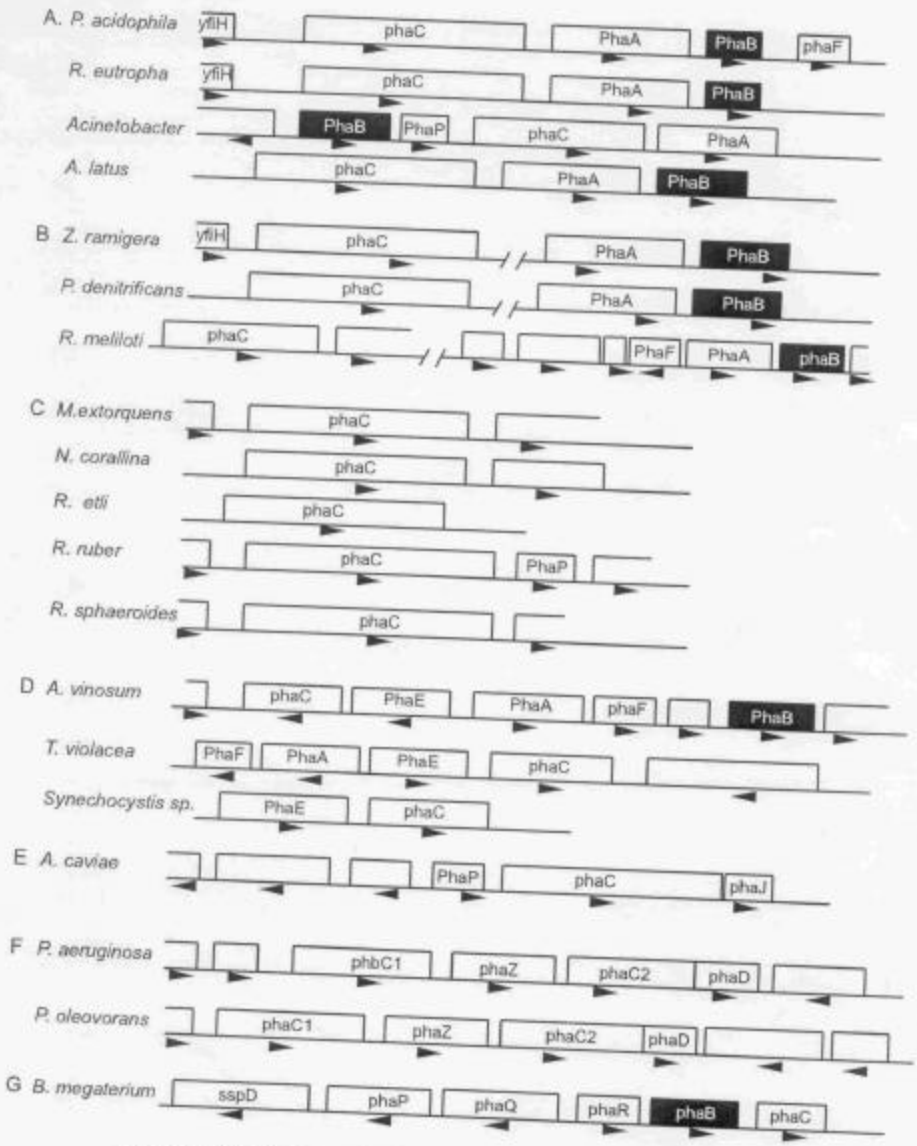

Fig.1.8 Molecular organization of genes relevant for biosynthesis of PHA (Madison and Huisman 1999, McCool and Cannon 2001)

Steinbüchel 1992, Liebergesell and Steinbüchel 1993, Hein *et al.* 1998, Liu and Steinbüchel 2000). But arrangement of PHA synthesizing genes is different amongst these organisms. In *A. vinosum* and *T. violacea*, *phaAB* and *phaEC* are in one locus but divergently oriented (Liebergesell and Steinbüchel 1992, Liebergesell and Steinbüchel 1993). *Synechocystis pha* genes are localized in different sections of the genome with arrangement similar to that in *Z. ramigera* (Taroncher-oldenburg 2000). The *pha* loci in *A. vinosum, P. acidophila, R. eutropha, R. meliloti* and *T. violacea* have an additional gene, *phaF*, of a hitherto unknown function in PHA metabolism (Madison and Huisman 1999).

In *Methylobacterium extorquens, Nocardia corallina, Rhizobium etli, Rhodococcus ruber* and *Rhodobacter sphaeroides*, only the PHA synthase gene has been identified thus far (Pieper and Steinbüchel 1992, Hustede and Steinbüchel 1993, Valentin and Steinbüchel 1993, Cevallos *et al.* 1996, Ji-Hoe and Lee 1997, Hall *et al.* 1998). In *Chromobacterium violaceum* and *Comamonas acidovorans phaC* and *phaA* are arranged in an operon (Sudesh *et al.* 1998, Kolibachuk *et al.* 1999).

In *Aeromonas caviae* PHA synthase gene is flanked by *phaJ* encoding for R-specific enoyl-CoA hydratase (Fukui and Doi 1997). In msc-PHA producing *P. oleovorans* and *P. aeruginosa*, the *pha* loci contain two *phaC* genes separated by the *phaZ* gene, which encodes an intracellular PHA depolymerase (Huisman *et al.* 1991, Timm and Steinbüchel 1992). In *Bacillus megaterium* the *phaC* gene is similar to that of *Allochromatium vinosum* in size and sequence. However, unlike $phaC_{Av}$, $phaC_{Bm}$ requires $phaR_{Bm}$ for PHA accumulation, but not *phaE* (McCool and Cannon 1999, McCool and Cannon 2001). $PhaR_{Bm}$ is probably orthologous replacement for $phaE_{Bm}$.

1.7 Regulation of PHA metabolism

The regulation of PHA synthesis is quite complex. It is exerted at different physiological and genetic levels such as: (i) activation of *pha* gene expression due to specific environmental signals e.g. nutrient starvation, (ii) activation of the PHA biosynthetic enzymes by specific cell components or metabolic intermediates for PHA synthesis, (iii) inhibition of metabolic enzymes of competing pathways and therefore enrichment of required intermediates for PHA synthesis, or (iv) a combination of all these (Kessler and Witholt 2001).

1.7.1 Regulation of PHA production at the enzymatic level

It is well known that PHB synthesis is regulated at the enzymatic level (Senior and Dawes 1971). The intracellular concentration of acetyl-CoA and free coenzymeA play a central role in the regulation of polymer synthesis (Haywood *et al*. 1988b, Mothes *et al*. 1997). Both β-ketothiolases, the biosynthetic and the degradative, exhibit pronounced competitive inhibition by free CoA (Oeding and Schlegel 1973, Haywood *et al*. 1988a). PHB synthesis is stimulated by both high intracellular concentrations of NAD(P)H and high ratios of NAD(P)H/NAD(P) (Lee *et al*. 1995a, Chohan and Copeland 1998). Citrate synthase activity is significantly inhibited by NADH and NADPH, further affirming that PHB accumulation is enhanced by facilitating the metabolic flux of acetyl-CoA to the PHB synthetic pathway. Citrate synthase emerges as a potentially important control point in the whole PHB synthesis process by virtue of its ability to control the availability of CoA (Henderson and Jones 1997), which in turn regulates the activity of β-ketothiolase. An isocitrate dehydrogenase-leaky mutant of *R. eutropha* which exhibits low TCA cycle activity and produces PHB at a faster rate than the wild type organism (Kessler and Witholt 2001), supports this statement. These findings, underline the importance of the redox balance in the cell in the control of PHB formation. Jung *et al*. (2000) have shown that in recombinant *R. eutropha* the biosynthesis rates of PHB were controlled by β-ketothiolase and acetoacetyl-CoA reductase, and especially by β-ketothiolase condensing acetyl-CoA. However, the PHB synthase is the most critical enzyme in determining the accumulation of intracellular PHB.

Limited knowledge is available about the regulation of PHA production in *Pseudomonas*. Many pseudomonads synthesize PHAs either through fatty acid biosynthesis when grown on gluconate or through fatty acid degradation when grown on fatty acids (Huijberts *et al*. 1992). Based on *in vitro* studies it was concluded that (R)-3-hydroxyacyl-CoA are the most likely ultimate substrate for the PHA synthase in *Pseudomonas oleovorans* (Kraak *et al*. 1997). But detailed information is not available as to which intermediates of the β-oxidation serve as substrates for conversion into (R)-3-hydroxyacyl-CoA. *E. coli*, harboring PHA synthase gene from *Pseudomonas* sp., have to have the ongoing β-oxidation slowed down by addition of acrylic acid (Qi *et al*. 1998) to produce PHB. Alternatively, specific *E. coli* mutants deficient in certain steps of β-oxidation to produce PHAs (Langenbach *et al*. 1997, Qi *et al*. 1997). Apparently it

is suggestive of PHA synthase competing with the enzymes of the β-oxidation pathway for substrates.

In the cyanobacterium, *Synechococcus* sp. MA19 PHB synthase activity has been exclusively found in membrane fractions isolated from nitrogen deprived cells under light conditions. Miyake *et al*. (1997) further proposed that the PHB synthase is posttranslationally activated by acetyl phosphate. Moreover phosphotransacetylase, which converts acetyl-CoA to acetyl phosphate is regulated by the acetyl-CoA concentration and carbon to nitrogen ratio (C:N) in the cell. Therefore, acetyl phosphate could act as a signal of C:N balance affecting PHB metabolism.

1.7.2 Regulation of PHA production at the transcription level

In different microorganisms promoters have been either identified experimentally or postulated based on the sequence upstream of PHA biosynthetic genes (Huisman *et al*. 1991, Schubert *et al*. 1991, Liebergesell and Steinbüchel 1992, 1993, Timm and Steinbüchel 1992, Matsusaki *et al*. 1998, McCool and Cannon 1999). In *Acinetobacter* sp. *pha* operon appears to be transcribed by two promoters upstream of *phaB*, an apparent constitutive promoter, and a second promoter induced by phosphate starvation. (Schembri *et al*. 1995). Table 1.2 enlists the known regulatory proteins involved in PHA metabolism (Kessler and Witholt 2001).

Table 1.2 Regulatory proteins involved in PHA metabolism (Kessler and Witholt 2001, Wu *et al.* 2001)

Regulatory protein	Regulator family	Organism
PhaF	Histone H1-like	*Pseudomonas oleovorans* *Pseudomonas aeruginosa* *Pseudomonas putida*
$PhaR_{Ps}$	AraC/XylS	*Pseudomonas* sp. 61-3
$PhaR_{Pd}$	?	*Pseudomonas denitrificans*
PhaS	Two component systems	*Pseudomonas putida*
GacS	Two component systems	*Azotobacter vinelandii*
NtrB/NtrC	Two component systems	*Azotobacter brasilense* Sp7
LuxR	Quantum sensor	*Vibrio harveyi*
CydR	?	*Azotobacter vinelandii*

A transcription regulator, $PhbR_{Ps}$ was identified in *Pseudomonas* sp. 61-3. This regulator exhibits significant similarity to members of the AraC/XylS family of prokaryotic positive transcriptional regulators (Gallegoes *et al.* 1997, Matsusaki *et al.* 1998). *Pseudomonas* sp. 61-3 is capable of producing a PHB homopolymer and a random copolymer consisting of monomeric units of four to twelve carbon atoms. $PhbR_{Ps}$ activator however, is only involved in the transcriptional regulation of the genes required for synthesis of the PHB homopolymer. Interestingly, transcription of the $phaCAB_{Ps}$ genes in a recombinant *R. eutropha* PHB negative mutant was independent of the $PhbR_{Ps}$ regulator encoding gene, suggesting either another functional promoter upstream of the genes or a regulator of *R. eutropha* which can substitute $PhbR_{Ps}$.

In *P. putida* KT2442, a putative regulatory protein called PhaS was identified. At primary structure level PhaS shows homology to the sensor component of the two component regulatory systems, containing a histidine protein kinase and response regulator domain. The two-component system homologous to the sensor kinase/response regulator couple LemA-GacA was found to regulate PHA synthesis in this strain (Hrabak and Willis 1992, Rich *et al.* 1994, Madison and Huisman 1999). In *Azotobacter vinelandii*, GacS transmembrane sensor kinase homologue regulates the alginate and PHB polymer production (Castaneda *et al.* 2000). The effect of *gacS* mutation on alginate and PHB production indicates that GacS plays a positive role in the regulation of polymer synthesis in *A. vinelandii*. This role is likely due to the kinase activity of GacS that results in phosphorylation of GacA, leading to activation of alginate and PHB genes. GacA must mediate signal transduction between GacS and GDP-mannose dehydrogenase, the key enzyme in the alginate biosynthetic pathway encoded by *algD*. Homolog of GacA has also been found in *A. vinelandii*.

In *P. aeruginosa*, the pathway from gluconate is strictly controlled by RpoN, the σ^{54} subunit of RNA polymerase, while the pathway from fatty acids is completely σ^{54} independent (Timm and Steinbüchel 1992). In addition, an ORF whose putative translational product exhibits similarity to the primary structure of AlgP, a member of the prokaryotic histone H1-like family of DNA binding regulators was identified adjacent to the PHA gene cluster in *P. aeruginosa* PAO1 (Timm and Steinbüchel 1992). In *P. oleovorans*, PhaF, again a member of the histone H1-like group of proteins has been shown to be involved in transcriptional regulation of *pha* gene expression (Prieto *et al.* 1999). Along with its regulatory function the PhaF protein also has a structural

function i.e. binding to PHA granules. As per the model suggested by Prieto *et al.* (1999) PhaF binds to DNA in the absence of substrates for PHA production, thereby inhibiting transcription of the *pha* genes. When PHA granules are formed, DNA binding is reduced and *pha* genes are expressed. A gene homologous to *phaF*, called *GA2*, has also been found in *P. putida* (Valentin *et al.* 1998), however, only its structural function has been demonstrated.

Granule associated proteins of *R. eutropha* and other bacteria are only synthesized when the cells accumulate PHB. Only those amount of proteins are synthesized that can be bound to the granules, since these were never detected in soluble state. Autoregulation of the expression of genes encoding granule-associated proteins has been proposed. However, the mechanism is not yet known. In *Paracoccus denitrificans*, $PhaR_{Pd}$, encoding for specific regulatory protein, was located downstream of the genes coding for the PHB synthase and a granule associated protein (Maehara *et al.* 1999). In recombinant *E. coli* $PhaR_{Pd}$ was shown to be involved in the regulation and gene expression of the granule associated proteins. One of the most abundant granule associated proteins of *Bacillus megaterium*, designated PhaP, is assumed to be a storage protein that is degraded under certain circumstances as a source of amino acids (McCool and Cannon 1999).

The synthesis of P(3HB) in *Vibrio harveyi*, is regulated by a regulatory protein LuxR (Sun *et al.* 1994). In general LuxR is activated on growth of cells to a high density in a confined environment due to the accumulation of an excreted N-acyl homoserine lactone autoinducer in the media. Mutants of *V. harveyi* defective in the production of LuxR were found to lack PHB. In *Azospirillum brasilense* SP7, *ntrB* and *ntrC* genes, encoding the two-component sensor-activator regulatory system, have been shown to be involved in the regulation of PHB synthesis by ammonia (Sun *et al.* 2000). The *Azospirillum brasilense* SP7 *ntrBC* and *ntrC* mutants were able to grow and accumulate PHB simultaneously in the presence of high concentration of ammonia, while little PHB was produced in the wild type during active growth phase. However, the target genes for NtrB-NtrC two-component system are yet to be identified. In *Azotobacter vinelandii* CydR, an oxygen responsive transcription factor, has been shown to control PHB biosynthesis (Wu *et al.* 2001). β-ketothiolase and acetoacetyl-CoA reductase were overexpressed in the CydR mutant of *Azotobacter vinelandii* leading to P(3HB)

accumulation throughout the exponential growth phase, unlike the wild-type strain that only accumulated P(3HB) during stationary phase (Wu *et al.* 2001).

1.7.3 Regulation of PHA biodegradation

It has been assumed that the synthesis of PHA and PHB depolymerases is highly regulated. Most PHA degrading bacteria repress PHA depolymerase gene expression in the presence of a soluble carbon source that permits high growth rates. After exhaustion of nutrients, the synthesis of PHA depolymerases is derepressed in many microorganisms (Jendrossek 1998). However, in contradiction to those data are the recent findings that inhibition of protein synthesis did not influence PHA degradation in *P. oleovorans*. It was suggested thst PHA depolymerase is always present and active, and that PHA is synthesized and degraded simultaneously (Kessler and Witholt 2001). A similar conclusion was reported for PHB metabolism in *R. eutropha* by Doi *et al.* (1992).

In PHB 'leaky' mutants of *R. eutropha*, it has been shown that the leaky phenotype is caused by inactivation of the genes *phbI* and *phbH*, encoding homologs of the *E. coli* phosphoenolpyruvate phosphotransferase system (PEP-PTS) (Pries *et al.* 1991). Pries *et al.* (1991) proposed that the 'leaky' phenotype of these mutant could actually be caused by aberrant regulation of the P(3HB) degradation pathway and suggested that the activity of the P(3HB) degrading enzymes was controlled by phosphorylation through metabolic signaling that involves a PEP-PTS. However, this model being hypothetical, needs to be proven experimentally.

1.8 Potential PHA production systems

Potential PHA production systems include:

Production of PHAs by natural organisms,

PHA production by recombinant bacteria,

Metabolic engineering of PHA biosynthetic pathways in higher organisms,

In vitro biosynthesis of PHAs.

1.8.1 Production of PHAs by natural organisms

Ralstonia eutropha has been most extensively used for PHB and PHBV production. The strains used extensively are the original PHB producer H16 (ATCC 17699) and its glucose utilizing mutant known as 11599 in the NCIMB collection. Other

strains are ATCC 17697^T, *R. eutropha* SH-69, and a natural isolate, *Alcaligenes* sp. strain AK201.

To achieve high productivity of a desired bioproduct, fed-batch cultures are usually grown with the controlled nutrient feeding, monitored dissolved oxygen (DO), pH, or carbon source as a feedback parameter (Kim *et al.* 1999, Ryu *et al.* 1997). Kim *et al* (1994) reported that a fed-batch culture of *R. eutropha* NCIMB 11599 with a glucose concentration control and nitrogen limitation gave a production of 121 g PHB/l. When nitrogen limitation is used to induce PHB accumulation, NaOH solution is added to the medium for pH control. However, due to the significant cell lysis caused by the toxicity of NaOH solution high-cell-density fermentation is impossible. In addition, it is very important to maintain phosphate and magnesium ion levels above 0.35 g/l and 10 mg/l respectively (Asenjo *et al.* 1995). Ryu *et al.* (1997), therefore, adopted phosphate limitation strategy to induce PHB accumulation where the pH is controlled by addition of NH_4OH. Under these conditions, PHB levels of 232 g/l were obtained.

In *R. eutropha* copolyester of 3HB and 3HV was synthesized if propionate or velerate was provided as the sole carbon source. Under these conditions the 3HV amounted to 45 or 90 mol% respectively (Doi *et al.* 1988). The molar fractions of 3HV dropped if a second carbon source like acetate or butyrate was provided in addition to propionate or valerate. *R. eutropha* H16 and *R. eutropha* NCIMB 11599 were compared for 3HV incorporation when butyrate and valerate were used as the carbon source. NCIMB 11599 was able to direct more 3HV monomer to P(3HB-3HV) (90% 3HV) than H16 (75%). Also, the molecular weight of the polymer produced by NCIMB 11599 was consistently higher. Further, it was observed that the fatty acids were converted to P(3HB-3HV) without undergoing complete degradation to acetyl-CoA and propionyl-CoA. This means, either (*S*)-3-hydroxyacyl-CoA or 3-ketoacyl-CoA is directly converted into monomer. Interestingly, this pathway operates in the presence of a nitrogen source, in contrast to the pathway from fructose (Doi *et al.* 1987). Probably inhibition of thiolase during active metabolism of carbohydrates prevents P(3HB) formation during growth whereas a pathway that involves only reductase and polymerase is insensitive to this inhibition (Madison and Huisman 1999). Similarly, for glucose utilizing mutant of *Alcaligenes eutrophus* DSM 545, production of co-polymer P(3HB-3HV) was enhanced when nitrogen feeding at limiting rates was used as opposed to nitrogen depletion (Aragao *et al.* 1996)

At high concentrations, short chain fatty acids such as propionate and valerate are toxic for *R. eutropha.* Alternative strategies have been developed to overcome this toxic effect. In *R. eutropha* NCIMB 11599 the cell growth inhibitory effect of propionic acid was minimized by pH adjustment of the fermentation broth (Chung *et al*. 1997). Propionyl-CoA is an intermediate in the degradation pathway of threonine, valine and isoleucine, and strains of *R. eutropha* with mutations in these pathways were tested for P(3HB-3HV) production. *R. eutropha* R3 is a prototrophic revertant of an isoleucine auxotroph of *R. eutropha* H16 and accumulates P(3HB-3HV) with up to 7% 3HV on fructose, gluconate, succinate, acetate and lactate (Steinbüchel and Pieper 1992). *R. eutropha* H16, when resuspended in medium deficient in Na^+- or O_2 – and with threonine as the sole carbon source, accumulated 6% PHA with 5% PHV (Nakamura *et al.* 1992). However, *R. eutropha* NCIMB 11599 does not incorporate 3HV from threonine and incorporates only up to 2% from isoleucine or valine (Yoon *et al*. 1995). These experiments prove that alternative, cell-derived substrates can be used for P(3HB-3HV) synthesis and that supplementation of carbon sources for alternative PHA monomers can be circumvented.

During PHBV production in fed-batch culture by *R. eutropha* H16, the mole fraction of HV units in copolymer can be controlled within the range of 0 - 40 mole% by adjusting the mole fraction of butyric acid in the feed medium (Ishihara *et al*. 1996). Continuous culture studies have shown that at dilution rates varying from 0.06-0.32 of fructose-valerate mixture, the 3HV content increased from 11 to 79% (Koyama and Doi 1995). Since the toxicity of propionate is pH dependent, varying pH of the culture can produce PHBV copolymers with different 3HV contents (Chung *et al.* 1997). *R. eutropha* SH-69 accumulates maximal P(3HB-3HV) in a medium with 2-3% glucose and a dissolved oxygen concentration of at least 20%. But, 20% 3HV content is obtained only with 6% glucose (Rhee *et al.* 1993). *R. eutropha* DSM 545 produces P(3HB-3HV) from glucose and propionate in fed-batch fermentation with nitrogen limitation and low dissolved-oxygen concentrations (Lefebvre *et al.* 1997). Though the yield of P(3HB) on glucose is apparently independent of the dissolved-oxygen concentration, the HV content is lower at high than at low dissolved-oxygen concentrations (Lefebvre *et al.* 1997).

R. eutropha H16 accumulates small amounts of poly(4HB) homopolyester in a medium with 4-hydroxybutyrate and 1.0 or 1.5% (w/v) potassium citrate (Nakamura *et al.* 1992). It accumulates copolyesters of 3HB and 4HB from mixtures of butyrate and

4HB or mixtures with 4-chlorobutyrate, 1,4-butanediol or γ-butyrolactone (Kunioka *et al.* 1989). PHA levels upto 40% of the cell dry weight with 4HB levels upto 37% were obtained with above mixtures of carbon sources. Mixtures of glucose, propionic acid and either 4-hydroxybutyric acid or γ-butyrolactone as carbon sources in fed-batch cultures of *R. eutropha* led to production of a P(3HB-4HB-3HV) terpolymer (Madden *et al.* 2000). Certain *R. eutropha* mutants accumulated copolyester with up to 96% 3HV and 84% 4HB (Madison and Huisman 1999). *Alcaligenes* sp. accumulated 50 to 60% P(3HB) of the cell dry weight on dicarboxylic acids in C_4 to C_9 range (Akiyama and Doi 1993).

Mixed or co-culture systems have been recognized to be effective for certain fermentations. In two-stage system xylose was converted first to lactate using *Lacticoccus lactis*, and then the lactate was converted to PHB by *R. eutropha*. Glucose was converted to lactate by *Lactobacillus delbrueckii* and then lactate to poly-β-hydroxybutyrate by *Ralstonia eutropha* in one fermentor (Tohyama and Shimizu 1999, Tohyama *et al.* 2000).

R. eutropha NCIMB 11599 when grown on tapioca hydrolysate (90% glucose) as a potential cheap carbon source, PHB concentrations of only 61 g/l were obtained, probably due to presence of toxic compounds, possibly cyanate (Kim and Chang 1995). PHB content of 0.33 g-PHB/g-dry cell has been reported when degraded alginic acid was used as carbon source (Seki *et al.*1994). The lower PHB content was probably due to heterogenous hydrolysis of alginic acid.

Methylobacterium

Methanol is a cheap substrate and its use in PHA synthesis would help to reduce significantly the production costs of PHB. Methanol is, in addition, a non-food substrate. It can also be considered as a renewable substrate since it could be derived from woody materials or from natural gas obtained after anaerobic digestion of organic substances. *Methylobacterium rhodesianum, M. extorquens, M. organophilum, M. rhodenium, M. zatmanii, M. radiotolerans, Mycoplana rubra, Paracoccus denitrificans* (Fölliner *et al.* 1995, Ueda *et al.* 1992a) and *Proteomonas extorquens* (Suzuki *et al.* 1986a, b, c, 1988) have been studied PHB accumulation with methanol as a carbon source. In a fully automated fed-batch culture PHB levels of 136 g/l were obtained in 121 h with *Proteomonas extorquens* (Suzuki *et al.* 1986b). After 170 h of incubation PHB concentration reached to 149 g/l.

Methylobacterium extorquens, with methanol concentration of 1.7 g/l, and addition of complex nitrogen source, accumulated 30% P(3HB) of the cell dry weight with a molecular mass of 250,000 Da (Bourque *et al.* 1992). *Methylobacterium* sp. strain KCTC0048 accumulates P(3HB-3HV), P(3HB-4HB) and poly(3-hydroxybutyrate-*co*-3-hydroxypropionate) [P(3HB-3HP)] to 30% of the cell dry weight with fractions of 3HV up to 0.7%, 4HB up to 0.13% and 3HP up to 0.11% (Kang *et al.* 1993).

Paracoccus denitrificans synthesizes P(3HB) from ethanol. When n-pentanol was used as growth substrate, homopolyester poly(3-hydroxyvalerate) was synthesized, whereas copolyester poly(3-hydroxybutyrate-co-3-hydroxyvalerate) accumulated during bacterial growth on mixture of methanol and n-pentanol (Yamane *et al.* 1996). Under controlled growth conditions with pentanol as the only growth substrate, *P. denitrificans* accumulates PHV as a homopolymer up to 55% of its cell dry weight (Yamane *et al.* 1996). *M. extorquens* synthesizes 50% more P(3HB-3HV) than *P. denitrificans*, while the latter incorporates twice as much 3HV on methanol-pentanol mixtures (Ueda *et al.* 1992b).

Pseudomonas

Pseudomonas putida efficiently incorporates monomers in the range of C_8 – C_{10} during PHA synthesis. Since long-side chain fatty acids such as oleate ($C_{18:1}$) need to be converted in multiple rounds of β-oxidation pathway before the resulting C_8 and C_{10} monomers can be incorporated, these substrates are less efficiently converted to PHA than octanoate. Oleic acid has to yield 4 acetyl-CoA molecules before a C_{10} monomer can be incorporated. This conversion yields 20 ATP equivalents in the reduction steps, which is unlikely to occur at a time when excess energy cannot be dissipated. In contrast, decanoic acid and octanoic acid yield 2 ATP equivalents before being incorporated into msc-PHA. As a consequence, the polymer yields per cell are often high when medium-chain fatty acids are used (Madison and Huisman 1999). Unfortunately, medium-chain fatty acids are generally more expensive and therefore a balance needs to be struck between substrate price and conversion yield.

Inexpensive substrates like tallow, low rank coal liquefaction products, palm kernel oil etc. (Cromwick *et al.* 1996, Tan *et al.* 1997, Füchtenbusch and Steinbüchel 1999) have been tested for PHA production by *Pseudomonas* sp. Continuous production of PHA by *P. oleovorans*, using octanoate as a carbon source in a one-stage culture was shown to produce 0.15 g/l/h (Ramsay *et al.* 1991). In continuous cultivation systems, *P.*

oleovorans is reported to produce 0.58 g/l/h PHAs (Preusting *et al.* 1993) while 1.56 g/l/h of PHAs yield is reported for *Pseudomonas putida* (Hazenberg and Witholt 1997).

Using pure oxygen and octanoic acid *P. oleovorans* cell density of 42 g/l/h, accumulating 37% PHA with a productivity of 0.35 g/l/h could be achieved (Madison and Huisman 1999). In an experiment where cells were pregrown on a rich medium followed by resuspending in nitrogen-free minimal medium with octanoate, Hori *et al.* (1994) examined the effect of several physiological parameters on PHA production by *P. putida*. The rate of PHA formation is highest at 30°C with octanoate concentration of 3.5 mM and pH of 7.8. While, PHA accumulation was upto 50% of the cell dry weight. Kim *et al.* (1997) studied the effects of different carbon sources on growth and PHA production and reported that simultaneous supply of glucose and octanoic acid resulted in 35.9 g of PHA/l (65% of the cell dry weight) with the productivity of 0.92 g/l/h (Kim *et al.* 1997). From the preceding it appears that mixtures of cheap growth substrates and more expensive substrates for product formation provide a valuable means of lowering PHA production costs.

Several studies have shown that *P. putida* and *P. aeruginosa* strains are able to convert acetyl-CoA to medium-chain-length monomers for PHA synthesis (Huijberts *et al.* 1992, Hoffman *et al.* 2000). PHAs that are formed from gluconate or related sugars have a different composition from the PHAs produced from fatty acids. Whereas the latter PHAs have 3-hydroxyoctanoate as the main constituent, sugar grown cells accumulate PHAs in which 3-hydroxydecanoate is the main monomer and small amounts of unsaturated monomer are present (Haywood *et al.* 1990, Timm and Steinbüchel 1990, Huijberts *et al.* 1992).

1.8.2 PHA production by recombinant bacteria

Whereas natural PHA producers have become accustomed to accumulating PHA during evolution, they often have a long generation time, relatively low optimal growth temperature, are often hard to lyse and contain pathways for PHA degradation. This led to development of recombinant PHA producers capable of high PHA accumulation and/or free of PHA degradative pathways.

Recombinant natural PHA producers

Recombinant *R. eutropha* cells overexpressing *phaCAB* genes from a plasmid, showed increase in P(3HB) levels from 33-40% of the cell dry weight (Park *et al.* 1995).

Although the increase was insignificant, recombinant *R. eutropha* strains could reduce the fermentation time by 20% while maintaining the same productivity (Park *et al.* 1997). This is significant from commercial production point of view, since the overall productivity of a P(3HB) plant would be 20% higher. *Alcaligenes latus* transformed with it's own cloned *phaC* gene, exhibited increased rate of PHB biosynthesis and increase in PHB content as well (Lee *et al.* 2000b). The maximum concentration and content of PHB in recombinant *A. latus* increased significantly from 3.1-3.7 g/l and from 50.2-65% of cell dry weight, respectively, as compared to the untransformed *A. latus*.

In a *Rhizobium meliloti* PHB^- mutant strain, P(3HB) accumulation was restored to wild type level by the introduction of a plasmid encoded *R. meliloti phaC* gene (Tombolini *et al.* 1995). In *Paracoccus denitrificans*, an additional *phaC* gene on a plasmid doubles the wild-type PHA levels in a pentanol-grown parent strain (Ueda *et al.* 1996).

The *phaCAB* operon from *R. eutropha* was expressed in *Pseudomonas* strains that normally do not accumulate P(3HB). P(3HB) accumulation was observed in recombinant *P. aeruginosa, P. putida, P. oleovorans, P. syringe* and *P. fluorescems*, while *P. stutzeri* was unable to synthesize P(3HB) with the *R. eutropha* genes (Steinbüchel and Schubert 1989). *R. eutropha phaC*$^-$ strain accumulated about 85% P(3HB) of cell dry weight with gluconate as a carbon source, when transformed with *phaC* gene from *C. violaceum*, however, *P. putida phaC* did not accumulate PHA even when transformed with the *C. violaceum phaC* (Kolibachuk *et al.* 1999). *Synechococcus* sp. harboring PHA biosynthetic genes from *R. eutropha* accumulated PHB up to 25% of cell dry weight (Takahashi *et al.* 1998).

Recombinant E. coli as PHA producer

Natural producers like *R. eutropha* produces high levels of P(3HB), but they have certain limitations. *R. eutropha* grows slowly, it is difficult to lyse and is genetically not well characterized which impedes genetic manipulations. As against this, *Escherichia coli* is genetically well characterized. Not being a natural PHA accumulator, PHA production has to be metabolically engineered in *E. coli* and it does not have any depolymerase activity to degrade accumulated PHA.

The expression of PHA biosynthetic genes of *R. eutropha* in *E. coli* for P(3HB) synthesis opened up the avenues for PHA production by recombinant organisms (Schubert *et al.* 1988, Slater *et al.* 1988, Peoples and Sinskey 1989b). A variety of

polymers, such as P(3HB-3HV), P(3HB-4HB), P(4HB) and P(3HO-3HH), have been synthesized by genetically and metabolically engineered *E. coli.*

The pH-stat fed-batch culture of XL1-Blue achieved a PHB concentration of 88.8 g/l in 42 h (Kim *et al.* 1992a). Similarly, a PHB concentration of 81.2 g/l was obtained in 39 h by fed-batch culture of XL1-Blue harboring a stable high-copy-number plasmid (Lee *et al.* 1994b). However, fed-batch culture of XL1-Blue harboring a stable medium-copy-number plasmid resulted in only 30.5 g/l of PHB in 41 h. Suggesting that a high gene dosage obtained by using high-copy-number plasmid is required for the higher accumulation of PHB in recombinant *E. coli* (Lee and Chang 1995). Lee and Chang (1995) transformed a number of *E. coli* strains with a stable high-copy-number plasmid containing the *R. eutropha* PHB biosynthesis genes, and compared these for their ability to synthesize and accumulate PHB. *E. coli* JM109 accumulated 85% PHB of cell dry weight, while XL1-Blue acuumulated 81% PHB of cell dry weight. However, yield per gram of glucose was higher for XL1-Blue (0.37 g PHB/g glucose) than for JM109 (0.3 g PHB/g glucose). Cultivation of recombinant *E. coli* in defined medium led to reduced levels of PHB accumulation. Supplementation of complex nitrogen sources to defined media restores the normal levels of PHB accumulation (Lee and Chang 1995). For cost reduction purpose, cheap carbon sources such as molasses, whey etc. have been used for PHA production (Zhang *et al.* 1994, Kim 2000). For enhanced PHA production in defined media by recombinant *E. coli*, *ftsZ* gene was expressed in *E. coli* to suppress the filamentation (Lee and Lee 1996).

During PHBV production, incorporation of 3HV by recombinant *E. coli* requires the function of *ackA* (acetate kinase) and *pta* (phosphotransacetylase) genes (Rhie and Dennis 1995). Propionate was provided for the PHBV production in recombonant *E. coli*. Since *E. coli* does not easily import propionate, cultures were adapted on acetate and then a glucose-propionic acid mixture was added (Slater *et al.* 1992). Further, this system was improved by using *E. coli* strains that have constitutive expression of the *ato* operon and *fad* regulon to fully express fatty acid utilization enzymes (Fidler and Dennis 1992, Slater *et al.* 1992). The 3HV fraction in the copolymer was dependent on the percentage of propionate used during the fermentation. *E. coli* is resistant to 100 mM propionate while 30 mM is already toxic to *R. eutropha* (Ramsay *et al.* 1990), making PHBV fermentations more efficient with *E. coli* strains (Slater *et al.* 1992). Recombinant *E. coli* cells harboring *A. latus* PHA biosynthetic genes were grown in fed-batch culture with the pH-stat feeding strategy in a chemically defined medium (Choi

and Lee 1999b). An improved nutrient feeding strategy to obtain glucose and propionic acid concentration at 110 mM and 20 mM respectively, acetic acid induction and oleic acid supplementstion led to high level of PHBV production, 2.88 g of P(3HB-3HV)/l/h (Choi and Lee 1999b).

Expression of *R. eutropha* PHA synthase gene and the *Clostridium kluyveri orfZ* encoding for 4-hydroxybutyrate-CoA transferase in *E. coli* led to the accumulation of P(4HB) homopolymer, when grown in presence of glucose. In the absence of glucose, a P(3HB-4HB) copolymer accumulated with up to 72% 3HB incorporation, even though *phbA* and *phaB* were absent. This indicates that in *E. coli* an unknown pathway allows the conversion of 4HB to 3HB (Hein *et al.* 1997). Valentin and Dennis (1997) coexpressed succinate degradation pathway from *Clostridium kluyveri* and PHB biosynthetic pathway from *R. eutropha* in *E. coli*. Resulting recombinant accumulated 46% P(3HB-4HB) of cell dry weight with 1.5% 4HB incorporation. To provide 4(HB)-CoA for poly(3HB-4HB) synthesis from glutamate, an acetyl-CoA:4-hydroxybutyrate CoA transferase from *C. kluyveri*, a 4-hydroxybutyrate dehydrogenase from *R. eutropha*, a γ-aminobutyrate:2-ketoglutarate transaminase from *Escherichia coli* and glutamate decarboxylases from *Arabidopsis thaliana* or *E. coli* were expressed in succinic semialdehyde mutant *E. coli* CT 101 (Valentin *et al.* 2000). In the recombinant P(3HB-4HB) was accumulated with only 1.2 to 2 mol% 4HB. Nevertheless, these observations are important since in plant plastids, succinyl-CoA is not available at high levels but glutamete being the intermediate of amino acid metabolism is present at sufficiently high levels. Subsequent studies in this direction will be useful to obtain a desirable polyester composition and in sufficient amounts in plants, since plants have the potential to generate PHAs at lower costs (Valentin *et al.* 2000).

Expression of *Pseudomonas aeruginosa* PHA synthases, *phaC1* and *phaC2* in *E. coli fadB* mutant resulted in msc-PHA accumulation when grown in presence of C_8-C_{14} fatty acids (Langenbach *et al.* 1997, Qi *et al.* 1997). The *E. coli fadB* mutant LS1298 containing $phaC1_{Pa}$ gene accumulated 21% PHA of cellular dry weight composed of mainly 3-hydroxydecanoate (72.5 mol%) and 3-hydroxyoctanoate (20 mol%) when grown in LB medium containing decanoate (Langenbach *et al.* 1997). Using acrylic acid, intermediates of fatty acid synthesis were chanelled to PHA synthesis in recombinant *E. coli* harboring the $phaC1_{Pa}$ gene (Qi *et al.*1998). The recombinant *E. coli fadR* mutant RS3097 grown in presence of 0.24 mg/ml acrylic acid and decanoate

revealed msc-PHA accumulation contributing to about 60% of cellular dry weight. For msc-PHA synthesis from gluconate, *E. coli* JMU193 was engineered for expression of $phaC_{Po}$ and thioesterase I leading to a maximum msc-PHA accumulation of 2.3% of cell dry weight (Klinke *et al.* 1999).

1.8.3 Metabolic engineering of PHA biosynthetic pathways in higher organisms

Saccharomyces cerevisiae

The $phaC_{Re}$ gene of the *R. eutropha* was used to construct a yeast plasmid, which enabled expression of the functional synthase enzyme in *S. cerevisiae.* These cells accumulated only up to 0.5% of cell dry weight as PHB, with accumulation occurring in the stationary phase of batch growth. In these recombinant yeast cells, PHB synthesis is catalyzed by native cytoplasmic acetoacetyl-CoA thiolase, a native β-oxidation protein processing D-3HB-CoA dehydrogenase activity and heterologous *pha* synthase. Low levels of β-ketoacyl-CoA thiolase and acetoacetyl-CoA reductase were detected. This eukaryotic system probably needs elevation of these activities for enhanced PHB production (Leaf *et al.* 1996).

Insect Cells

PHB synthesis by *Spodoptera frugiperda* cell lines has been reported by simultaneous transfection of mutant form of the rat fatty acid synthase and PHA synthase from *R. eutropha.* Approximately 1 mg of PHB was isolated from a one-litre culture of these cells corresponding to 0.16% of cell dry weight. Though not an efficient system, however, provides an example of alternative, eukaryotic enzymes for the generation of P(3HB) intermediates (Williams *et al.* 1996).

Plants

Plants are interesting targets for expression of PHA biosynthetic genes. Transgenic plants could produce PHAs directly from CO_2 and solar energy and at least theoretically at costs which are comparable to those of other biopolymers already obtained from plants. PHA biosynthetic genes have been expressed in *Arabidopsis thaliana* (Poirier *et al.* 1992) and in agricultural crops such as *Brassica napus, Gossypium hirsutum, Nicotiana tabacum, Solanum tuberosum* and *Zea mays* (John and Keller 1996, Hahn *et al.* 1999, Houmiel *et al.* 1999, Nakashita *et al.* 1999, Steinbüchel 2001)

Synthesis of PHA in plants was initially explored by the expression of PHA biosynthetic genes of the bacterium *R. eutropha* in the well-studied plant *Arabidopsis thaliana* (Poirier *et al.* 1992). But PHB accumulation of only 0.1% of the plant dry weight was achieved. Moreover, the growth of the transgenic plants was severely reduced probably due to depletion of one or more essential substrates for growth (Poirier *et al.* 1992). To overcome this problem, PHB biosynthetic pathway was targeted to the plastids of *A. thaliana* through an N-terminal transit peptide. This resulted in PHB accumulation of up to 14% of the dry weight with no deleterious effects on plant growth (Nawrath *et al.* 1994). Recently all three genes necessary for PHB biosynthesis were transformed to *A. thaliana* in a single transformation event (Bohmert *et al.* 2000). These plants accumulated more than 4% of their fresh weight (approximately 40% of their dry weight) of PHB in leaf chloroplasts. Accumulation of high levels of PHB in transgenic *A. thaliana* plants was not accompanied by any appreciable change either in the composition or the amount of fatty acids. Substantial changes were, however, observed in the levels of various organic acids, amino acids, sugars and sugar alcohols (Bohmert *et al.* 2000). Synthesis of msc-PHAs in *A. thaliana* was explored with the transformation of $phaC1_{Pa}$ from *Pseudomonas aeruginosa* modified for peroxisome targeting by addition at the carboxyl end of the protein with 34 amino acids from the *Brassica napus* isocitrate lyase (Mittendorf *et al.* 1998). Transgenic *A. thaliana* accumulated approximately 4 mg of msc-PHA per g of dry weight. The plant PHA contained saturated and unsaturated 3-hydroxyalkanoic acids ranging from 6-16 carbons with 41% of the monomers being 3-hydroxyoctanoic acid and 3-hydroxyoctenoic acid (Mittendorf *et al.* 1998). This achievement indicates that the β-oxidation of plant fatty acids can generate a broad range of saturated and unsaturated (R) – hydroxyalkanoate monomers that can be used to synthesize msc-PHA. *Arabidopsis* and *Brassica* were engineered to produce PHBV in leaves and seeds respectively, by transforming threonine deaminase gene from *E. coli* and PHB biosynthetic genes from *R. eutropha* (Slater *et al.* 1999).

Expression of $phaB_{Re}$ and $phaC_{Re}$ in tobacco and potato led to accumulation of very low levels of PHB (Poirier and Nawrath 1998, Nakashita *et al.* 1999). *R. eutropha* PHA biosynthetic genes were targeted to plant peroxisomes in Black Mexican Sweet maize suspension culture by adding a carboxy-terminal targeting sequence (Hahn *et al.* 1999). Up to 2 mg/g fresh weight PHB was produced in suspension cultures. P(3HB) up to 7.7% of fresh seed weight of mature seeds was accumulated in oilseed leucoplasts of

Brassica napus when PHA biosynthetic genes from *R. eutropha* were expressed (Houmiel *et al.* 1999). The novel perspective on the use of PHA synthesis was uncovered by the expression of the *R. eutropha* PHB biosynthetic pathway in cotton fiber cells (John and Keller 1996). Analysis of the transgenic fibers showed accumulation of PHB up to 0.3% of dry weight and they exhibited better insulating properties.

1.8.4 *In vitro* biosynthesis of PHAs

Six different systems have been described in the literature for *in vitro* biosynthesis of PHAs employing purified PHA synthases and other enzymes (Steinbüchel 2001). These include: (i) one step system for synthesis of poly(3HB) employing the PHA synthase of *Ralstonia eutropha* (Gerngross and Martin 1995), (ii) two step system for synthesis of poly(3HB) employing the propionyl-CoA transferase of *Clostridium propionicum* plus the PHA synthase of *C. vinosum* (Jossek and Steinbüchel 1998), (iii) three-step system for synthesis of poly(3HB) employing the acetyl-CoA synthetase of *Saccharomyces cerevisiae*, propionyl-CoA transferase of *Clostridium propionicum* plus the PHA synthase of *C. vinosum* (Jossek and Steinbüchel 1998), (iv) one-step system for synthesis of poly(3HV) employing the PHA synthase of *R. eutropha* (Steinbüchel 2001), (v) three-step system for PHA_{SCL} consisting of 3HB, 4HB and/or 4HV employing the butyrate kinase of *C. acetobutylicum*, phosphotransbutyrylase of *C. acetobutylicum*, plus the PHA synthases of *C. vinosum* or *Thiocapsa pfennigii* (Liu and Steinbüchel 2000), (vi) two-step system for synthesis of poly(3HD) employing an acyl-CoA synthetase of *Pseudomonas* sp. plus the PHA synthase of *P. aeruginosa* (Qi *et al.* 2000). Advantages and limitations of *in vitro* synthesis and production processes are listed in Table. 1.3.

1.9 Commercial status

Bacterial PHA were first commercialized way back in the early 1960s by an American company W. R. Grace. In 1970s ICI Bioproducts, UK produced PHBV on industrial scale from glucose utilizing mutant of *R. eutropha* with a glucose/propionic acid substrate mixtures as the carbon source and under phosphate limiting conditions (Steinbüchel 1991). The copolymer was marketed under the trade name 'Biopol' and used for the manufacture of biodegradable films and bottles. Pilot plant polymer was offered at US$ 30/kg and material from a 5000 tons/year semi-commercial plant was

projected to go down to US $ 8-10/kg. Fermentations with recombinant *E. coli* harboring *A. latus* PHA biosynthetic genes coupled with recovery method of simple alkali digestion, the production cost has been estimated to be US $ 3.32/kg PHB (Lee and Choi 2000). Another company involved in PHA fermentations was Austrian Chemie Linz (Wilke 1999).

Table 1.3 The pros and cons of *in vitro* metabolic engineering (Steinbüchel 2001)

Advantages and Prospects	**Limitations and Problems**
✕ **Detailed biochemical studies** through characterization of the enzyme (s) becomes possible	✕ **Purified PHA synthase is required**
✕ **Application of non natural substrates** PHAs with new constituents may be produced	✕ **Additional enzymes must be available in a purified stage**
✕ **Rapid shifts of substrates applicable** PHAs with a novel order of constituents may be produced (e. g. blockcopolyesters)	
✕ **Cell-free system** Production is limited by the volume of the reactor and not by the volume of the cytoplasm	✕ **All enzymes must be stable**
Increase of PHA produced per volume is possible	✕ **Coenzymes must be recycled**
Synthesis may be performed in the presence of compounds or under conditions which are inhibiting cells	
No expression required	
Amount of enzymes can be easily varied	
✕ Only few components required downstream processing will be easier	
✕ Requires less efforts than *in vivo* metabolic engineering fast evaluation of the feasibility of a strategy and the functionality of a “designed” pathway	
✕ Special applications *in situ* PHA formation becomes possible	

Zeneca, earlier the part of ICI Bioproducts, UK sold its Biopol business in 1996 to Monsanto who continued the plant-based production approach to PHAs. In May 2001, Metabolix Inc. has purchased Monsanto's Biopol assets (http://www.metabolx.com/). Metabolix Inc., Cambridge, Massachusetts founded in 1992, is actively involved in developing efficient technologies for PHA production (Williams and Peoples 1996). In a world with shrinking petroleum reserves and increasing environmental issues, PHA is a definitely a potential candidate that deserves further exploration.

SCOPE OF PRESENT THESIS:

Streptomyces aureofaciens NRRL 2209, which produces chlortetracycline has been earlier reported to accumulate PHB upto 1.1% of the cell dry weight (Kannan and Rehacek 1970). Although *Streptomyces* sp. is able to accumulate PHA, no molecular data is available on genes involved in PHA biosynthesis. Present study is the first report on PHB biosynthesizing genes from streptomycetes.

The objective of the present work has been:

(i) Isolation and cloning of PHA synthesizing genes from *Streptomyces aureofaciens* NRRL 2209,

(ii) Sequencing and Sequence analysis of a ~5.0 kb genomic DNA fragment of *Streptomyces aureofaciens* harboring the PHB biosynthetic genes,

(iii) Heterologous expression of PHB synthesizing genes from *Streptomyces aureofaciens* NRRL 2209 in *Escherichia coli*.

CHAPTER 2

ISOLATION AND CLONING OF THE PHA SYNTHESIZING GENES FROM *STREPTOMYCES AUREOFACIENS* NRRL 2209

2.1 INTRODUCTION

Among prokaryotes, actinomycetes constitute an important part of the microbial community responsible for degradation and recycling of natural substrates. These bacteria can be separated into different taxa based on partial sequencing of 16S ribosomal ribonucleic acids. These include Actinobacteria, Nocardioforms, Actinoplanetes, Thermomonosporas, Maduromycetes, Streptomycetes and multilocular sporangia forming Actinomycetes (Williams 1989). Actinomycetes are Gram positive bacteria characterized by the formation of substrate and aerial mycelia on solid media, the presence of spores and a high G+C content of the DNA (50–80 mol %). The majorities of these are soil bacteria and constitute a very important class that produce a number of varied and complex molecules such as antibiotics (Berdy 1984) and enzymes (Edwards 1993).

Streptomyces belong to the streptomycetes group in that they are Gram positive, have a high G+C ratio (69-78 mol %) and have complex colony morphology resulting in the formation of arthospores. Their ability to produce a very diverse range of metabolic products, some of which have important roles in medicine and veterinary science, has excited the interest of biologists. The most common among these are antibiotics, immune system modulators and enzyme inhibitors. Of the approximately 9000 known antibiotics about 6000 were identified in actinomycetes, of which 5000 were identified from streptomycetes alone. About 500 new antibiotics are identified every year and still a very healthy proportion are from the streptomycetes. This incredible diversity of biologically active natural molecules is reflected in the great importance of streptomycetes to the pharmaceutical industry (Hodgson 2000). Streptomycetes have certain biological characteristics in common with eukaryotic organisms such as the presence of reiterated sequences within the chromosomes e.g. in *S. reticuli* and *S. coelicolor*. (Schrempf 1982). Streptomycetes also have DNA repair system akin to the eukaryotes (Stonesifer and Baltz 1985).

Accumulation of Polyhydroxyalkanoates (PHAs) is reported in nine different strains of *Streptomyces* (Kannan and Rehacek 1970, Manna *et al*. 1999). These have been implicated in the supply of two-carbon units for the biosynthesis of polyketide derived phenolic metabolites such as actinorhodin in *Streptomyces coelicolor* A 3 (2) or antimycin in *Streptomyces antibioticus* (Kannan and Rehacek 1970, Packter and

Flatman 1983). PHB (Poly-3-hydroxybutyrate) accumulation in *Streptomyces venezuelae* mycelia precedes the period of rapid chloramphenicol synthesis. This is suggested to indicate, that the stored polymer might be one of the sources of acetoacetyl-CoA for the synthesis of dichloroacetyl substituent of chloramphenicol (Ranade and Vining 1993). However, PHB might not be the sole source for acetoacetyl-CoA.

Streptomyces aureofaciens NRRL 2209, a chlortetracycline producer, has been reported to accumulate upto 1.1% PHB of the cell dry weight (Kannan and Rehacek 1970). However, very little is known about the biosynthesis of the polymer in these bacteria. Also no molecular data is available about the *Streptomyces* genes involved in the process. Hence, *S. aureofaciens* was chosen as an experimental system for the present study.

Among actinomycetes, gene encoding for PHA synthase has been reported from *Nocardia corallina* (now known as *Gordonia rubripertinctus*) and *Rhodococcus ruber* (earlier known as *Nocardia rubra*). Both belong to the group Nocardioforms. Remaining taxa of actinomycetes are as yet unexplored with regards to the PHA synthesizing genes. *Gordonia* sp. may cause opportunistic infections, in particular bacteremia and endocarditis, in patients with severe underlying diseases and indwelling central catheters (Lesens *et al.* 2000), while *R. ruber phaC* gene can not be expressed in *E. coli*, thus limiting its exploitation for biotechnological purpose (Pieper and Steinbüchel 1992). Present study is the first report on isolation and characterization of PHA synthesizing genes from *Streptomyces aureofaciens* NRRL 2209, a nonpathogenic member of the streptomycete.

Among the strategies employed to identify genes involved in PHA biosynthesis screening of the genomic libraries is the most successful and widely applied (Rehm and Steinbüchel 1999). Other strategies include use of homologous or heterologous gene probes, short consensus oligonucleotide hybridizations or PCR technique (Timm *et al.* 1994, Rehm and Steinbüchel 1999, Solaiman 2000, Solaiman *et al.* 2000, Zhang *et al.* 2001).

The 5' coding region of the *phaC* gene from *R. eutropha* has been shown to be dispensable for PHB synthesis (Schubert *et al.* 1991). In the PHA synthase genes from *Alcaligenes latus* and *R. eutropha* the only major difference is the replacement of 560 nucleotides at its 5' end by 160 non-homologous nucleotides in *A. latus* (Genser *et al.* 1998). Hence, in the present study the *Sau*3A I partial genomic library of *S.*

aureofaciens was screened using radiolabelled 3' region of *R. eutropha* PHA synthase gene as the hybridization probe (Peoples and Sinskey 1989b).

2.2 MATERIALS AND METHODS

2.2.1 Materials

Agarose, ampicillin, Tris, IPTG, X-gal, PEG-8000, SDS, bovine serum albumin, Nile blue A, benzoic acid, EDTA and poly-β-hydroxybutyrate were purchased from Sigma-Aldrich, USA. Restriction enzymes, T4 DNA ligase, Rnase A, lysozyme and pronase were obtained from GIBCO-BRL (USA), Promega (USA) and Amersham (UK). pGEM-3Z, pGEM-5Z vectors were purchased from Promega (USA). Megaprime labelling kit and Hybond-N membrane were obtained from Amersham (UK). Radiolabelled [α-^{32}P]-dCTP was obtained from BARC, India. X-ray films were obtained from Konika (Japan) or Kodak (USA). All other chemicals used were of analytical grade and obtained from HIMEDIA, Qualigens Fine Chemicals and E. Merck Laboratories, India.

2.2.2 Bacterial culture conditions

For routine maintenance, *E. coli* cells were grown at 37°C with shaking at 200 rpm in Luria Bertani medium (Sambrook *et al*. 1989). For medium scale plasmid DNA preparation, recombinant *E. coli* cells were grown in Terrific broth (TB) supplemented with appropriate antibiotic. *S. aureofaciens* NRRL 2209 cells were grown at 28°C in MGYP broth (Hopwood *et al*. 1985) in baffeled flasks, with shaking at 200 rpm. For genomic DNA isolation, *S. aureofaciens* cells were grown in YEME broth (Hopwood *et al*. 1985).

Media compositions

Luria-Bertani (LB)	g/l	LB-agar
Bacto-tryptone	10	1.5 % agar
Bacto-yeast extract	5	
NaCl	10	
	pH 7.0	

MGYP	g/l
Malt extract	3
Glucose	10
Yeast extract	3
Peptone	10
pH 7.0	

YEME	g/l
Yeast extract	3
Peptone	5
Malt extract	3
Glucose	10
Sucrose	340
After autoclaving add:	
2.5 M $MgCl_2$	2 ml
20 % Glycine	25 ml

TB	g/l
To 900 ml of deionized water, add	
Bacto-tryptone	12
Yeast extract	24
Glycerol	4 ml

To sterilized medium add 100 ml of 0.17 M KH_2PO_4, 0.72 M K_2HPO_4. (This solution is made by dissolving 2.31 g of KH_2PO_4 and 12.54 g of K_2HPO_4 in 90 ml of deionized water. After the salts have dissolved make up the volume to 100 ml with deionized water and sterilize by autoclaving).

S. aureofaciens vegetative inoculum was prepared by incubating spores from MGYP agar culture (Hopwood *et al.* 1985) in the following medium (Kannan and Rehacek 1970) for 24 h.

	g/l
Glucose	10
Soybean meal	2
Peptone	10
Soybean oil	10
pH 7.2	

Kannan-Rehacek medium (Kannan and Rehacek 1970) was used for PHA accumulation in *S. aureofaciens*:

	g/l
Glucose	20
$NH_4(SO)_4$	5
Yeast extract	2.5
KCl	3
$CaCO_3$	4
Soybean meal extract	100 ml (prepared by suspending 10g soybean meal in 1 liter distilled water at 4°C for 24 h)
pH 6.8	

For PHA accumulation in *E. coli*, the recombinant cells were grown in **basal medium**

	g/l
Yeast extract	5
Peptone	5
Na_2HPO_4	1
$MgSO_4$	0.2
pH 7.2	
Glycerol	10 ml (used as a carbon source)

Ampicillin to a final concentration of 100 µg/ml was added to the medium post sterilization. The bacterial strains and plasmids used are listed in Table 2.1.

Culture of *Streptomyces aureofaciens* for microscopy

For examination of *S. aureofaciens* cells by fluorescence microscopy, the culture method developed to obtain growth of *Streptomyces* sp. for scanning electron microscopy was used (Williams and Davies 1967). Briefly, coverslips were sterilized by autoclaving. Each coverslip was then inserted at an angle of about 45° into solidified medium in a petri dish until about half the coverslip was in the medium. An inoculum of *Streptomyces* was then spread along the line where the upper surface of the coverslip met the medium, using a fine wire needle. The plates were incubated at 28°C for different time periods. After incubation the mycelia grow both on the medium and in a line across the upper surface of the coverslips. This line of growth remained attached to the coverslips when carefully withdrawn from the medium and was used for fluorescence microscopic study.

Table 2.1 Bacterial strains and plasmids used in this study

Strain or Plasmid	**Important features (reference or source)**
S. aureofaciens NRRL 2209	Source of PHA synthesizing genes (ATCC 10762)
E. coli JM109	F'; *lac* for blue/white screening (Promega)
E. coli DH5α	F'; *lac* for blue/white screening (Promega)
pGEM-3Z	Cloning vector (Promega)
pGEM-5Z	Cloning vector (Promega)
pSa240	pGEM-3Z + 4.8 kb *S. aureofaciens* insert
Recombinant *E. coli* JM109 harboring pSa240	ATCC: PTA-1579

2.2.3 The nucleic acids

2.2.3.1 Plasmid DNA isolation

Solutions

Solution I	**Solution II**
50 mM glucose	0.2 N NaOH
25 mM Tris-Cl (pH 8.0)	1% SDS
10 mM EDTA (pH 8.0)	

Solution III		**TE pH 8.0**
5 M potassium acetate	60 ml	10 mM Tris-Cl (pH 8.0)
Glacial acetic acid	11.5 ml	1 mM EDTA (pH 8.0)
Distilled water	28.5 ml	

The resulting Solution III is 3 M with respect to potassium and 5 M with respect to acetate

Plasmid DNA from *E. coli* was isolated by alkaline lysis method (Sambrook *et al.* 1989). A single bacterial colony was inoculated into 2 ml of LB medium containing the appropriate antibiotic. The culture was incubated at 37°C with vigorous shaking for 18 h. The culture was centrifuged at 12,000 x *g* for 30 s at 4°C. The bacterial pellet was resuspended in 100 µl of ice-cold Solution I and kept on ice for 5 min. Then 200 µl of freshly prepared Solution II was added with vigorous mixing and stored on ice for 10 min. This was followed by addition of 150 µl of ice cold Solution III. The tube was stored on ice for 5 min and then centrifuged at 12,000 x *g* for 5 min at 4°C. The supernatent was transferred to a fresh tube and extracted with an equal volume of phenol:chloroform (1:1), followed by chloroform:isoamyl alcohol (24:1). Two volumes of ethanol was added to the aqueous phase and the mixture was allowed to stand for 2 min at room temperature. The tube was centrifuged at 12,000 x *g* for 5 min at 4°C. The pellet was rinsed with 70 % ethanol, air-dried and suspended in 50 µl TE (pH 8.0) containing RnaseA (20 µg/ml). The tube was stored at room temperature for 30 min. The solution was extracted with phenol:chloroform (1:1) and chloroform:isoamyl alcohol (24:1). To the aqueous phase, 2.5 volume of 10 M ammonium acetate and 2 volumes of ethanol was added. The tube was kept at room temperature for 10 min. The

plasmid DNA was precipitated by centrifugation at 12000 x *g* for 5 min at 4°C. The pellet was rinsed with 70% ethanol, dried and dissolved in 30 µl of TE (pH 8.0) .

For medium scale preparations of plasmid DNA (50 ml culture grown in TB) after RnaseA treatment (as described above), equal volume of 1.6 M NaCl containing 13% (w/v) polyethylene glycol (PEG 8000) was added. The tube was centrifuged at 12,000 x *g* for 5 min at 4°C. The pellet was dissolved in 400 µl TE (pH 8.0). The solution was extracted with phenol:chloroform (1:1) and chloroform:isoamyl alcohol (24:1). To the aqueous phase 100 µl of 10 M ammonium acetate and 2 volumes of ethanol was added. The tube was kept at room temperature for 10 min. The plasmid DNA was precipitated by centrifugation at 12000 x *g* for 5 min at 4°C. The pellet was rinsed with 70% ethanol, dried and dissolved in 500 µl of TE (pH 8.0).

2.2.3.2 *Streptomyces aureofaciens* NRRL 2209 genomic DNA isolation

S. aureofaciens cultures were centrifuged at 8,000 x *g* for 10 min at 4°C and washed twice with STE buffer (0.3 M sucrose, 25 mM Tris-HCl and 25 mM EDTA, pH 8.0). 1.0 g (wet weight) mycelium was resuspended in 8.55 ml STE buffer and 950 µl lysozyme (20 mg/ml STE buffer) was then added. After protoplast formation, which took about 20-30 min, 500 µl 10% SDS (w/v) and 50 µl of pronase (20 mg/ml) were added, and the mixture held at 37°C for 1 h. Next 1.8 ml 5 M NaCl was added with gentle mixing followed by 1.5 ml of 10 % (w/v) CTAB in 0.7 M NaCl and incubated for 20 min at 65°C. After the addition of CTAB, all the steps were carried out at room temperature. The lysate was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, v/v) and centrifuged at 12,000 x *g* for 10 min. The aqueous phase was transferred to a fresh tube and the above step repeated. The aqueous phase was finally extracted with chloroform:isoamyl alcohol (24:1, v/v) and DNA spooled out after 10 min following addition of 0.6 volume of isopropanol. Alternatively, it was recovered by centrifugation at 12,000 x *g* for 10 min. The pellet was washed twice with 70% ethanol, vacuum dried and dissolved in 2 ml TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). RnaseA (50µg/ml) was added with incubation at 37°C for 2 h. After digestion the sample was extracted with phenol as described above. DNA was precipitated from the aqueous phase with addition of 0.1 volume 3 M sodium acetate (pH 5.3) and 0.6 volume Isopropanol. The DNA pellet was washed with 70% ethanol, dried and dissolved in TE buffer.

The above method employed for DNA isolation from *Streptomyces* sp. is a modification of the method of Murray and Thompson (1980) for isolation of nucleic acids from plants and it works well with Gram positive and Gram negative bacteria (Tripathi and Rawal 1998).

2.2.3.3 Screening of partial genomic library of *Streptomyces aureofaciens* NRRL 2209

Solutions

1 X TAE	**Gel loading buffer (6 X)**
0.04 M Tris-acetate	0.25 % bromophenol blue
0.001 M EDTA	0.25 % xylene cyanol FF
	40 % (w/v) sucrose in water
IPTG stock solution	200 mg/ml dissolved in sterile distilled water
X-gal stock solution	20 mg/ml dissolved in dimethyl formamide
Ampicillin stock	100 mg/ml dissolved in sterile distilled water

S. aureofaciens NRRL 2209, genomic DNA was digested with restriction endonuclease *Sau*3A I (0.07 units of *Sau*3A I per μg of genomic DNA) at 37°C for 40 min. The resulting DNA fragments were mixed with 1/6th volume of gel loading buffer and electrophoresed on 1.2% low melting point agarose gel in 1X TAE running buffer by applying a voltage of 50V. Bromophenol blue and xylene cyanol in the gel loading buffer acted as the tracking dyes. After completion of electrophoresis, the gel was stained with ethidium bromide (0.5 μg/ml) and DNA visualized using a hand held long wavelength UV transilluminator. DNA fragments ranging from 2-8 kb in size were purified from the agarose gel. The region in the low melting agarose gel with the DNA bands of interest was cut out with a razor blade and the agarose slice transferred to a microfuge tube. Five volumes of 20 mM Tris-Cl (pH 8.0) was added to the tube followed by incubation at 65°C for 5 min. The solution was cooled to room temperature and an equal volume of phenol, equillibriated to pH 8.0 with 0.1 M Tris-Cl was added. The mixture was vortexed for 20 s and the aqueous phase was recovered by centrifugation at 4,000 x *g* for 10 min at 20°C. The aqueous phase was reextracted with phenol:chloroform (1:1), followed by chloroform:isoamyl alcohol (24:1). To the

aqueous phase 0.2 volume of 10 M ammonium acetate and 2 volumes ethanol was added. The mixture was stored at room temperature for 10 min and the DNA was recovered by centrifugation at 12,000 x *g* for 10 min at 20°C. The pellet was rinsed with 70 % ethanol, air dried and dissolved in TE (pH 8.0) (Sambrook *et al.* 1989).

The purified DNA fragments were ligated to *Bam*H I digested pGEM-3Z plasmid vector and later introduced into *E. coli* JM109 cells. Different molar ratios of vector to insert DNA (1:1, 1:3, 2:3, 3:2 and 3:1) were used for ligation. Typical ligation conditions used were 100 ng of the vector DNA, 1 µl of T4 DNA ligase buffer, insert DNA and 1 Weiss unit of T4 DNA ligase in a 10 µl total volume. Fresh competent *E. coli* cells were prepared using calcium chloride (Sambrook *et al.* 1989). Single *E. coli* colony from a plate freshly grown for 16 to 20 h at 37°C was inoculated into 100 ml of LB broth. The culture was incubated at 37°C for about 3 h with shaking at 200 rpm. The culture was transferred to ice-cold centrifuge tubes and kept on ice for 10 min. The cells were recovered by centrifugation at 2,000 x *g* for 10 min at 4°C. Pellet was resuspended in 10 ml of ice-cold 0.1 M $CaCl_2$ and stored on ice for 10 min. Cells were recovered by centrifugation at 2,000 x *g* at 4°C for 10 min. The pellet was resuspended in 2 ml of ice-cold 0.1 M $CaCl_2$. The suspension of competent cells was transferred to sterile microfuge tube in 200 µl aliquots. DNA (no more than 50 ng in a volume of 10 µl or less) was added to each tube, mixed and kept on ice for 30 min. Tubes were then incubated at 42°C for 90 s. To each tube 800 µl of LB broth was added and further incubated at 37°C for 45 min. About 100 µl of transformed competent cells was spread onto LB plates containing appropriate antibiotic, IPTG and X-gal (Sambrook *et al.* 1989).

2.2.3.4 Colony blotting

A nylon membrane of appropriate size was selected and placed on the agar surface in a petri dish bearing *E. coli* colonies. The membrane and the agar plate were marked using a sterile needle to mark for orientation of the colonies. The membrane was peeled off after 1 min and placed with the colony side up on a pad of 3MM Whatman paper soaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH). After 7 min the membrane was removed and placed on a separate pad soaked in neutralizing buffer (1.5 M NaCl, 1.0 M Tris-HCl, pH 7.4). After 3 min the blot was removed and kept on a filter paper soaked in 2 X SSC (Section 2.2.3.6). The blot was then transferred to a dry filter

paper and air dried with the colony side up. The membrane was baked at 80°C for 2 h to heat fix the DNA.

2.2.3.5 Probe preparation

The 430 bp *Sac* I/*Stu* I fragment spanning the 3' region of the *phaC*$_{Re}$ gene of *Ralstonia eutropha* (Fig. 2.1) was used as the hybridization probe to screen the *Sau*3A I partial genomic library of *S. aureofaciens*. The DNA probe was labeled with [α-^{32}P]-dCTP using the Megaprime DNA labeling system (Feinberg and Vogelstein 1983).

Reaction was set up (50 µl) as follows:

25 ng DNA probe	5.0 µl
Primer solution (Random hexanucleotides) (3.5 A260 units)	5.0 µl
Above mixture was kept in boiling water-bath for 10 min for denaturation and then allowed to come to room temperature slowly to facilitate annealing of the primer to the template DNA.	
10 X reaction buffer (500 mM Tris-HCl, pH 7.5; 100 mM $MgCl_2$; 10 mM DTT; 0.5 mg/ml acetylated BSA)	5.0 µl
0.5 mM dATP, dGTP, dTTP solution (333 mM Tris-Cl, pH 8.0; 33.3 mM $MgCl_2$; 10 mM 2-mercaptoethanol)	12.0 µl (4 µl each)
[α-^{32}P]-dCTP (Sp. activity 3000 Ci/mmole)	5.0 µl
sterile deionized water	16 µl
Exonuclease free Klenow fragment (2 Units/µl)	2.0 µl
Total Volume	50.0 µl

The reaction was carried out at 37°C for 45 min. For reaction termination and probe denaturation the reaction mix was kept in a boiling water bath for 10 min and snap chilled on ice.

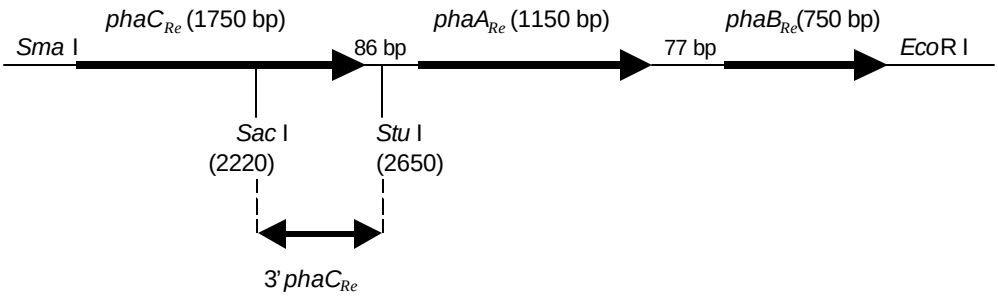

Fig. 2.1 3' *Sac* I/*Stu* I fragment of *phaC*$_{Re}$ gene of *Ralstonia eutropha*: hybridization probe used in colony and Southern hybridization studies.

2.2.3.6 Hybridization and washing

Solutions

20 X SSC

3 M NaCl

0.3 M Sodium Citrate, pH 7.0

Hybridization buffer

1 % BSA

1.0 mM EDTA, pH 8.0

0.5 M Sodium phosphate, pH 7.2

7 % SDS

Low stringency wash buffer	**Moderate stringency wash buffer**
5 X SSC	2 X SSC
0.1 % SDS	0.1 % SDS

Prehybridization of the nylon membrane(s) was carried out at 55°C in hybridization buffer for 6-8 h in a hybridization incubator (Robin Scientific, USA). The amount of buffer added was 0.1 ml/cm^2 of the membrane. The prehybridization buffer was decanted off and fresh hybridization buffer added along with the denatured radiolabelled probe. Hybridization was carried out at 55°C for 14-18 h. The solution was decanted and the membrane washed with low stringency wash buffer first at 28°C and then at 55°C for 15 min each.

2.2.3.7 Southern hybridization

The DNA blots were prepared according to Sambrook *et al*. (1989). The DNA blotting technique was originally described by Southern (1975). The DNA samples with 1X gel loading buffer were electrophoresed in an agarose gel in 1 X TAE buffer. The gel was stained with ethidium bromide (0.5 μg/ml) and photographed over a UV-transilluminator. The DNA in the gel was depurinated by treatment with 0.25 N HCl for 10 min. The gel was rinsed with deionized water and then immersed in a denaturation solution (1.5 M NaCl, 0.5 M NaOH). Denaturation was carried out for 30 min with gentle shaking. The gel was again rinsed with deionized water and immersed in

neutralization buffer (1.5 M NaCl, 1.0 M Tris-HCl, pH 7.4) for 15 min. The gel was then set up for capillary transfer of DNA to solid membrane support.

A tray was filled with the transfer buffer (20 X SSC). A platform was made and covered with a wick, made from 2 sheets of Whatmann 3MM paper saturated with transfer buffer and the gel was placed on it. It was then surrounded with a cling film to prevent the transfer buffer from being absorbed directly by the paper towels to be stacked above. A sheet of Hybond-N^+ membrane (Amersham, UK) of the exact gel size was wetted in deionized water followed by transfer buffer and placed on top of the gel. Any air bubble trapped beneath the membrane was removed. Two pieces of 3MM Whatmann paper wetted with 2 X SSC were placed on the membrane. A stack of absorbent paper towels was placed on top of the 3MM paper. A glass plate was kept on top of the paper towel and a 0.5 kg weight put on top. Transfer of DNA fragments was allowed to proceed for about 18 h. The membrane was then marked for orientation, removed carefully and washed with 6 X SSC. The membrane was air dried and baked for 2 h at 80°C to immobilize DNA onto the nylon membrane. Hybridization was carried out as described earlier (section 2.2.3.6) with the denatured radiolabelled probe (section 2.2.3.5). The membrane was washed with low stringency wash buffer first at 28°C and then at 55°C for 15 min each. Then it was washed with moderate stringency wash buffer at 28°C and then at 55°C for 10 min each.

2.2.3.8 Autoradiography

The moist blot(s) was wrapped in saran-wrap and exposed to X-ray film at -70°C in a cassette with intensifying screen.

2.2.4 PHA

2.2.4.1 *In vivo* detection of PHA granules by fluorescence microscopy

Heats fixed smears of bacterial cells (*S. aureofaciens* and recombinant *E. coli*) were stained with 1.0% aqueous solution of Nile blue A at 55°C for 10 min. The slides were washed with tap water to remove excess stain and then with 8 % aqueous acetic acid for 1 min. The stained smear was again washed with water, blotted dry, remoistened with tap water and covered with a glass cover slip. The coverslip was necessary, since immersion oil extracts some of the fluorescent dye and obscures the field with a general yellow fluorescence. The slide was examined under a Leica

fluorescence microscope fitted with a camera. Fluorescence was observed at an excitation wavelength of 460 nm (Ostle and Holt 1982).

2.2.4.2 Gas chromatographic (GC) analysis of PHAs

S. aureofaciens cells were cultured in Kannan-Rehacek medium (section 2.2.2) at 28°C with constant shaking at 200 rpm and harvested at different time points for PHA analysis.

Recombinant *E. coli* cells were harvested after 48 h of incubation in basal medium at 37°C with constant shaking at 200 rpm.

The cells were centrifuged at 8,000 x *g* for 10 min at 20°C. The cell pellet was washed with saline and freeze dried.

Esterification of PHAs for GC analysis

The method reported by Riis and Mai (1988) was used for esterification of PHAs. About 20 – 40 mg of freeze dried bacterial cell mass was weighed in tightly sealable vials (volume 10 ml). Two ml of 1,2-Dichloroethane (DCE), 2 ml n-Propanol containing hydrochloric acid (HCl) (1 volume concentrated HCl + 4 volume n-Propanol) and 200 µl internal standard (2.0 g benzoic acid in 50 ml n-Propanol) were added. The mix was incubated for 4 h in a water bath at 85°C. The mixture was shaken intermittently. After cooling to room temperature, 4 ml water were added and the mixture shaken for 20 – 30 s. The heavier DCE-Propanol phase was collected and injected directly into the gas chromatograph. Quantitative evaluation was affected by means of the peak areas of hydroxybutyric acid and benzoic acid. PHB content was defined as the ratio of PHB to cell dry weight and expressed as a percentage.

GC conditions

BP 1 capillary column (J & W Scientific Co., USA), 25 m in length with 0.32 mm inner diameter was used. The temperature of the injection port and the flame ionization detector (FID) port was set at 250°C. Following temperature profile was used: 5 min at 80°C, followed by 7°C/min rise to reach a final temperature of 200°C. Nitrogen (5 ml/min) was used as the carrier gas. Injection: splitless, and volume 0.6 µl.

A computer controlled gas chromatograph (Model GC 17-A, Shimadzu) equipped with double FID was used for analysis.

Calibration

Approximately 200 mg of PHB was dissolved by heating in a small volume of DCE using a calibrated 10-ml flask. After cooling to room temperature, the solution was made up to 10 ml. Volumes of 200. 400, 600, 800 and 1000 µl of this solution were esterified as described above and subjected to GC analysis. The relationship between the peak area and the quantity of PHB was observed to be linear up to 3.0 µg of PHB (Fig. 2.2).

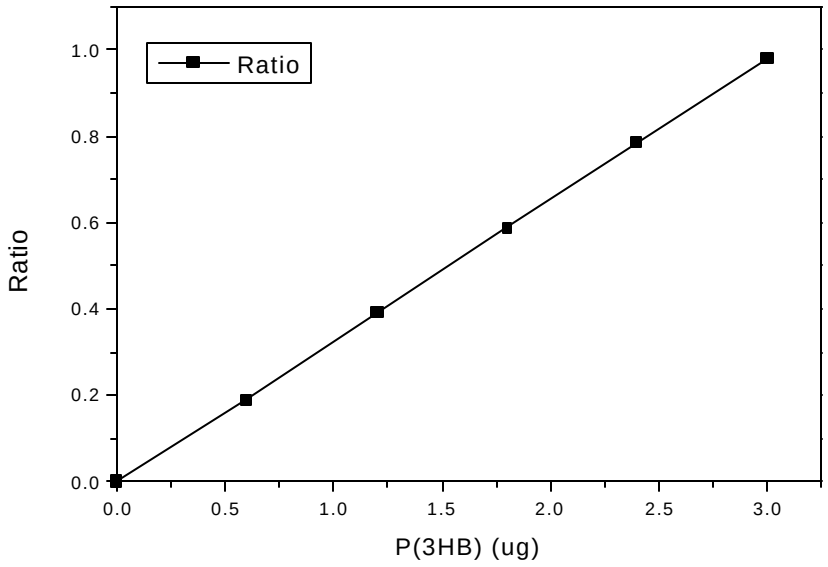

Fig. 2.2 Calibration of the gas chromatographic determination of PHB. The amount of PHB per injected sample was plotted versus the ratio of the concentrations of 3-hydroxybutyryl propyl ester and benzoic acid propyl ester.

2.3 RESULTS AND DISCUSSION

2.3.1 PHA accumulation by *Streptomyces aureofaciens* NRRL 2209

Earlier reports by Kannan and Rehacek (1970) and Ranade and Vining (1993) have shown maximum PHB accumulation in *S. aureofaciens* to occur between 16 to 18 h of incubation. Hence, *S. aureofaciens* cells grown in Kannan–Rehacek medium were harvested after 14, 16, 18 and 24 h of incubation. These time points were selected by bracketing the incubation time for maximum PHB accumulation in *S. aureofaciens* NRRL 2209. An aliquot of the harvested cells when stained with Nile blue-A and observed under a fluorescence microscope at an excitation wavelength of 460 nm, showed characteristic orange fluorescence indicating intracellular accumulation of PHA granules (Fig. 2.3). To specifically determine the composition of the synthesized PHA, the freeze dried cell material and commercial P(3HB) were subjected to esterification. The propyl esters formed were analyzed by gas chromatography. Benzoic acid propyl ester was used as the internal standard. The peak corresponding to propyl ester of 3-hydroxybutyric acid was observed in the gas chromatogram for PHA from *S. aureofaciens* (Fig. 2.4). This indicated that the PHA accumulated by *S. aureofaciens* to be poly(3-hydroxybutyrate), P(3HB). There was a gradual increase in cell mass as is evident by increase in cell dry weight of 0.283 mg at 14 h to 0.312 mg at 24 h. However, a maximum accumulation of 2.4% P(3HB) of cell dry weight (CDW) was obtained in 16 h of incubation (Fig. 2.4). Thereafter P(3HB) levels dropped to 2.05 % of CDW at 18 h and to 0.85 % of CDW at 24 h of incubation. The pH of the medium increased from 6.8 to 7.4 at 16 h of incubation and then decreased to 7.2 after 24 h of incubation. Growth, pH changes and PHB accumulation observed in *S. aureofaciens* at different time intervals is presented in Table 2.2.

Table 2.2: Growth and PHB content in flask culture of *Streptomyces aureofaciens* NRRL 2209 grown in Kannan-Rehacek medium

Time (h)	Dry weight (mg)	PHB % cell dry weight	pH
14	0.283 ± 0.003	2.31 ± 0.028	7.32 ± 0.028
16	0.284 ± 0.003	2.40 ± 0.014	7.40 ± 0.014
18	0.295 ± 0.003	2.05 ± 0.031	7.33 ± 0.042
24	0.312 ± 0.013	0.85 ± 0.127	7.23 ± 0.042

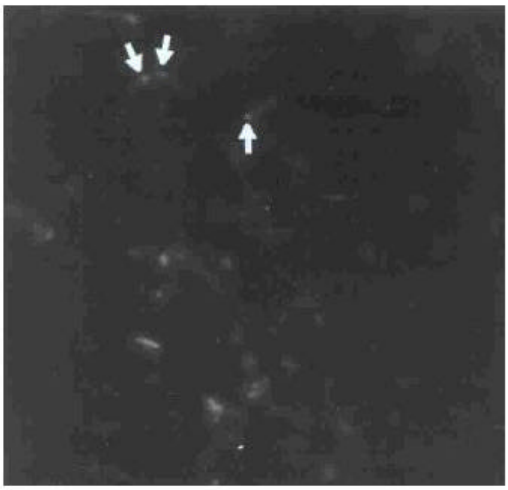

Fig. 2.3 Fluorescence micrograph of *Streptomyces aureofaciens* stained with Nile blueA showing PHA accumulation. Magnification. Ca. X 400.

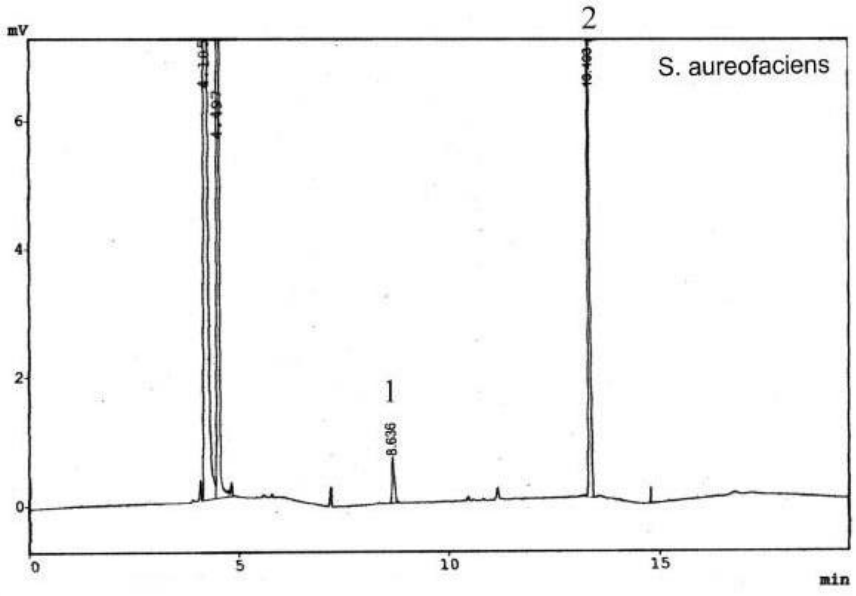

Fig. 2.4 GC analysis of 3-hydroxybutyric acid propyl ester obtained by the hydrochloric acid propanolysis of freeze dried *S. aureofaciens* cells
Peak1: propyl ester of 3-hydroxybutyric acid,
Peak 2: propyl ester of benzoic acid (internal standard).

Maximum accumulation of PHB in *S. aureofaciens* observed at 16 h of incubation is in agreement with the earlier report of Ranade and Vining (1993). However, as against a 2.4% PHB accumulation observed in the present study, Kannan and Rehacek (1970) and Ranade and Vining (1993) had reported a maximum accumulation of only 1.1 %. This difference could be attributed to the more specific and accurate gas chromatographic method (Riis and Mai 1988) for PHB estimation used in the present study as against the spectrophotometric methods (Law and Slepecky 1961) used by earlier authors. In the latter method the PHB must be extracted from the cells prior to determination. The extraction process itself contributes inherent errors.

2.3.2 Screening of *Streptomyces aureofaciens Sau*3A I partial genomic library

The *Sau*3A I partial genomic library of *Streptomyces aureofaciens* NRRL 2209 was constructed in the *Bam*H I site of pGEM-3Z plasmid vector and maintained in *E. coli* JM109 (Tripathi 1999).

The 'N' terminal region of $phaC_{Re}$ gene has been shown to be variable and dispensable for PHB synthesis (Genser *et al.* 1998, Schubert *et al.* 1991). Hence, the *Sau*3A I partial genomic library of *S. aureofaciens* was initially screened by colony hybridization using radiolabelled 3' region of *R. eutropha* PHA synthase gene as the probe (Fig. 2.1) (Peoples and Sinskey 1989b). Twelve positive clones were obtained. These were designated as pSa series with numbers 2, 5, 9, 36, 49, 51, 63, 69, 179, 240, 278 and 281. The representative colony blot is shown in Fig. 2.5.

These twelve positive clones were further analyzed by Southern hybridization studies.

2.3.3 Southern hybridization with the 3' region of $phaC_{Re}$

Plasmid DNA prepared from the twelve *phaC* positive clones was digested with *Eco*R I/*Pst* I to release the insert DNA and separated by agarose gel electrophoresis (Fig. 2.6). Insert DNA size varied from about 1.5 kb to 7.0 kb. The digested and electrophoresed

DNA samples were transferred onto nylon membrane and hybridized with the 3' region of $phaC_{Re}$ gene probe. Three positive clones *viz* pSa9 (with ~ 5 kb insert DNA), pSa69 (with ~ 7 kb insert DNA) and pSa240 (with ~ 5 kb insert DNA) were obtained (Fig. 2.7). The recombinant *E. coli* JM109 cells harboring these three clones were next analyzed for PHA accumulation by fluorescence microscopy and gas chromatography.

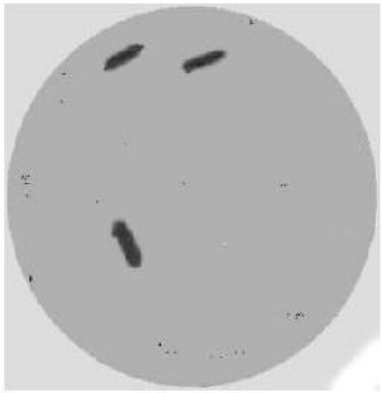

Fig. 2.5 Colony hybridization representative autoradiogram for screening of partial genomic library of *Streptomyces aureofaciens* NRRL 2209 hybridized with [α-^{32}P]-dCTP labeled 3' *Sac I*/*Stu* I fragment of $phaC_{Re}$ gene of *Ralstonia eutropha*.

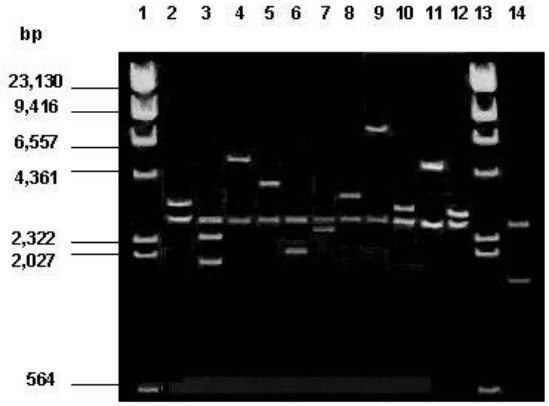

Fig. 2.6 Agarose gel electrophoresis of *Eco*R I/*Pst* I digested 12 selected pSa series clones: Lanes 2-12 and 14: pSa clones 2, 5, 9, 36, 49, 51, 63, 69, 179, 240, 278 and 281 respectively.
Lane1 and 13: Bacteriophage λ DNA digested with *Hin*d III was used as molecular size standards.

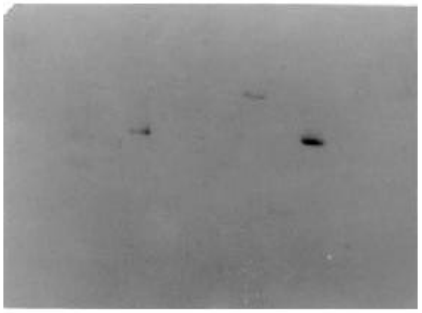

Fig. 2.7 Southern hybridization analysis of the pSa series clones restriction digested with *Eco*R I/*Pst* I hybridized with [α-^{32}P]-dCTP labeled 3' *Sac* I/*Stu* I fragment of *phaC*$_{Re}$ gene of *Ralstonia eutropha*.
Three positive clones were obtained *viz* pSa9, pSa69 and pSa240.

2.3.4 *In vivo* detection of PHA granules by fluorescence microscopy

Recombinant *E. coli* JM109 cells harboring pSa9, pSa69 and pSa240 clones were screened for PHA accumulation by fluorescence microscopy. The cells were stained with Nile blue A and observed using fluorescence microscope at an excitation wavelength of 460 nm. *E. coli* cells harboring the ~ 5 kb *S. aureofaciens* genomic DNA clone pSa240 showed characteristic orange fluorescence indicating PHA accumulation (Fig. 2.8). *E. coli* cells harboring pSa9 and pSa69 did not show any fluorescence. It was, hence, apparent that the presence of pSa240 plasmid clone, which harbors an ~ 5.0 kb genomic DNA fragment from *S. aureofaciens*, supports PHA accumulation in the recombinant cells. The recombinant *E. coli* was next subjected to gas chromatography analysis to determine the type of PHA accumulated.

2.3.5 Gas chromatographic (GC) analysis of recombinant *E. coli* cells harboring pSa240

After 48h of culture growth, both recombinant and nonrecombinant *E. coli* JM109 were harvested and esterified as described earlier. The propyl esters formed were analyzed by gas chromatography. Propyl ester of benzoic acid was used as an internal standard. The gas chromatogram for PHA obtained from recombinant *E. coli* showed presence of a peak corresponding to the propyl ester of 3-hydroxybutyric acid and a peak corresponding to the internal standard, benzoic acid propyl ester. This indicated that the type of PHA accumulated in the recombinant *E. coli* cells to be PHB (Fig. 2.9b). This peak was absent in the gas chromatogram of esterified nonrecombinant *E. coli* JM109 cells (Fig. 2.9a).

Thus, pSa240 which contains an approximate 5.0 kb *Sau*3A I genomic DNA fragment from *S. aureofaciens* NRRL 2209, apparently carries all the necessary genetic information to order and direct poly(3-hydroxybutyric acid) synthesis in recombinant *E. coli* harboring it. This ~ 5.0 kb DNA fragment and the recombinant *E. coli* harboring it were further characterized.

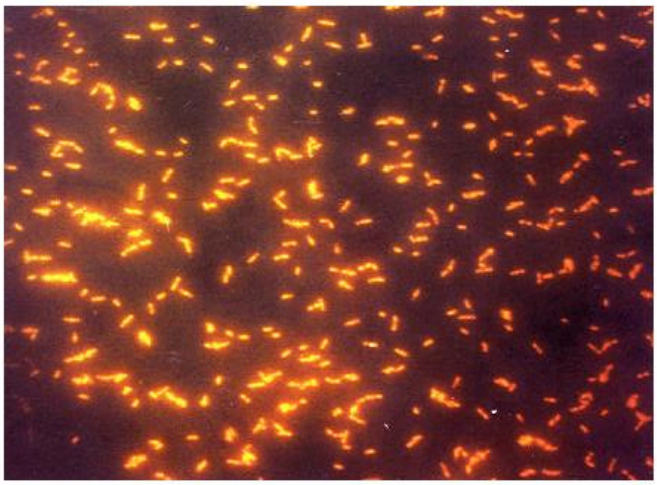

Fig. 2.8 Fluorescence micrograph of recombinant *pha*$_{Sa}^{+}$ *Escherichia coli* cells harboring pSa240 stained with Nile blueA showing PHA accumulation. Magnification. ca. X 400.

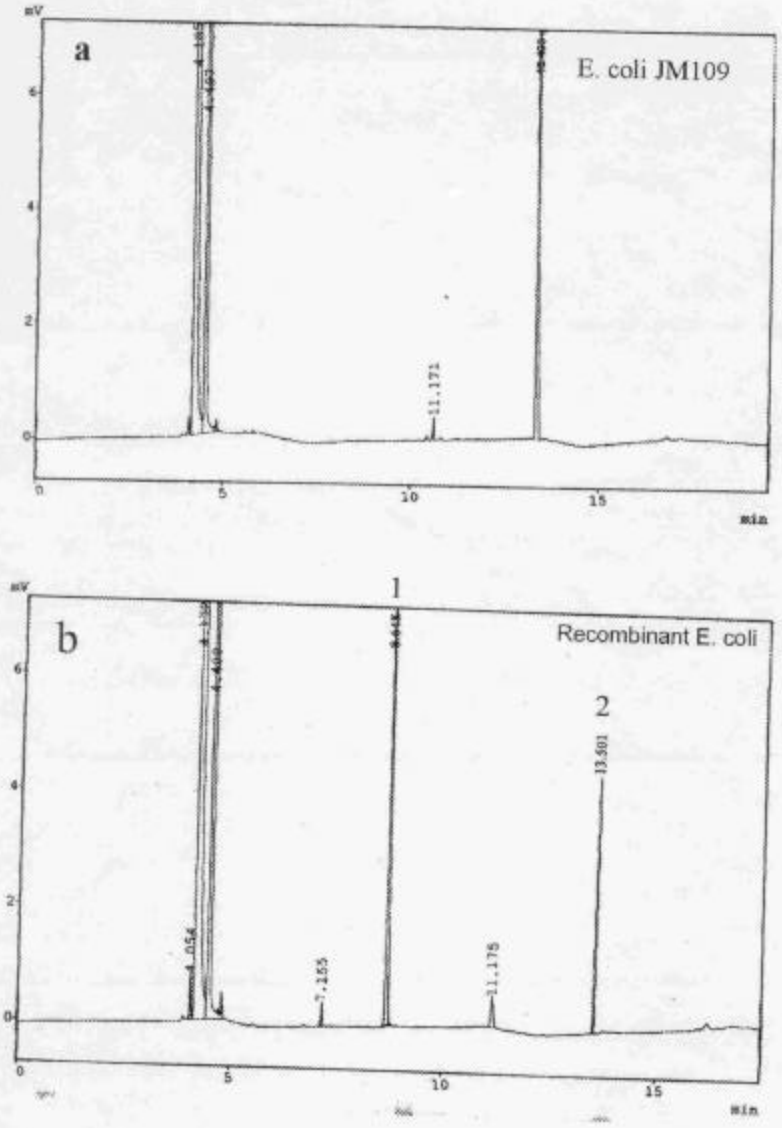

Fig. 2.9 GC analysis of 3-hydroxybutyric acid propyl ester obtained by the hydrochloric acid propanolysis of freeze dried
(a) *Escherichia coli* JM109 and (b) recombinant *Escherichia coli* cells harboring pSa240 cells.
Peak1: propyl ester of 3-hydroxybutyric acid,
Peak 2: propyl ester of benzoic acid (internal standard).

CHAPTER 3

SEQUENCING AND SEQUENCE ANALYSIS OF A ~5.0 KB GENOMIC DNA FRAGMENT OF *STREPTOMYCES AUREOFACIENS* HARBORING THE PHB BIOSYNTHETIC GENES

3.1 INTRODUCTION

The knowledge of physiology, biochemistry and molecular genetics of PHA biosynthesis in bacteria provides sound basis for a thorough evaluation of the biochemistry and catalytic mechanisms of PHA synthesis. The formation of PHA inclusions in the cytoplasm and the design of pathways for the synthesis of known or novel PHAs, not only in recombinant bacteria but also in transgenic plants and other eukaryotic organisms would also be evaluated.

The PHA biosynthesis genes were first identified, cloned, and characterized in *Zoogloea ramigera* by Peoples *et al.* (1987), and in *Ralstonia eutropha* by Schubert *et al.* (1988), Slater *et al.* (1988) and Peoples and Sinskey (1989b). Several more representatives of these genes have been identified and cloned in other organisms (Peoples and Sinskey 1989b, c, Huisman *et al.* 1991, Liebergesell and Steinbüchel 1992, Timm and Steinbüchel 1992, Liebergesell and Steinbüchel 1993, Schembri *et al.* 1994, Schembri *et al.* 1995, Yabutani *et al.* 1995, Fukui and Doi 1997, Hein *et al.* 1998, Matsusaki *et al.* 1998, Umeda *et al.* 1998, Taroncher-olderberg *et al.* 2000). Biosynthesis of P(3HB), the most extensively studied amongst PHAs, involves three sequential enzymatic reactions catalyzed by three distinct enzymes. The first enzyme of the pathway, β-ketothiolase (encoded by *phaA* gene), catalyzes reversible condensation of two acetyl-CoA moieties to form acetoacetyl-CoA. NADPH dependent acetoacetyl-CoA reductase (encoded by *phaB* gene) subsequently reduces acetoacetyl-CoA to R-(-)-3-hydroxybutyryl-CoA, which is then polymerized by the action of PHA synthase (encoded by *phaC* gene) to form PHB.

In order to reduce the confusion over the years a nomenclature has been evolved to depict the genes involved in PHAs synthesis. Hence, genes encoding for enzymes involved in the biosynthesis of PHA are referred to in an alphabetical order as *phaA* (β-ketothiolase), *phaB* (acetoacetyl-CoA reductase), *phaC* (PHA synthase), *phaG* (3-hydroxyacyl-acyl-carrier protein-coenzyme A transferase), *phaJ* (enoyl-CoA hydratase) and so on, whereas the genes required for the degradation are referred to in a reverse alphabetical order such as *phaZ* for PHA depolymerases, *phaY*, *phaX*, *phaW*, etc. The genes for phasins are referred to as *phaP*. The origin of a gene or a protein is indicated by the first letter of the genus and the species, which is added as a subscript (Rehm and

Steinbüchel 1999). Thus PHA synthase gene, *phaC*, from *Ralstonia eutropha* will be depicted as $phaC_{Re}$.

Based on the data available for PHA synthesizing genes from various organisms, different types of arrangements of the genes on the genome have been observed (Madison and Huisman 1999, McCool and Cannon 2001). In *Acinetobacter* sp., *Burkholderia* sp., *Alcaligenes latus, Pseudomonas acidophila* and *R. eutropha*, the *phaC, phaA* and *phaB* genes are placed in tandem on the chromosome although not necessarily in the same order (Schembri *et al.* 1995, Peoples and Sinskey 1989a, b, Umeda *et al.* 1998, Madison and Huisman 1999, Rodrigues *et al.* 2000,). The genes may also be unlinked as *phaAB* and *phaC* loci in *Paracoccus denitrificans*, *Rhizobium meliloti* and *Zoogloea ramigera* (Peoples and Sinskey 1989c, Tombolini *et al.* 1995, Yabutani *et al.* 1995, Lee *et al.* 1996, Ueda *et al.* 1996).

In *Allochromatium vinosum* (formerly called *Chromatium vinosum*), *Thiocystis violacea, Thiocapsa pfennigii* and *Synechocystis* the PHA polymerase is a two-subunit enzyme encoded by the *phbE* and *phbC* genes (Liebergesell and Steinbüchel 1992, Liebergesell and Steinbüchel 1993, Hein *et al.* 1998, Liu and Steinbüchel 2000). But, the arrangement of PHA synthesizing genes is different amongst these organisms. In *A. vinosum* and *T. violacea*, *phbAB* and *phbEC* are in one locus but divergently oriented (Liebergesell and Steinbüchel 1992, Liebergesell and Steinbüchel 1993). However, in *Synechocystis pha* genes are localized in different sections of the genome with arrangement similar to that in *Z. ramigera* (Taroncher-oldenburg 2000). The *pha* loci in *A. vinosum, P. acidophila, R. eutropha, R. meliloti* and *T. violacea* have an additional gene, *phaF*, of a hitherto unknown function in PHA metabolism (Madison and Huisman 1999).

The PHA synthase gene in *Aeromonas caviae* is flanked by *phaJ* encoding for R-specific enoyl-CoA hydratase (Fukui and Doi 1997). In msc-PHA producing *P. oleovorans* and *P. aeruginosa*, the *pha* loci contain two *phaC* genes separated by *phaZ*, which encodes an intracellular PHA depolymerase (Huisman *et al.* 1991, Timm and Steinbüchel 1992). In *Bacillus megaterium* the *phaC* gene is similar to that of *A. vinosum* in size and sequence. However, unlike $phaC_{Av}$, $phaC_{Bm}$ requires $phaR_{Bm}$ for PHA accumulation, but not *phaE* (McCool and Cannon 1999, McCool and Cannon 2001). $PhaR_{Bm}$ is probably an orthologous replacement for $phaE_{Bm}$.

In *Methylobacterium extorquens, Rhizobium etli* and *Rhodobacter sphaeroides* only the PHA synthase gene (*phaC*) has been identified thus far (Hustede and

Steinbüchel 1993, Valentin and Steinbüchel 1993, Cevallos *et al.* 1996, Ji-Hoe and Lee 1997). The PHA synthase gene of *Rhodococcus ruber* and *Nocardia corallina* is not clustered with genes encoding β-ketothiolase, NADPH dependent acetoacetyl-CoA reductase or PHA depolymerase (Pieper and Steinbüchel 1992, Hall *et al.* 1998). In *Chromobacterium violaceum* and *Comamonas acidovorans*, *phaC* and *phaA* genes are arranged in an operon (Sudesh *et al.* 1998, Kolibachuk *et al.* 1999).

Identification and characterization of PHA synthesizing genes from different organisms will help in *in vitro* and *in vivo* metabolic engineering for efficient biosynthesis of known as well as novel PHAs. Knowledge of new PHA synthase genes would also help to elucidate the evolution of PHA synthase genes (Hein *et al.* 1998). Furthermore, the availability of sequence information and the cloned *phaC* genes from different organisms makes possible the application of gene shuffling (Stemmer 1994) and directed evolution (Moore *et al.* 1997) to engineer new or improved PHA synthase(s) for the synthesis of novel biodegradable polymers. Insight gained at molecular genetic level will provide necessary tools for developing the biopolymer engineering approach to produce these materials in recombinant organisms and it would aid in their *in vitro* production as well.

The present chapter of the thesis will describe (a) restriction mapping of the pSa240 DNA insert from the genomic DNA of *S. aureofaciens* NRRL 2209, (b) the sequencing of the pSa240 DNA insert and (c) sequence analysis of the pSa240 insert.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Agarose, ampicillin, kanamycin, Tris and EDTA were purchased from Sigma-Aldrich, USA. All restriction enzymes, T4 DNA ligase and Rnase A were obtained from GIBCO-BRL (USA), Promega (USA) and Amersham (UK). pGEM-3Z, pGEM-5Z vectors were purchased from Promega, USA. All other chemicals used were of analytical grade and obtained from HIMEDIA, Qualigens Fine Chemicals and E. Merck Laboratories, India.

3.2.2 Bacteria and Plasmids

Different bacterial strains and plasmids used are described in Chapter 2, Table. 2.1. The bacterial culture conditions were used as described in Chapter 2, section 2.2.2. Recombinant *E. coli* cells harboring pSa240 DNA (*pha*$_{Sa}^{+}$ recombinant *E. coli*) with transposon Tn5 insertion were grown in media supplemented with kanamycin (50 µg/ml).

3.2.3 Plasmid DNA isolation

Plasmid DNA from *E. coli* was isolated by alkaline lysis method followed by polyethylene glycol precipitation (Sambrook *et al*. 1989; also refer Chapter 2, section 2.2.3.1).

3.2.4 Restriction endonuclease mapping of pSa240

Solutions

TAE and gel loading buffer were prepared as described in Chapter 2, section 2.2.3.7.

pSa240 plasmid DNA was restriction digested with various restriction enzymes singly or in combinations. Restriction enzymes used include *Eco*R I, *Apa* I, *Bam*H I, *Eco*R V, *Hin*d III, *Kpn* I, *Nsi* I, *Not* I, *Pst* I, *Sal* I, *Sma* I, *Xba* I, *Stu* I and *Pvu* II. The reaction conditions used were as recommended by the manufacturer. The restriction enzyme digested DNA samples were electrophoresed in 1% agarose gel in 1 X TAE buffer.

3.2.5 Subcloning of pSa240

Based on the restriction endonuclease map of pSa240, the insert DNA was digested with different restriction endonucleases and electrophoresed in agarose gel. The resulting DNA fragments were purified from agarose slices and ligated to corresponding restriction sites in pGEM-3Z or pGEM-5Z plasmid vectors. Competent cells were prepared by $CaCl_2$ method and the cells transformed using the protocol described by Sambrook *et al*. (1989) (also refer Chapter 2, section 2.2.3.2). Nine subclones pLHSa1 to pLHSa9 were obtained, which spanned the entire pSa240 insert sequence.

3.2.6 DNA sequencing and sequence analysis

DNA sequencing reactions were performed using ABI prism BigDye Terminator Cycle Sequencing kit from PE ABI. The primer extension products were separated by gel electrophoresis on an ABI Sequencer. None of the clones could be sequenced fully since in all instances the sequencing reaction did not proceed beyond about 100 nucleotides and severe sequence compaction was encountered. Therefore transposon insertion system was used to generate frequent and overlapping DNA sequences. Transposons Tn5 were randomly inserted in pSa240 using EZ::TN Kan-2 insertion kit from Epicentre Technologies, USA. Plasmid DNA was purified from randomly chosen kanamycin/ampicillin resistant colonies and sequenced using Forward (KAN – F) and/or Reverse (KAN – R) primers. The ABI prism BigDye Terminator Cycle Sequencing kit from PE ABI was used for DNA sequencing. The primer extension products were separated by gel electrophoresis on an ABI Sequencer. Sequences generated from each clone were assembled using Sequencher software. Nucleotide sequence of both DNA strands was analyzed, using FramePlot method for predicting protein coding region of bacterial DNA with high G+C content (Ishikawa and Hotta 1999). Sequence comparisons and alignments were performed with the Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information) and the Clustal X Multiple sequence alignment program (Baylor College of Medicine).

3.2.7 Gas chromatography analysis

GC analysis of the freeze dried *E. coli* cells was performed as described earlier using a GC 17-A Shimadzu make GC (see chapter 2, section 2.2.4.2).

3.2.8 Enzyme assays

E. coli cells were grown in following basal medium:

	g/l
Yeast extract	5
Peptone	5
Na_2HPO_4	1
$MgSO_4$	0.2
pH 7.2	
Glycerol	10 ml (used as a carbon source)

Ampicillin to a final concentration of 100 µg/ml and kanamycin to a final concentration 50 µg/ml was added to the medium used for cultivation of recombinant pha_{Sa}^+ *E. coli* cells harboring pSa240 and recombinant pha_{Sa}^+ *E. coli* cells harboring transpososn Tn5 insertion clones respectively. The cells were harvested by centrifugation (5,000 x *g*) and the resulting cell pellets were stored overnight frozen at -70°C. The frozen pellets were thawed on ice prior to resuspension in $1/10^{th}$ the original volume in Tris-buffer (1M Tris-HCl pH 7.5, 10mM β-mercaptoethanol). Upon resuspension, the cells were subjected to sonication on ice. The resulting crude cell extract was centrifuged for 10 min at 18,000 x *g* at 4°C and the supernatant was used for enzyme assays. β-ketothiolase and NADPH dependent acetoacetyl-CoA reductase activities were measured, respectively, by previously described methods (Nishimura *et al*. 1978, Belova *et al.* 1997).

β-ketothiolase activity was determined by measuring the decrease in absorbance at 303 nm. The reaction mixture (1 ml) contained: 50 µM Tris-HCl, pH 8.1, 40 µM $MgCl_2$, 0.05 µM CoA and an aliquot of the enzyme preparation. Reaction was started by addition of 0.05 µM acetoacetyl-CoA (AcAc-CoA) after incubation of the mixture at 30°C for 2 min. One unit of β-ketothiolase is defined as the enzyme required for cleavage of 1µmole of acetoacetyl-CoA in 1 min. The activity of NADPH-acetoacetyl-CoA reductase was determined by measuring the decrease in the absorption at 340 nm. The reaction mixture (1 ml) contained: 50 mM Tris-HCl, pH 7.5, 0.5 mM $MgCl_2$, 0.025 mM NADPH and an aliquot of the enzyme preparation. Reactions were started by addition of 0.025 mM AcAc-CoA after incubation of the mixture at 30°C for 1 min. One unit of acetoacetyl-CoA reductase was defined as the enzyme required for oxidizing 1mM of NADPH in 1 min.

3.2.9 Protein estimation

Protein content was determined by the method of Bradford (Bradford 1976).

Protein reagent: Coomassie Brilliant Blue-G 250 (100mg) was dissolved in 50 ml of 95% ethanol. To this solution 100 ml of 85% (w/v) phosphoric acid was added. The resulting solution was diluted to a final volume of 1 l. Final concentration in the reagent was 0.01% Coomassie Brilliant Blue G 250, 4.7% (w/v) ethanol, and 8.5% (w/v) phosphoric acid.

Solutions containing 1–10 μg BSA in a volume of upto 0.1 ml were dispensed into test tubes. The volume was adjusted to 0.1 ml with 0.9% NaCl. One ml of protein reagent was added to the test-tube and the contents were mixed. The absorbance at 595 nm was measured after 2 min against a reagent blank prepared from 0.1 ml of the 0.9% NaCl and one ml of protein reagent. The weight of protein was plotted against the corresponding absorbance resulting in a standard curve (Fig. 3.1) to be used to determine the protein concentration in unknown samples.

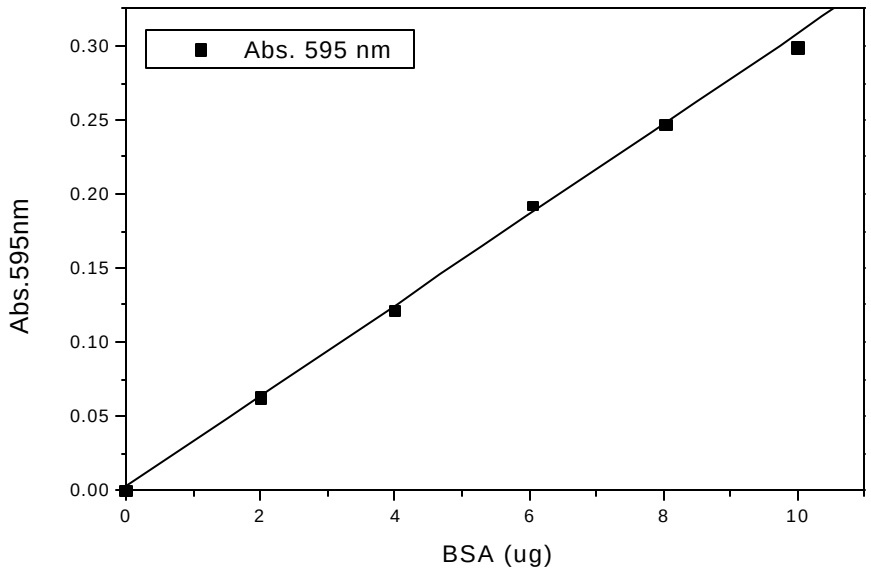

Fig. 3.1 Bradford's method for protein estimation: Protein dye binding response pattern for BSA.

3.3 RESULTS AND DISCUSSION

3.3.1 Restriction endonuclease mapping of pSa240 plasmid DNA

pSa240 plasmid DNA carrying the ~ 5.0 kb insert from *S. aureofaciens* was digested with restriction endonucleases *Eco*R I, *Bam*H I, *Hin*d III, *Kpn* I, *Nsi* I, *Not* I, *Pst* I, *Sal* I, *Sma* I, *Xba* I, *Stu* I, *Apa* I and *Pvu* II, singly or in combination and electrophoresed on 1% agarose gel. The representative gel photograph is shown in Fig. 3.2. There is one site for restriction endonucleases *Stu* I and *Nsi* I. Three sites are present in pSa240 DNA insert for restriction endonuclease *Sal* I. No site in the pSa240 insert was detected for restriction endonucleases *Eco*R I, *Hin*d III, *Xba* I, *Bam*H I, *Kpn* I, *Not* I and *Pst* I. Five and six DNA fragments were obtained when pSa240 plasmid DNA was digested with *Sma* I and *Apa* I, respectively. Sites for these two restriction endonucleases (*Apa* I and *Sma* I) could not be placed on the map. The *Stu* I/*Nsi* I 1.4 kb DNA fragment was ligated to *Eco*R V/*Nsi* I digested pGEM-5Z. The resulting DNA fragment was digested with *Pvu* II to release three fragments of size 0.5 kb, 1.0 kb and 2.7 kb (not shown); Accordingly *Pvu* II site is placed on the restriction map of pSa240 (Two *Pvu* II sites are present in the vector pGEM-5Z). The pSa240 insert restriction endonuclease fragments are tabulated in Table 3.1 and the map generated is shown in Fig. 3.3.

Table 3.1 The pSa240 insert restriction endonuclease fragments

Restriction endonucleases used for pSa240 digestion	Approximate sizes of the DNA fragments released (kb)
Sal I	0.5, 3.4, 3.7
Stu I	7.6 (linearized)
Nsi I	7.6 (linearized)
*Eco*R I	7.6 (linearized, NS*)
*Bam*H I	7.6 (linearized, NS*)
*Hin*d III	7.6 (linearized, NS*)
Kpn I	7.6 (linearized, NS*)
Not I	NS*
Apa I	0.3, 0.4, 0.6, 0.9, 1.1, 4.3
Pst I	7.6 (linearized, NS*)
Sma I	0.5, 0.6, 1.1, 2.4, 3.0
Xba I	7.6 (linearized, NS*)
*Eco*R I + *Hin*d III	2.7, 4.9
Nsi I + *Stu* I	1.4, 6.2
*Eco*R I + *Stu* I	2.0, 4.6
*Eco*R I + *Nsi* I	3.4, 4.2
Stu I + *Hin*d III	2.9, 4.7
Sal I + *Hin*d III	0.5, 3.4, 3.7
*Eco*R I + *Sal* I	0.5, 1.0, 2.7, 3.4

* NS: No site detected in the insert.

3.3.2 Subcloning

Based on restriction endonuclease map of the pSa240 insert, the DNA was digested with different restriction enzymes to get subclones of the insert. The DNA was digested with: *Eco*R I + *Sal* I (1.0 kb); *Sal* I + *Stu* I (1.0 kb); *Stu* I + *Nsi* I (1.4 kb); *Nsi* I + *Sal* I (1.0 kb); *Sal* I + *Hin*d III (0.5 kb); *Sal* I + *Nsi* I (2.4 kb); *Nsi* I + *Hin*d III (1.5 kb). The resulting DNA fragments were ligated to pGEM-3Z or pGEM-5Z and transformed

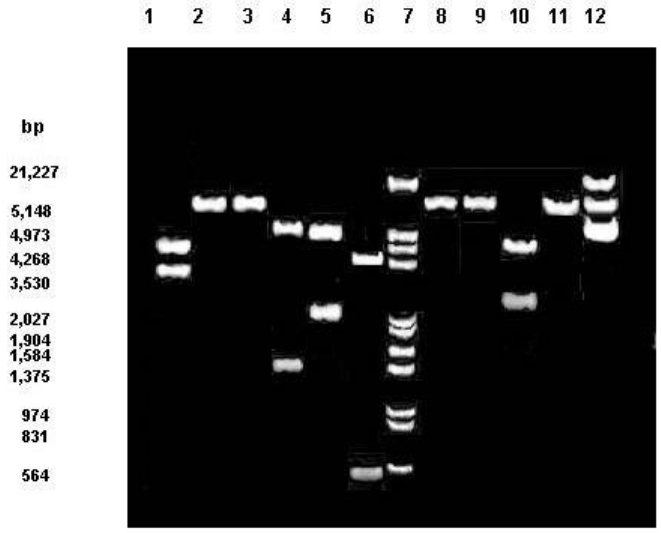

Fig. 3.2 Agarose gel electrophoresis of pSa240 digested with restriction endonuclease (s): Lane 1: *Eco*R I + *Nsi* I; Lane 2: *Eco*R I; Lane 3: *Stu* I; Lane 4: *Nsi* I + *Stu* I; Lane 5: *Eco*R I + *Stu* I; Lane 6: *Sal* I; Lane 8: *Nsi* I; Lane 9: *Pst* I;Lane 10: *Eco*R I + *Hin*d III; Lane 11: *Xba* I; Lane 12: *Not* I.
Lane7: Bacteriophage λ DNA digested with *Hin*d III + *Eco*R I was used as molecular size standards.

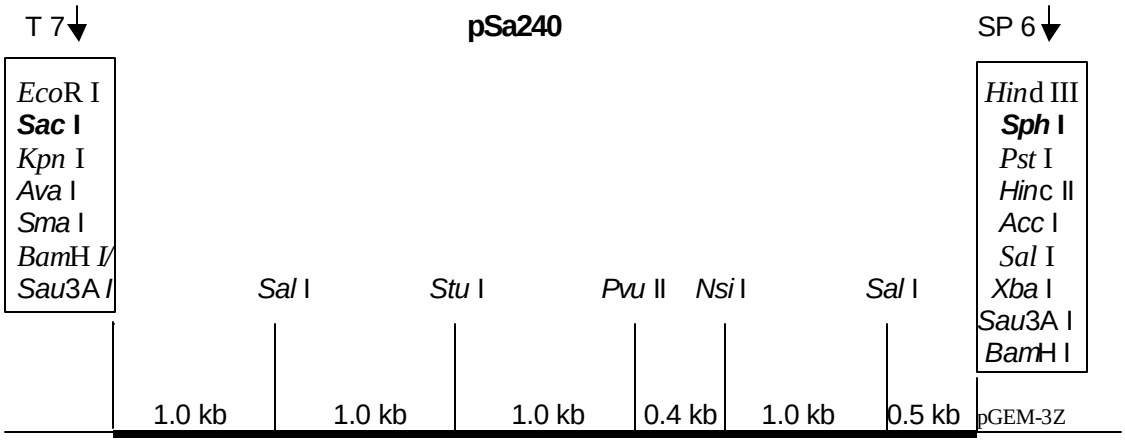

Fig. 3.3 ***Restriction endonuclease map of ~ 5.0 kb insert DNA of pSa240***

into competent JM109 or DH5α *E. coli* cells. Seven subclones generated and designated as pLHSa 1 to pLHSa 7 are shown in Table 3.2 and line diagram (Fig. 3.4). pLHSa 3 was digested with *Pvu* II, out of resulting three fragments, ~ 0.5 kb and ~1.0 kb fragments were purified and ligated to *Eco*R V digested pGEM-5Z independently to get pLHSa 8 and pLHSa 9, respectively (shown in Table 3.2 and Fig. 3.4). Nine subclones were digested with appropriate restriction endonucleases to release the insert DNA fragment and electrophoresed on 1% agarose gel for clone verification (Fig. 3.5).

Table 3.2 Subclones of pSa240 obtained for sequencing and the plasmid vector used.

Subclone	Flanking RE sites	Fragment size (kb)	Plasmid vector
pLHSa 1	*Eco*R I / *Hin*d III	1.0	pGEM-3Z f(-)
pLHSa 2	*Eco*R I + *Hin*d III	1.0	pGEM-3Z f(-)
pLHSa 3	*Sph* I / *Nsi* I	1.4	pGEM-5Z f(+)
pLHSa 4	*Nsi* I + *Sal* I	1.0	pGEM-5Z f(+)
pLHSa 5	*Eco*R I + *Hin*d III	0.5	pGEM-3Z f(-)
pLHSa 6	*Sal* I + *Nsi* I	2.4	pGEM-5Z f(+)
pLHSa 7	*Eco*R I + *Hin*d III	1.5	pGEM-3Z f(-)
pLHSa 8	*Sph* I + *Nsi* I	0.5	pGEM-5Z f(+)
pLHSa 9	*Sph* I + *Nsi* I	1.0	pGEM-5Z f(+)

3.3.3 Sequencing and sequence analysis

Sequencing of the subclones pLHSa1 to pLHSa9 was attempted using ABI prism BigDye Terminator Cycle Sequencing kit from PE ABI. The primer extension products were separated by gel electrophoresis on an ABI Sequencer. However, in each instance the sequencing reaction did not usually proceed beyond about 100 nucleotides due to severe band compactions. Generally templates containing high G+C content can produce shorter readable sequences than other templates do. *Streptomyces* sp. are known to have a high (69-78%) G+C ratio (Hodgson 2000). GC rich regions in DNA are known to cause secondary structures, which create problems for a polymerase reaction during (i) the denaturation of the template, (ii) annealing of the primers to the template and, (iii) extension of the primers by polymerase (Choi *et al*. 1999). Band compressions are caused by anomalies in the migration behavior of certain DNA fragments in the

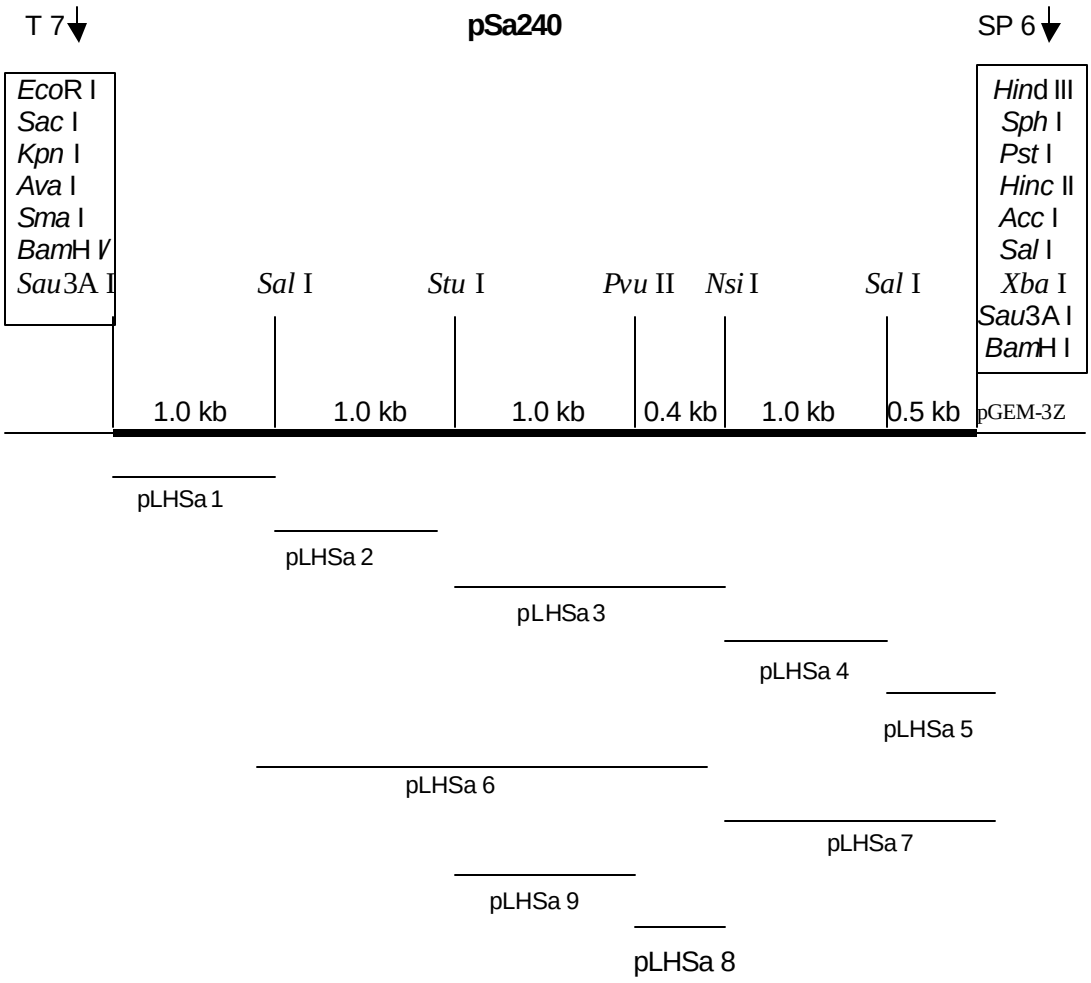

Fig. 3.4 Strategy for subcloning of ~ 5.0 kb insert DNA of pSa240

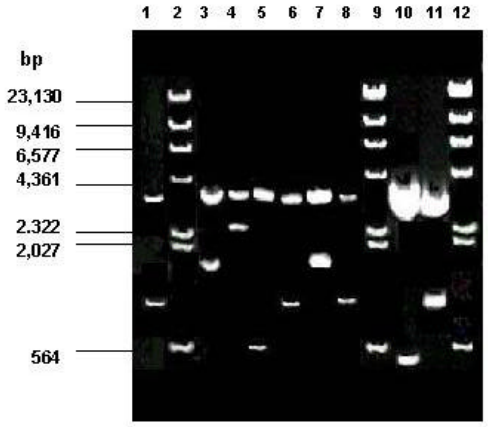

Fig. 3.5 Agarose gel electrophoresis of pLHSa subclones digested with different restriction endonucleases: Lane 1: pLHSa4 (*Nsi* I + *Sal* I); Lane 3: pLHSa3 (*Sph* I + *Nsi* I); Lane 4: pLHSa6 (*Sal* I + *Nsi* I); Lane 5: pLHSa5: (*EcoR* I + *Hin*d III); Lane 6: pLHSa 1 (*EcoR* I + *Sal* I); Lane 7: pLHSa7 (*EcoR* I + *Hin*d III); Lane 8: pLHSa2 (*EcoR* I + *Hin*d III); Lane 10: pLHSa8 (*Sph* I + *Nsi* I); Lane 11: pLHSa9 (*Sph* I + *Nsi* I). Bacteriophage λ DNA digested with *Hin*d III (lanes 2, 9 and 12) was used as molecular size standards.

polyacrylamide gel because of intramolecular base pairing between guanine and cytosine residues (Motz et al. 2000) resulting in unreadable sequencing gel profiles.

Goryshin and Reznikoff (1998) developed a simple yet elegant in vitro Tn5 transposition system. The system permits very high efficiency random insertions of a Tn5 transposon into a DNA molecule in vitro. In the present study a EZ::TN Kan-2 insertion system was used for random Tn5 transposon insertion to generate shorter overlapping DNA sequences. Plasmid DNA isolated from randomly selected Kan^r E. coli colonies was sequenced using forward (KAN-F) and reverse (KAN-R) primers and ABI BigDye terminator cycle sequencing kit followed by gel electrophoresis on an ABI sequencer (Fig. 3.6). Sequences generated from each clone were assembled using Sequencher software.

The 4826 bp sequence obtained for pSa240 DNA insert from *S. aureofaciens* (Fig. 3.7) was deposited with the GenBank under accesssion number AY032926. The 3' *Sac* I/*Stu* I fragment (430 bp) of the $phaC_{Re}$ gene of *Ralstonia eutropha* (Chapter 2, Fig. 2.1) which was used as a probe to screen recombinant *E. coli* colonies (Chapter 2, Fig. 2.5) and in Southern hybridization (Chapter 2, Fig. 2.7) of the *phaC* positive clones showed ~ 53% sequence identity with the 3' end of the ORF 3 (GTG^{275}-TGA^{2443} described later) pSa240 nucleotide sequence (Fig. 3.8).

Open reading frames (ORFs) and Codon Usage

The 4826 bp pSa240 DNA fragment (Fig. 3.7) had a G+C content of 74.6 mol%. *Streptomyces* genome has been reported earlier to have a G+C content of 69 to 78% (Hodgson 2000). Thus the G+C content of the isolated insert was in conformity with earlier reports.

While ATG is the commonest translation initiation codon in *E. coli*, GTG as an initiation codon is also found, though at low frequency (about 3%) (Hopwood *et al.* 1986). Amongst *Streptomyces* sp. genes characterized so far, GTG as the initiation codon is more frequently encountered than in *E. coli*. The *argG* gene of *Streptomyces clavuligerus* (Rodriguez-Garcia *et al.* 1995), gene encoding for chymotrypsin-like serine protease from *Streptomyces lividans* 66 (Binnie *et al.* 1996), gene for chitinase (*chi*B) from *Streptomyces lividans* (Miyashita *et al.* 1997) and the gene encoding for OtrB (Tet347) tetracycline efflux protein from *Streptomyces rimosus* (Mcmurry and Levy 1998) are few examples where GTG serves as the translation start codon.

Nucleotide sequence analysis of both DNA strands, using FramePlot method for predicting protein coding region of bacterial DNA with high G+C content (Ishikawa and

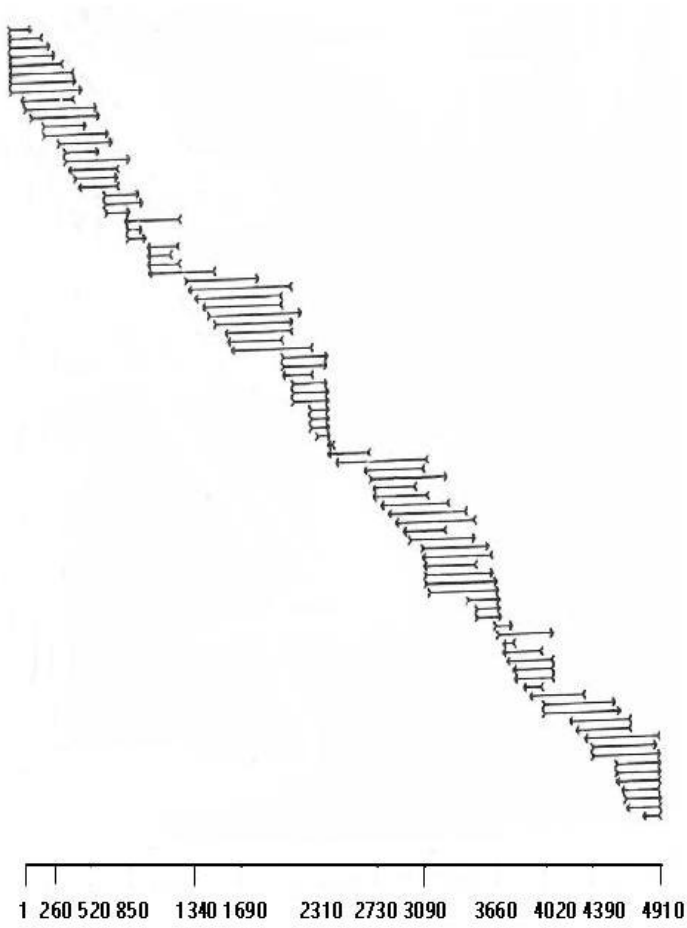

Fig. 3.6 DNA sequencing strategy: DNA sequences generated by transposon mediated insertions. The arrows indicate the direction of DNA sequencing

GATCGGCGGCCGGTCGGCGGTGCTGGCCGCGGTGACCCTGGGGGCGC TGGCCGCTCCGGCGGTGCTGCT
GCGCCGGGGGCTGGCGGCCACCGCGGAGGCGCTGGCGGCGCTGGCCCTGGTGCTGACGCTGCTGGACG
TGTACGCGGTCCACGCGGTGGCCGCGCCGGACACCGACGGACTCGGCTTCACGGCCCTCGCGTCGGCGG
TGCTCGCGGCGCTGTGGACGGCGTACGGGCTGGCGCTGGGCAAGCTGCGCCTGCCGTTGCCGGCCGCCG
TGGTGCTGGCCCAGTGGCCGCTGCTGTTCTGGGCCTGGGCCGTGGGCGCACCGGCGCCGGTGGTCGGGT
GGGCGCTGCTGGCCACCGCGGTGCTGGACGGGGCGATCGCCCTGTGGGGCAAGGGCGCCGGGGTGCGG
GTCACGGCGTGCGTCGGTGGAGCGGTGATGGGCTTCTCGGCCCTGATGGTGGGCCTGGCGCTGTCCCTG
ACGGCCCCGGGGCCGCTCGGGGCGGTGGCTCCGGGCGTGCTGCTGCTGACGGCCTCGGCGGCGGCCGT
GGCCGGGGCGTGGCGCGCGCCGAAGGGTTTCGCGCGGACGGGTGGTGCGGTGGCGGGGCTCGCGGCGG
TGGCGGCCGTCGGCGGCGTACCGGCGGCGGCGCTCCCGGCGGGCTGGCGGGTGCTCGCGTACCTGCTGT
GCGGTCTCGCGTTGACGGCGGTCGTCCGTTCCCGGCTGCCGGGCCACGCCGCGCGCGGGGTACTGGCGG
CGTCGGGGGCGGTGGTGGCCGGCGCGCTGGTGTGGGCGCTGCCGCCGCTCGCGGCGGTGCTGCTGGGG
CCGGTGACGGTGCTGTCGGACGTGTGGGCGGGGACGCCGGACGGCTTCCGGTCCGCGCTGGGGTCGAC
GCTGCCCTGGTCGGAGCTGGCCGCGGCCCCGGTGGTGCTCGCGCTGGTGGCCGGGCATGCTGGGGGCG
AGCGTAACCGGAGGTGGCCGTCGGTCGTCCGGCTCCGGCGCCGTTGGCCGGTCCTTCTGGCTCGACGCC
GGCCCCCCGGCAGCACCGGCAGCGGGAGCCCCGGGCACGGATGCGCCGGGCGCGGCCGGGGGCGCTGC
GCCGTGGCCCGGCTGGTCCGGCTGGTCCGGCCGGCCCGGTGCGGGGGCCGGTGGTCGCGGGCGGCCTTC
CGCGGCGACGCTGCGCGGGGTCGTCGGCGCGGGCGCGGTGGCGCTCGGCTGGGGGGCCCTCCTGCTGG
CCGGCGCGCTGCTGGACGTGCCCCACGCGCTCGCGCTGGCCGGGGAGACGGCTCTGGTGGGCGTCCTGC
TCGCCCTGGCGGTCCGGGGTGGCGGCGCCGAGCGGGGCGCGACGGCGATGCCGGTGACCGCTCTGGTG
GCTTCGGTGGCCGGGGCGGTGAGCGCCGGGCTGCTGTCGCTGGCGTCCGAGGGGGCCTCGTACGCGGT
GTTCGGCGCGCTGGCGGCGCTGTTCGCCGGGGCCGCTCTGCGGGCGGGCGCCGGGGTGCCGCGTGCGGT
GTTCGCGGTCGCCGCGGTGGTCTGGGGCACCGTGATCACGGGGTTGGCGGGCCGGTCCCTGGGGCTCGC
CCCGCACGAGGCCGCCCCGCTGATGCTGCTGGTGCCGGCGCTGACGGTGCTGCTCGGGGCACGACTGCG
GCGGAACCCGGTGGCCTTGCCCGTGGAGCTGACGGGAGCGCTGGGCGCGCTCGTCGCCGTGGGGCTCG
CGGTGTCCGACGCGCCGTTCCTGGCCCTGGTGCTGGCGCTGTGCGGGGTGCTGGCGGCGGGGACGGCGG
TGCGGCCGGAGCGGCGGCCGGTGGCGGGCTACCTGGCGGCGACGCTGTTCGTGCTGGCCACGTGGGTG
CGGCTGGCGGCCTCGGAGGTGTCGTTCCCGGAGGCGTACACGCTGCCGGTGACGGTGCCCGCGCTGCTG
GTCGGTGCGGCGCGGCGGCGCCGGGACCCGGAGGCCTCGTCGTGGACGGCGTACGGGCCGGGGCTCGC
GGCGACGCTGCTGCCCAGCCTGGCGGTCGCCTGGACCGACCCGGACTGGCTCAGGCCGTTGCTGCTGGG
GACGGCGGCGCTGGTGATCACCCTGCTCGGCGCGCGCCACCGGCTCCAGGCGCTGCTGCTGCTCGGCGG
GACGGTGCTGGCACTGGTCGGCCTGCACGAGCTGGCGCCGTACGTGGTGCAGGTCGCGGGTGCGCTCCC
CCGCTGGCTCCCGCCCGCCCTGGCCGGGCTGTTGTTGCTGGTGGTCGGAGCGACGTACGAGCAGCGGCT
GCGGGACGCCCGCCGTCTGAAGGACGCGCTGGGGCGGATGCGGTGAGCCGTGCCCGGTCCGGGGGCGC
GCAGGTCACGGCGTCCCCGGGCCGGGCGCCAGTGGCGTGGGCAACGCAGAGGGCCCGGCCCTCTGTCC
GGGTGGGCGATACTGGGTTCGAACCAGTGACCTCTTCGGTGTGAACGAAGCGCTCTCCCACTGAGCTAA
TCGCCCGGGCGCACCGCAAACATTACCCCATGTCAGCGGTGCTCCCGGACCGTCCCCGGGCTACTCGCT
GATCTTCCACGGCATGGTGAGCCCGAACTTCCAGACGTAGATCCCGGCCAGCACCGCCATGATCACGAG
CCCGAGCGTGGTGAGGATGATGTTGCGCCGCCGGACCTTGGGATCGAGGGCCCGCTGCGCCGCTTCGGT
GACCTTGCGCTTGGTCCAGCGCAGCACCAGCTGGGCCCAGACGAACTCGGTCGCCCAGATCGCCATGCC
GCCGAAGATCACCAGCCAGCCGGGGCCCGGCAGCACCAGCATGAGCACACCCGCGATCACCACGCCGA
GACCGACGATGAAGACACCGACCTGCCAGCTCAGGTGGAGCGCCTTGGACGCCTTGATGAAACCCGGC
GCCCGCGAGCCCAGCGCGCGTTCCTCCCGGTCCGATTCCCCCGTGGCGGATACCGGGGACGCCTGCTCG
GCGACCTTGCTCCGCTCGTCACTCTCCGCGTTCATGAAGCTCAACTTACCCGACCTGTCTCCGTCACTGG
AATGGGCGCATAACTCAAAGTTACACGCCGCTGAGCGGGGGACCCGAAGCGTCACAAATGGGTCAGAG
GGGTTTACAACGCCACCGTAGGTGGCATGTCGATTTCGCCGACGTGCGAATCCCCGAGCGCACACTGAG
CGAAAGGCCCTGGCGCTTATGAACACCACGGTCAGCTGCGAGCTGCACCTGCGCCTCGTTGTGTCGAGC
GAGTCCTCACTGCCTGTACCCGCGGGCCTGCGGTATGACACGGCCGATCCCTATGCCGTGCACGCCACC
TTCCACACCGGAGCGGAGGAGACGGTCGAATGGGTATTCGCCCGCGACCTCCTTGCCGAGGGGCTGCA
CCGGCCCACCGGCACCGGAGACGTCCGCGTCTGGCCATCTCGTAGTCACGGTCAAGGCGTCGTATGCAT
CGCCCTGAGCTCCCCAGAGGGAGAAGCCCTGCTCGAAGCCCCGGCGCGGGCCCTGGAGTCGTTCCTGA
AGAGGACCGACGCCGCGGTTCCGCCCGGCACCGAGCATCGTCACTTCGATCTCGACACGGAGCTCTCCC
ACATCCTGGCCGAGAGCTGAGCCAGGCAGAGAGCCGCTCTACGCCGTCCGACTCGGGGCGACGGCGTC
GTGCTGACAACCGCATAGGGCAGACACCGGCGCCGTCGTCGCGGAATCCACCGCGACGACGGCGCCGG
CGCGTTCCCCGCCGCGCCGCCGGAGGGGTCCGTTCCGCTCTCCGCCGGGCCCGCACCGGGCCCGGCACC
GGCCGGCCGAGCCAGTAGAGTCAGCCGCCATCGGCAGGCGCCCGCCCGGCGGAAGGCCAGGGAGCGA
AGCGTGCTGATCCCTCACGACACCCGGATCGCCCTCGACGCGGTGGT CGATCTGGTGAACACCGCACCG
GAGAGCGAGCCGCCGGGGGACGACCCCGGCGACAGACACGCGGGCGGGCCCGAGGACGGTCTCCCCG
ACATCGCCGCGCTGTACGCCTTCGCGGAGCGCCATCTCATCAGCGGGGTCGGCACCCTCGGCGAGAAGG
ACCTCGGCGCCGTGCGCGACGTCCGGGCCCGCTTCGCCGAGGTC4089

Fig. 3.7 pSa240 insert DNA sequence (4,826 bp)

TTCGCGGCGCCCGACGCCCGCGTCGCCGCCGACCTGGTCAACCGGCTCGTCGCGGCGGCCGGGACCACC
CCGCAGCTCACGGACCACGACGGCTACGACTGGCACGTGCACTACTTCGCCCCGGACGCCTCGATCGCC
GACCATCTCGCGGCCGACTGCGGCATGGCGCTGGCCTTCATCATCGTGGCGGGCGAGCAGGAGCGGCT
GCGGCGCTGCGAGGCCCCGGACTGCGGGCACGCGTTCGTCGACCT GTCGCGCAACCGCTCCCGCCGCTA
CTGCTCCAGCCGTACGTGCGGGAACCGGCTCCACGTCGCGGCGTACCGGGCCCGGCGCAAGGAAGCCG
CGGGCTGACGCCCGGCACGGTGGCGCGAGGCGTCACAGCACGAAGAGATCGTGCAGCGCGGCCATCAG
CAGCAGGCCCCCGATCACCGTCAGGAAGATCATCAGGGGCGGCTGGGAGAGCGCGAAAAGACAGCCG
CGGGCCTCTTCGGCGGGGGGTGCGGGGGCATCGCCCCGGGAAGTGTCCACCATCTCGGGGTGATCATG
ACGCACCGGCGGCGGTGTTGGCGATCAACCGGCTTCATTCTCCCGGGAGTTCACCGTCCCGTGGCCATC
GATATTCGCTCCGGCGTACGGGGAGCCGTCAGACATTCGGACCGCCGCCCGGAACGCACGCCGGCGGG
GCCGGCCGACGCCTCGGACGCCGCGCTTCTCAGATGCCGTGCTTCTTGAGGATC

Fig. 3.7(contd.) pSa240 insert DNA sequence (4,826 bp)

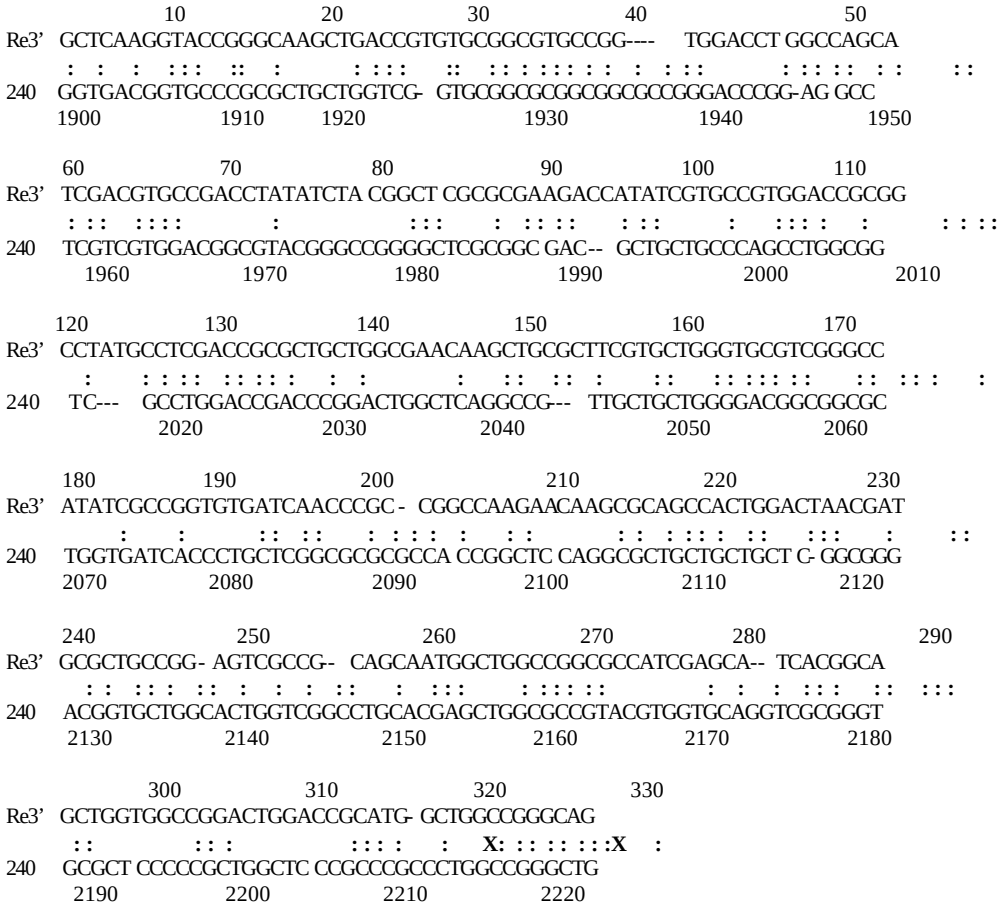

Fig. 3.8 52.8% sequence identity observed in the stretch of 339 nucleotides (Local alignment using LFasta).
Re3': The nucleotide sequence of 3' *Sac* I/*Stu* I fragment (430 bp) of the *phaC*$_{Re}$ gene from *Ralstonia eutropha*.
240: The nucleotide sequence of pSa240 DNA insert (4826 bp) from *Streptomyces aureofaciens* NRRL 2209.

Hotta 1999), revealed six open reading frames (ORFs) with GTG as start codon. These were:

ORF 1: 2153bp – 414bp; ORF 2: 3266bp – 2529bp; ORF 3: 20bp – 2443bp; ORF 4: 3760bp – 3188bp; ORF 5: 1203bp – 2309bp; ORF 6: 4398bp – 3607bp (Fig. 3.9).

Four ORFs were also identified with ATG as start codon. These were:

ORF 7: 2596bp – 3108bp; ORF 8: 440bp – 2443bp; ORF 9: 948bp – 2309bp; ORF 10: 4233bp – 3607bp (Fig. 3.9).

Analysis of ORFs

In *Streptomyces*, amongst 139 promoter sequences complied, only 29 sequences show presence of consensus −35(TTGACPu) and −10 (TAgPuPuT) boxes (Strohl 1992). The −10 and −35 box sequences in pSa240 sequence were identified using the −10 and −35 sequences reported for these 29 genes (Table 3.3). Remaining 110 promoters identified either by S1 nuclease mapping or primer extension analysis, do not display either typical −10 box or the −35 region or both (Strohl 1992). The nucleotide sequence at the 3' end of 16s rRNA of *S. aureofaciens* is 5'ACGAAGTCGT AACAAGGTAG CCGTACCGGA AGG3' (GenBank accession no. AB045881).

Though ORF 3 which starts at nucleotide position 20 shows the presence of a ribosome binding site (^{10}CCGGT^{14}) was detected upstream of the GTG start codon. However, a small upstream sequence rules out the presence of any promoter sequence. Moreover, ORF 3 reads in opposite orientation with respect to the *lacZ* gene and its promoter and hence, would not be driven by the *lacZ* promoter. An internal region of ORF 3 (275 to 2443 nucleotides) may encode for a gene product of approximate M_r. 69500 (Table 3.4a). In the PHA synthase genes reported till-date size variation is observed from 1068 bp in *Ectothiorhodospira shaposhnikovii* (GenBank accession no. AF307334) to 2022 bp in *Rhodospirillum rubrum* (GenBank accession no. AF178117). Correspondingly the molecular mass of PHA synthase varies from approximately 40000 to 73000. In the same reading frame ATG is present at position 440 (ORF 8, Fig. 3.9). This translational codon is also in frame. The deduced translational product of ORF 8 has M_r. of ~ 64000**.**

The ORF which starts with ^{275}GTG as the initiation codon, shows presence of a ribosome binding site (RBS) ^{266}**CCGG**^{269}. In *Streptomyces* sp. the distance of RBS from initiation codon usually ranges from 6 to 12 nucleotides (Strohl 1992, Hopwood *et al.* 1986). In the promoter region the important sequences are −10 and −35 boxes

pSa240

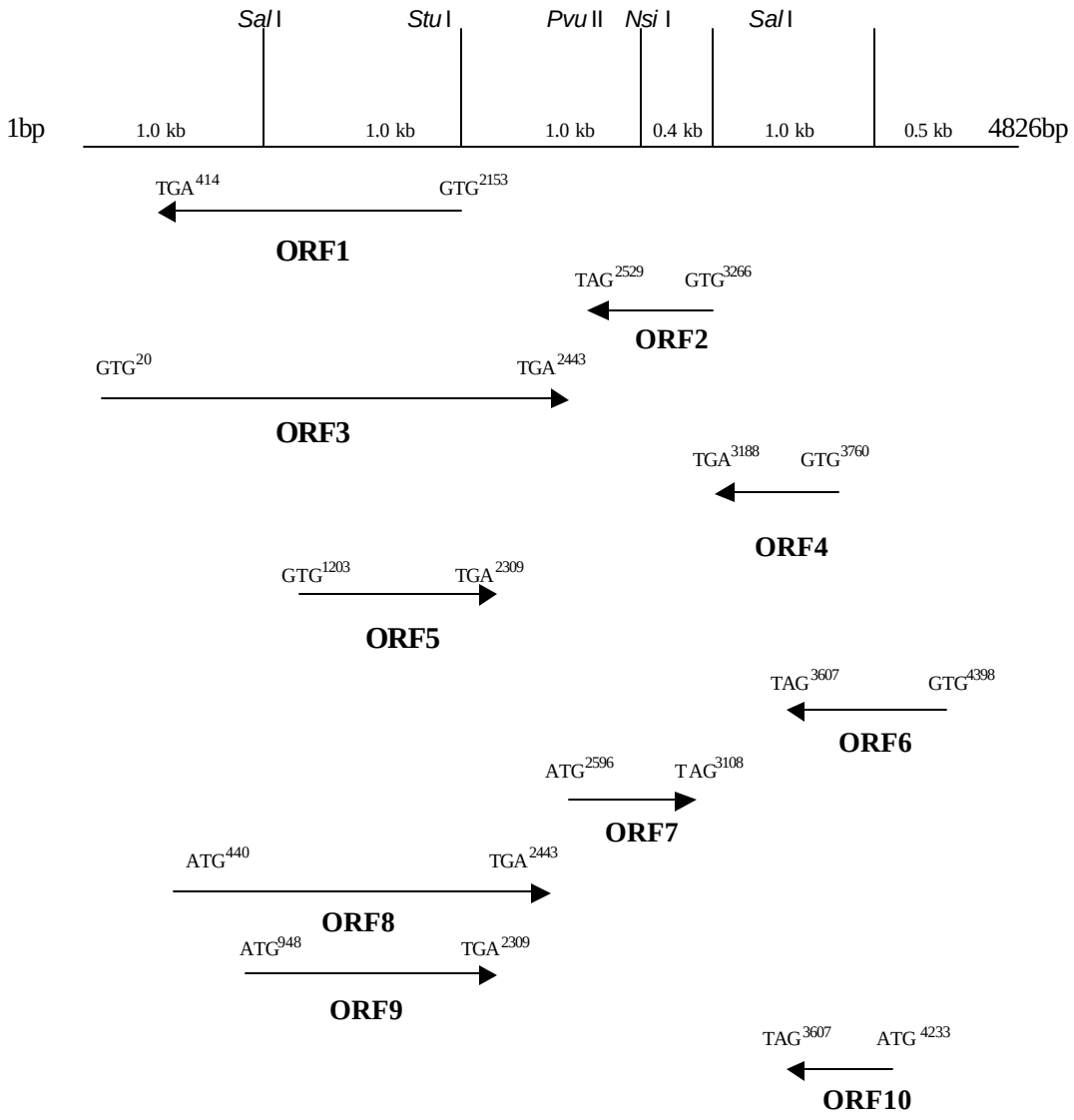

Fig. 3.9 Positions and orientations of open reading frames (ORFs) probably representing coding DNA**.**

Table 3.3 Compilation of –10 and –35 promoter sequences of *Streptomyces* sp. promoters

Promoter	**-35 box**	**-10 box**
Consensus	*TTGACPu*	*TagPuPuT*
SEP3	TTGCAA	CATCAT
redD-pr1	TGGTGT	CATCAT
vph-Pa1	TGGAAT	GTTGTT
rrnD-p2	TTGACA	TAGATT
Tra-p(pIJ101B)	TTGACA	CAGTAT
mmr-p	TTGACA	TTCAGT
agal-p	TTGATT	TAGGGT
Korp	TTGCGC	CAGGAT
SEP8	TTGACG	CATACT
gly-p1	TTGACG	GAGACT
amlv-p	TTGACC	TACGGT
aml-p	TTGACC	TACGGT
amySG-P	TTGACC	TACGGT
XP55-p	TTGACG	TGCAAT
SEP6	TGGACA	TTATAT
rrnD-p1	GTGCAT	TAGTGT
ssi-p2	GTGAGT	CAGACT
choP-p	TTGACA	TACGGT
dag-p4	TTGTCA	TAGCAT
ermE-p2	TTGACG	GAGGAT
SEP2	TTGACG	TAAAAT
ermSF-p	TTGCGC	TACCGT
KilB-p1(pIJ101A)	TTGGTC	TACAAT
parc-p	TTGCCG	TAGCGT
cerfD-p	TTGAAG	CAGAAT
mP1c-p	TTGACG	CACACT
aacC7-p1	GTGCCG	TACCTT
npr-p	GTGACA	TAAAGT

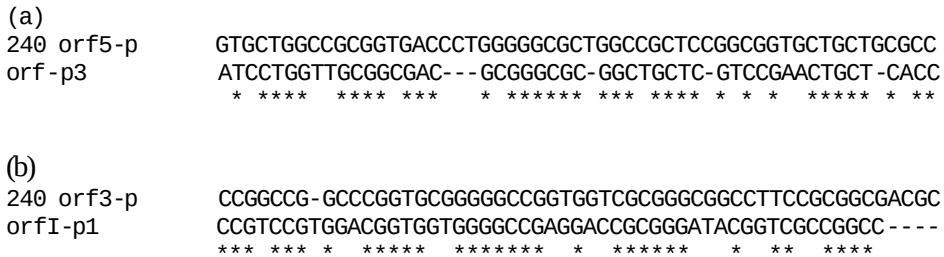

Fig 3.10 Putative promoter sequences upstream of ORF 3 (a) and ORF 5 (b) based on sequence identity with the reported *Streptomyces* sp. promoter sequences

Table 3.4a Amino acid composition of deduced translation product of putative *phaC*$_{Sa}$ gene (http://www.expasy.ch/tools/protparam.html)

Number of amino acids: 722

Molecular weight: 69443.1

Theoretical pI: 12.20

Amino acid composition:

Ala (A)	162	22.4%
Arg (R)	103	14.3%
Asn (N)	1	0.1%
Asp (D)	24	3.3%
Cys (C)	8	1.1%
Gln (Q)	5	0.7%
Glu (E)	6	0.8%
Gly (G)	159	22.0%
His (H)	8	1.1%
Ile (I)	1	0.1%
Leu (L)	50	6.9%
Lys (K)	2	0.3%
Met (M)	2	0.3%
Phe (F)	7	1.0%
Pro (P)	56	7.8%
Ser (S)	26	3.6%
Thr (T)	17	2.4%
Trp (W)	15	2.1%
Tyr (Y)	1	0.1%
Val (V)	69	9.6%

Total number of negatively charged residues (Asp + Glu): 30
Total number of positively charged residues (Arg + Lys): 105

that lie on the same face of the DNA helix and bind the RNA polymerase. There is often little sequence identity between promoters in the intervening and surrounding DNA sequences. However, about 110 promoter sequences from *Streptomyces* sp. do not display typical −10 and −35 regions. With this background, a nucleotide sequence spanning nucleotides 20 to 73 showing sequence similarity with the promoter sequence of the orf-p3 promoter of *Streptomyces* (Fig. 3.10a) (Strohl 1992) could serve as a promoter. In *Streptomyces*, a feature of a majority of translational start sites determined so far is the occurrence of a cytosine residue five nucleotides upstream of the translation start codon and the base immediately preceding this codon is either C or G (Hopwood *et al.* 1986). Both these features were observed for ORF 3 with the base preceding the start codon ^{275}GTG and the base at −5 position being C. The translation initiation codon GTG is also followed by another GTG as reported for SAM-P20D gene of *Streptomyces* sp. (Taguchi *et al.* 1997). PHA synthase has been reported to be a member of the prokaryotic lipase superfamily, which in turn is a member of the α/β hydrolase family (Jia *et al.* 2000). Like other reported PHA synthases, the deduced amino acid sequence of ORF 3 (275 - 2443) shows the presence of the conserved lipase box, **Gly^{399}-X-Ser-X-Gly-Gly^{404}** (Madison and Huisman 1999). While cysteine serves as a nucleophile in the active site of other reported synthases, in the *S. aureofaciens* putative PHA synthase, cysteine, the amino acid with uncharged polar side chain is replaced by serine, another amino acid with uncharged polar side chain. This makes it the first among the PHA synthases wherein serine acts as the nucleophile. Given the fact that the PHA synthases belong to the lipase superfamily and catalyzes ester bond formation, the presence of serine in the lipase box of the sequence in question should not be surprising, since serine indeed serve as the nucleophile in the active site of lipases (Brady *et al.* 1990, Winkler *et al.* 1990). The two histidine residues present as **Gly^{522}-His^{523}** and **Asp^{600}-His^{601}** dyads, probably form a part of the catalytic triad (Madison and Huisman 1999), needed for synthase function (Fig. 3.11a). The 26 PHA synthases studied till date among themselves show less than 3% sequence identity at amino acid level (Madison and Huisman 1999). ORF 3 (275 – 2443) which exhibits the attributes of an ester bond forming polymerase could be the putative gene encoding for PHA synthase (*phaC*$_{Sa}$) from *S. aureofaciens*. One might argue that there is high probability of occurrence of

Putative promoter

8 GGCCGGTCGGCG**GTGCTGGCCGCGGTGACCCTGGGGGCGCTGGCCGCTCCGGCGGTGCTGCTG

CGCCGGGGGCTGGCGGCCACCGCGGAGGCGCTGGCGGCGCTGGCCCTGGTGCTGACGCTGCTGGACGT

GTACGCGGTCCACGCGGTGGCCGCGCCGGACACCGACGGACTCGGCTTCACGGCCCTCGCGTCGGCGGT

RBS

GCTCGCGGCGCTGTGGACGGCGTACGGGCTGGCGCTGGGCAAGCTGCGCCTGCCGTTG**CCGG**CCGCC

Putative phaC →

GTGGTGCTGGCCCAGTGGCCGCTGCTGTTCTGGGCCTGGGCCGTGGGCGCACCGGCGCCG

V V L A Q W P L L F W A W A V G A P A P

GTGGTCGGGTGGGCGCTGCTGGCCACCGCGGTGCTGGACGGGGCGATCGCCCTGTGGGGC

V V G W A L L A T A V L D G A I A L W G

RBS ***Putative phaC*** →

AAGGGCGCCGGGGTGCGGGTCACGGCGTGCGT**CGGT**GGAGCGGTG**ATG**GGCTTCTCGGCC

K G A G V R V T A C V G G A V M G F S A

CTGATGGTGGGCCTGGCGCTGTCCCTGACGGCCCCGGGGCCGCTCGGGGCGGTGGCTCCG

L M V G L A L S L T A P G P L G A V A P

GGCGTGCTGCTGCTGACGGCCTCGGCGGCGGCCGTGGCCGGGGCGTGGCGCGCGCCGAAG

G V L L L T A S A A A V A G A W R A P K

GGTTTCGCGCGGACGGGTGGTGCGGTGGCGGGGCTCGCGGCGGTGGCGGCCGTCGGCGGC

G F A R T G G A V A G L A A V A A V G G

GTACCGGCGGCGGCGCTCCCGGCGGGCTGGCGGGTGCTCGCGTACCTGCTGTGCGGTCTC

V P A A A L P A G W R V L A Y L L C G L

GCGTTGACGGCGGTCGTCCGTTCCCGGCTGCCGGGCCACGCCGCGCGCGGGGTACTGGCG

A L T A V V R S R L P G H A A R G V L A

GCGTCGGGGGCGGTGGTGGCCGGCGCGCTGGTGTGGGCGCTGCCGCCGCTCGCGGCGGTG

A S G A V V A G A L V W A L P P L A A V

CTGCTGGGGCCGGTGACGGTGCTGTCGGACGTGTGGGCGGGGACGCCGGACGGCTTCCGG

L L G P V T V L S D V W A G T P D G F R

TCCGCGCTGGGGTCGACGCTGCCCTGGTCGGAGCTGGCCGCGGCCCCGGTGGTGCTCGCG

S A L G S T L P W S E L A A A P V V L A

CTGGTG GCCGGGCATGCTGGGGGCGAGCGTAACCGGAGGTGGCCGTCGGTCGTCCGGCTC

L V A G H A G G E R N R R W P S V V R L

CGGCGCCGTTGGCCGGTCCTTCTGGCTCGACGCCGGCCCCCCGGCAGCACCGGCAGCGGG

R R R W P V L L A R R R P P G S T G S G

AGCCCCGGGCACGGATGCGCCGGGCGCGGCCGGGGGCGCTGCGCCGTGGCCCGGCTGGTC

S P G H G C A G R G R G R C A V A R L V

CGGCTGGTCCGGCCGGCCCGGTGCGGGGGCCGGTGGTCGCGGGCGGCCTTCCGCGGCGAC

R L V R P A R C G G R W S R A A F R G D

GCTGCGCGGGGTCGTCGGCGCGGGCGCGGTGGCGCTCGGCTGGGGGGCCCTCCTGCTGGC

A A R G R R R G R G G A R L G G P P A G

CGGCGCGCTGCTGGACGTGCCCCACGCGCTCGCGCTGGCCGGGGAGACGGCTCTGGTGGG

R R A A G R A P R A R A G R G D G S G G

CGTCCTGCTCGCCCTGGCGGTCCGGGGTGGCGGCGCCGAGCGGGGCGCGACGGCGATGCC

R P A R P G G P G W R R R A G R D G D A

GGTGACCGCTCTGGTGGCTTCGGTGGCCGGGGCGGTGAGCGCCGGGCTGCTGTCGCTGGC

G D R S G G F G G R G G E R R A A V A G

GTCCGAGGGGGCCTCGTACGCGGTGTTCGGCGCGCTGGCGGCGCTGTTCGCCGGGGCCGC

V R G G L V R G V R R A G G A V R R [399]**G R**

TCTGCGGGCGGGCGCCGGGGTGCCGCGTGCGGTGTTCGCGGTCGCCGCGGTGGTCTGGGG

S A G G[404] R R G A A C G V R G R R G G L G

-lipase box

CACCGTGATCACGGGGTTGGCGGGCCGGTCCCTGGGGCTCGCCCCGCACGAGGCCGCCCC

H R D H G V G G P V P G A R P A R G R P

GCTGATGCTGCTGGTGCCGGCGCTGACGGTGCTGCTCGGGGCACGACTGCGGCGGAACCC

A D A A G A G A D G A A R G T T A A E P

GGTGGCCTTGCCCGTG GAGCTGACGGGAGCGCTGGGCGCGCTCGTCGCCGTGGGGCTCGC

G G L A R G A D G S A G R A R R R G A R

GGTGTCCGACGCGCCGTTCCTGGCCCTGGTGCTGGCGCTGTGCGGGGTGCTGGCGGCGGG
G V R R A V P G P G A G A V R G A G G G
GACGGCGGTGCGGCCGGAGCGGCGGCCGGTGGCGGGCTACCTGGCGGCGACGCTGTTCGT
D G G A A G A A A G G G L P G G D A V R
GCTGGCCACGTGGGTGCGGCTGGCGGCCTCGGAGGTGTCGTTCCCGGAGGCGTACACGCT
A [522]**G H**[523] V G A A G G L G G V V P G G V H A
GCCGGTGACGGTGCCCGCGCTGCTGGTCGGTGCGGCGCGGCGGCGCCGGGACCCGGAGGC
A G D G A R A A G R C G A A A P G P G G
CTCGTCGTGGACGGCGTACGGGCCGGGGCTCGCGGCGACGCTGCTGCCCAGCCTGGCGGT
L V V D G V R A G A R G D A A A Q P G G
CGCCTGGACCGACCCGGACTGGCTCAGGCCGTTGCTGCTGGGGACGGCGGCGCTGGTGAT
R L D R P G L A Q A V A A G D G G A G [600]**D**
CACCCTGCTCGGCGCGCGCCACCGGCTCCAGGCGCTGCTGCTGCTCGGCGGGACGGTGCT
H[601] P A R R A P P A P G A A A A R R D G A
GGCACTGGTCGGCCTGCACGAGCTGGCGCCGTACGTGGTGCAGGTCGCGGGTGCGCTCCC
G T G R P A R A G A V R G A G R G C A P
CCGCTGGCTCCCGCCCGCCCTGGCCGGGCTGTTGTTGCTGGTGGTCGGAGCGACGTACGA
P L A P A R P G R A V V A G G R S D V R
GCAGCGGCTGCGGGACGCCCGCCGTCTGAAGGACGCGCTGGGGCGGATGCGGTGAGCCGT
A A A A G R P P S E G R A G A D A V S R
GCCCGGTCCGGGGGCGCGCAGGTCACGGCGTCCCCGGGCCGGGCGCCAGTGGCGTGGGCA
A R S G G A Q V T A S P G R A P V A W A
ACGCAGAGGGCCCGGCCCTCTGTCCGGGTGGGCGATACTGGGTTCGAACCAGTGACCTCT
T Q R A R P S V R V G D T G F E P V T S
TCGGTGTGA **2443**
S V -

Fig. 3.11a Nucleotide and deduced amino acid sequence of putative *phaC*$_{Sa}$ gene. Lipase box region, DH, GH dyads; potential promoter region, RBS: potential ribosome binding site are underlined.

features such as the presence of cytosine five nucleotides upstream of the translation start codon or presence of G/C preceding the translation start codon, in G+C rich genome of *Streptomyces*. But other features observed such as its orientation opposite to *lacZ* gene promoter, occurrence of RBS and promoter upstream of it, and presence of catalytic domains supports its candidature as the putative PHA synthase gene.

The ORF 8 and ORF 3, both operate in same reading frame. The ORF 8, which starts with ^{440}ATG as the initiation codon shows presence of a ribosome binding site (RBS) ^{427}CGGT^{430}. The probable promoter region mentioned above spanning nucleotides 20 to 73 might serve as the promoter. The –35 box ^{263}TTGCCG^{268} was also identified. For ORF 8, the base preceding the start codon ^{440}ATG is G and the base at –5 position is C. ORF 8 being part of ORF 3, also has features such as the presence of lipase box and the **Gly^{522}-His^{523}** and **Asp^{600}-His^{601}** dyads. Hence, either of these two ORFs, ORF 3 or ORF 8 could serve the PHA synthase function. In other words, the *phaC$_{Sa}$* gene may commence its translation either from ^{275}GTG or ^{440}ATG.

The deduced translational product of ORF 5 (1203 – 2306 nucleotides) is of approximate M_r. 37000 (Table 3.4b). The base preceding the translation start codon is G in this ORF. ORF 5 shows presence of a ribosome binding site (RBS) ^{1186}**TCGT**^{1189}. About 400 bp upstream of the ^{1203}GTG, a –35 box ^{783}TGGTGT^{788} was identified (Strohl 1992). No sequence corresponding to –10 box was observed. A putative promoter sequence spanning nucleotides 1123 to 1176 was identified in the 5' UTR (untranslated region) of ORF 5. This promoter sequence is similar to the orfI-p1 promoter of *Streptomyces* (Fig. 3.10b) (Strohl 1992). Like other NADPH dependent acetoacetyl-CoA reductases (Olivier *et al.* 1988) the ORF 5 shows the presence of the NADP binding motif, **V^{1}-A-L-G-W-G-A-L-L-L-A-G-A-L-L-D-V-P-H-A-L-A-L-A-G-E-T-A-L^{29}** (Fig. 3.11b). The ORF 5 coding for 368 aa could be the putative *phaB$_{Sa}$*. The putative *phaB$_{Sa}$* gene coding for 368 aa is relatively longer than other *phaB* genes reported earlier. For example, *phaB* gene from *R. eutropha* codes for 246 aa with an approximate M_r. 26300 (Peoples and Sinskey 1989a); while *phaB* gene from *Azotobacter vinelandii* codes for 247 aa with an approximate M_r. 26700 (GenBank accession no. AF267243). The ORF 5 and ORF 9, both operate in same reading frame. ORF 9 starting from ^{948}ATG as the initiation codon shows presence of a possible ribosome binding site (RBS) ^{940}**GGC**^{942}. About 250 bp upstream of the ^{948}ATG, a –35 box ^{783}TGGTGT^{788} was observed (Strohl 1992). No sequence corresponding to –10 box

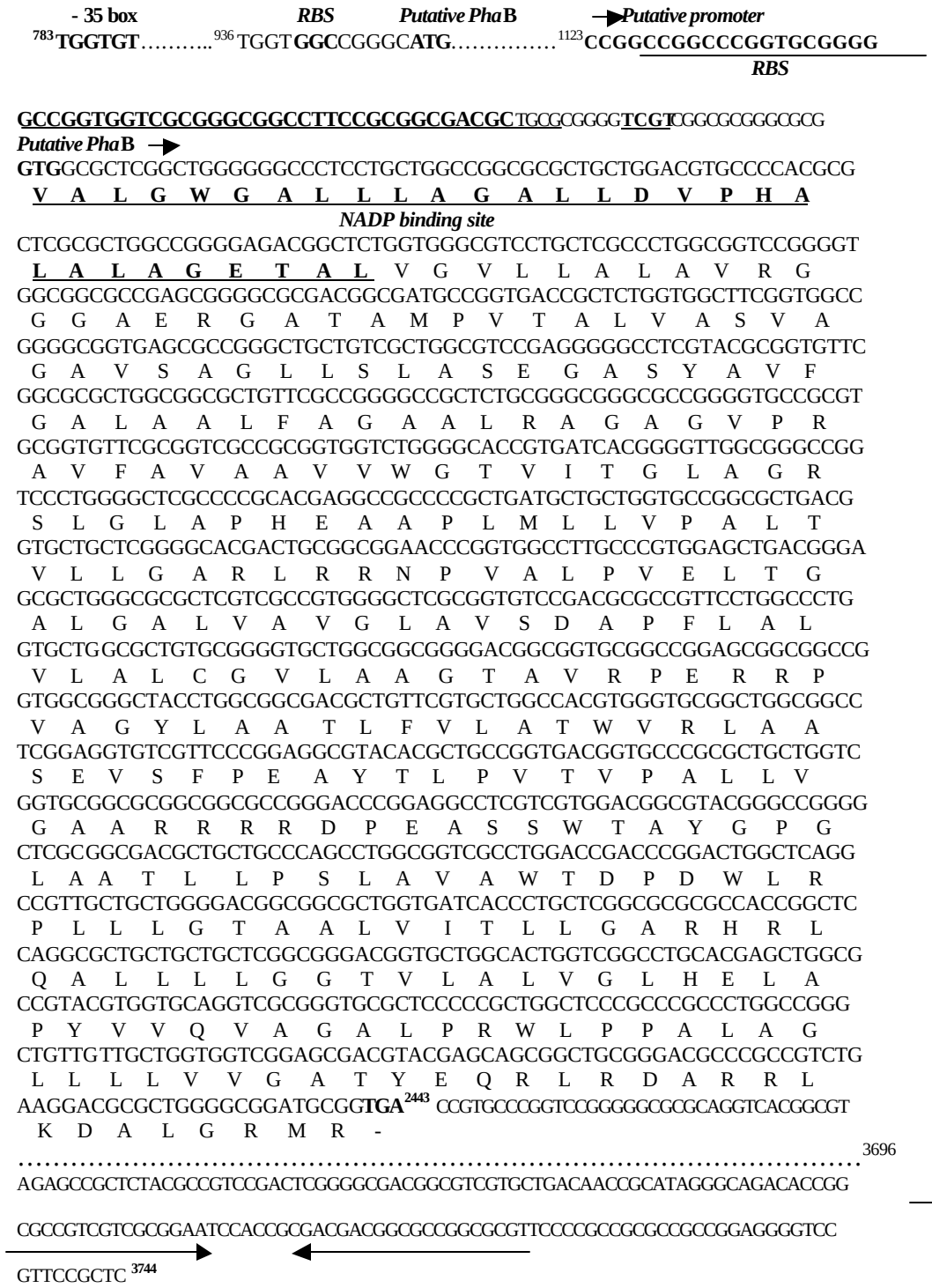

Fig. 3.11b Nucleotide and deduced amino acid sequence of putative *phaB*$_{Sa}$ gene. NADP binding domain, potential promoter region, RBS: potential ribosome binding site are underlined. Arrows: a potential transcription terminator

Table 3.4b Amino acid composition of deduced translation product of putative *phaB*$_{Sa}$ gene

Number of amino acids: 368

Molecular weight: 37172.2

Theoretical pI: 10.69

Amino acid composition:

Ala (A)	79	21.5%
Arg (R)	26	7.1%
Asn (N)	1	0.3%
Asp (D)	7	1.9%
Cys (C)	1	0.3%
Gln (Q)	3	0.8%
Glu (E)	11	3.0%
Gly (G)	39	10.6%
His (H)	4	1.1%
Ile (I)	2	0.5%
Leu (L)	76	20.7%
Lys (K)	1	0.3%
Met (M)	3	0.8%
Phe (F)	6	1.6%
Pro (P)	23	6.2%
Ser (S)	12	3.3%
Thr (T)	19	5.2%
Trp (W)	7	1.9%
Tyr (Y)	6	1.6%
Val (V)	42	11.4%

Total number of negatively charged residues (Asp + Glu): 18
Total number of positively charged residues (Arg + Lys): 27

was observed. For ORF 9, both, the base preceding the start codon ^{948}ATG and the base at −5 position were C. ORF 5 and ORF 9 being overlapping and along the same reading frame, have the NADP binding site present in both the ORFs. ORF 9 encodes for 453 aa with approximate M_r. of 45000 which is much higher than the reported acetoacetyl-CoA reductases. Presence of putative promoter region and comparatively smaller size are in favor of ORF 5 to be probable gene encoding for NADPH dependent acetoacetyl-CoA reductase. Unlike many of the other reported NADPH dependent-acetoacetyl-CoA reductases, where the NADPH binding site is found at the N-terminal end of the protein, the same would be out of context if ORF 9 was to be considered as the *phaB* gene.

Downstream of ORF 3, ORF 8 and ORF 5, inverted repeats were identified in the 3' UTR (ΔG = -54.2 kcal) which may serve as the putative transcription termination signals (Fig. 3.11b).

The codon usage data calculated using the gene sequences available for *S. aureofaciens* (http://www.kazusa.or.jp/codon/) (Nakamura *et al.* 2000) is shown in Table 3.5a. A strong bias towards the use of G or C in the third position of the codon was observed. Similarly, High G+C content and biased use of G or C in the third position of codon was observed for the putative $phaC_{Sa}$ and $phaB_{Sa}$ genes (Table 3.5b and 3.5c).

Based on sequence analysis, none of the other ORFs could be assigned the functions of β-ketothiolase gene (*phaA*). Apparently the 4826 bp insert from pSa240 does not carry this gene.

ORF 4 (3760 – 3188) encodes for a gene product of approximate M_r. 21000. It shows significant homology at amino acid level with the hypothetical regulatory protein of *Streptomyces coelicolor* (SWALL:Q9L268).

Remaining ORFs neither show homology with any of the proteins reported in protein databases nor did they show presence of any conserved motifs. Hence, functions could not be assigned to these ORFs. However, their involvement in PHB synthesis could not be ruled out considering the fact that genes relevant for PHA synthesis are usually clustered in many bacteria. Putative promoter sequences were identified upstream of ORF 6 (4563-4514), ORF 7 (2435-2475) and ORF 10 (4563-4514) (http://www.fruitfly.org/seq_tools/promoter.html Waibel *et al.* 1989). Upstream of ORFs 2 (GTGCCG), 6 (TTGCGC) and 10 (TTGCGC), only −35 box sequences were identified (Strohl 1992). While, upstream of ORFs 1 (CAGTAT) and 7 (GTTGTT) only

Table 3.5a Codon usage data for *Streptomyces aureofaciens* [gbbct]: 34 CDS's (12288 codons) (http://www.kazusa.or.jp/codon/) (Nakamura *et al.* 2000)

fields: [triplet] [frequency: per thousand] ([number])

Phe UUU 0.4(5)	Ser UCU 0.3 (4)	Tyr UAU 0.3(4)	Cys UGU 0.7 (8)
UUC 29.6 (364)	UCC 19.5 (240)	UAC 22.3 (274)	UGC 6.3 (78)
Leu UUA 0.1 (1)	UCA 0.7 (9)	Ter UAA 0.2 (3)	Ter UGA 2.3 (28)
UUG 2.9 (36)	UCG 15.0 (184)	UAG 0.2 (3)	Trp UGG 14.6 (180)
CUU 1.1 (13)	Pro CCU 1.5 (18)	His CAU 1.0 (12)	Arg CGU 5.5 (67)
CUC 38.8 (477)	CCC 24.0 (295)	CAC 22.2 (273)	CGC 44.3 (544)
CUA 0.2 (2)	CCA 0.7 (9)	CAA 0.7 (9)	CGA 1.5 (19)
CUG 57.2 (703)	CCG 34.5 (424)	CAG 26.9 (331)	CGG 28.8 (354)
Ile AUU 1.0 (12)	ACU 1.1 (13)	Asn AAU 0.3 (4)	Ser AGU 1.4 (17)
AUC 30.6 (376)	ACC 40.6 (499)	AAC 17.6 (216)	AGC13.2 (162)
AUA 0.2 (2)	ACA 0.7 (9)	Lys AAA 0.8 (10)	Arg AGA 0.6 (7)
Met AUG 16.0 (196)	ACG 15.7 (193)	AAG 25.3 (311)	AGG 3.3 (40)
Val GUU 1.2 (15)	Ala GCU 2.7 (33)	GAU 2.4 (29)	GGU 7.1 (87)
GUC 44.2 (543)	GCC 79.8 (980)	GAC 61.5 (756)	GGC 58.6 (720)
GUA 1.5 (18)	GCA 3.9 (48)	GAA 8.3 (102)	GGA 5.8 (71)
GUG 34.1 (419)	GCG 42.9 (527)	GAG 55.6 (683)	GGG 17.8 (219)

Table 3.5b Codon usage data for the putative *phaC*$_{Sa}$ gene (http://www.kazusa.or.jp/codon/countcodon.html) (Nakamura *et al.* 2000)

fields: [triplet] [frequency: per thousand] ([number])

Phe UUU 0.0 (0)	Ser UCU 8.3 (6)	Tyr UAU 0.0 (0)	Cys UGU 0.0 (0)
UUC 9.7 (7)	UCC 6.9 (5)	UAC 1.4 (1)	UGC 11.1 (8)
Leu UUA 0.0 (0)	UCA 0.0 (0)	Ter UAA 0.0 (0)	Ter UGA 1.4 (1)
UUG 1.4 (1)	UCG 12.4 (9)	UAG 0.0 (0)	Trp UGG 20.7 (15)
CUU 2.8 (2)	Pro CCU 16.6 (12)	His CAU 1.4 (1)	Arg CGU 18.0 (13)
CUC 15.2 (11)	CCC 18.0 (13)	CAC 9.7 (7)	CGC 53.9 (39)
CUA 1.4 (1)	CCA 6.9 (5)	CAA 0.0 (0)	CGA 11.1 (8)
CUG 48.4 (35)	CCG 36.0 (26)	CAG 6.9 (5)	CGG 56.7 (41)
Ile AUU 0.0(0)	ACU 4.1(3)	Asn AAU 0.0(0)	Ser AGU 0.0(0)
AUC 1.4(1)	ACC 4.1(3)	AAC 1.4(1)	AGC 8.3(6)
AUA 0.0(0)	ACA 0.0(0)	Lys AAA 0.0(0)	Arg AGA 0.0(0)
Met AUG 2.8(2)	ACG 15.2(11)	AAG 2.8(2)	AGG 2.8(2)
Val GUU 13.8 (10)	Ala GCU 78.8 (57)	Asp GAU 8.3 (6)	Gly GGU 55.3 (40)
GUC 26.3 (19)	GCC 53.9 (39)	GAC 24.9 (18)	GGC 89.9 (65)
GUA 9.7 (7)	GCA 8.3 (6)	Glu GAA 4.1 (3)	GGA 18.0 (13)
GUG 45.6 (33)	GCG 83.0 (60)	GAG 4.1 (3)	GGG 56.7 (41)

Table 3.5c Codon usage data for the putative *phaB*$_{Sa}$ gene (http://www.kazusa.or.jp/codon/countcodon.html) (Nakamura *et al.* 2000)

fields: [triplet] [frequency: per thousand] ([number])

Phe UUU 0.0 (0)	Ser UCU 0.0 (0)	Tyr UAU 0.0 (0)	Cys UGU 0.0 (0)
UUC 16.3 (6)	UCC 8.1 (3)	UAC 16.3 (6)	UGC 2.7 (1)
Leu UUA 0.0 (0)	UCA 0.0 (0)	Ter UAA 0.0 (0)	Ter UGA 2.7 (1)
UUG 13.6 (5)	UCG 19.0 (7)	UAG 0.0 (0)	Trp UGG 19.0 (7)
CUU 0.0 (0)	Pro CCU 0.0 (0)	His CAU 0.0 (0)	Arg CGU 5.4 (2)
CUC 40.7 (15)	CCC 16.3 (6)	CAC 10.8 (4)	CGC 10.8 (4)
CUA 0.0 (0)	CCA 0.0 (0)	CAA 0.0 (0)	CGA 2.7 (1)
CUG 151.8 (56)	CCG 46.1 (17)	CAG 8.1 (3)	CGG 48.8 (18)
Ile AUU 0.0 (0)	ACU 0.0 (0)	Asn AAU 0.0 (0)	Ser AGU 0.0 (0)
AUC 5.4 (2)	ACC 10.8 (4)	AAC 2.7 (1)	AGC 5.4 (2)
AUA 0.0 (0)	ACA 0.0 (0)	Lys AAA 0.0 (0)	Arg AGA 0.0 (0)
Met AUG 8.1 (3)	ACG 40.7 (15)	AAG 2.7 (1)	AGG 2.7 (1)
Val GUU 0.0 (0)	Ala GCU 10.8 (4)	Asp GAU 0.0 (0)	Gly GGU 8.1 (3)
GUC 27.1 (10)	GCC 67.8 (25)	GAC 19.0 (7)	GGC 40.7 (15)
GUA 0.0 (0)	GCA 5.4 (2)	Glu GAA 0.0 (0)	GGA 5.4 (2)
GUG 86.7 (32)	GCG 130.1 (48)	GAG 29.8 (11)	GGG 51.5 (19)

–10 box sequences were identified (Strohl 1992). The RBS was also identified for ORFs 1(GTACGGC); 2 (ACGGC); 4 (CCGG); 6 (CCGGTACG); 7 (CCGG) and 10 (TCG).

Phasins, low molecular weight proteins, are proposed to promote PHA synthesis in *Rhodococcus ruber* (Pieper-Fürst *et al.* 1994, Pieper-Fürst *et al.* 1995) and *Ralstonia eutropha* (Wieczorek *et al.* 1995, York *et al.* 2001). Three different mechanisms for the function of phasins have been proposed. Firstly these may enhance PHA production by binding to the granules and increasing the surface to volume ratio of the granules. Second, phasins may activate the rate of PHA synthesis by interacting directly with the PHA synthase. Thirdly, phasins may promote PHA synthesis indirectly by preventing growth defects associated with the binding of other cellular proteins to PHA granules (York *et al.* 2001). Interestingly the amino acid sequences of phasin proteins are dissimilar even in closely related bacteria (McCool and Cannon 1999). The ORF 7 and ORF 10 could be encoding for small molecular weight phasin proteins.

3.3.4 Organization of PHA biosynthesis genes in *Streptomyces*

In many of the bacteria analyzed thus far, *phaA*, *phaB* and *phaC*(E) genes form a single cluster in the genome (Rehm and Steinbüchel 1999). In some organisms, like *Synechocystis* sp., *Zoogloea ramigera*, *Aeromonas caviae*, *Nocardia corallina*, *Rhizobium meliloti*, *Rhodococcus ruber*, *Pseudomonas denitrificans*, *Methylobacterium extorquens*, *Rhodobacter sphaeroides*, *Rhodospirillum rubrum* and *Rhodobacter capsulatus* the genes for PHB biosynthesis, however, are not colocalized (Taroncher-oldenburg 2000). Of these *N. corallina* and *R. ruber* are members of the actinomycetes group of bacteria. In the present study with *S. aureofaciens*, also a member of the actinomycetes, the DNA sequence analysis, however, suggests that the putative $phaC_{Sa}$ and $phaB_{Sa}$ genes are indeed clustered (Fig. 3.9, 3.11a, b). This is the first instance of its kind where these two putative genes have been shown to share the same nucleotide sequence, transcribe in the same direction and overlap each other. The two genes, however, operate from two different reading frames.

3.3.5 b-ketothiolase activity is provided by the host *Escherichia coli*

It is a well established fact that synthesis and accumulation of PHB as a storage compound in *E. coli* requires heterologous and concerted expression of the β-

ketothiolase, acetoacetyl-CoA reductase and PHA synthase (Taroncher-oldenberg *et al.* 2000).

While the pSa240 insert DNA apparently does not carry the *phaA* gene (β-ketothiolase), the *E. coli* host harboring the pSa240, however, synthesizes and accumulates PHB. Then it should follow that the β-ketothiolase enzyme for PHB synthesis is provided by the host *E. coli*. To substantiate this observation the β-ketothiolase enzyme activity in recombinant *E. coli* harboring pSa240 and in the untransformed *E. coli* host was assayed after about 48 h of growth (this timepoint coincides with peak PHB accumulation). Both revealed similar activity levels. While the β-ketothiolase activity was 41.05 ± 5.05 U/mg of protein in the untransformed *E. coli* host, it was 38.70 ± 6.28 U/mg of protein in the recombinant cells. However, NADPH dependent acetoacetyl-CoA reductase activity in the recombinant *E. coli* (pSa240) was 2.1 ± 0.55 U/mg of protein, no activity for this enzyme was detected in the untransformed cells (Table 3.6). These results substantiate the observation that the pSa240 DNA fragment from *S. aureofaciens* probably does not carry the *phaA* gene and this function is provided by the host *E. coli.* At the same time the absence of NADPH-dependent acetoacetyl-CoA activity in the untransformed host *E. coli* cells and its presence in the recombinant cells suggests that this function is provided by the $phaB_{Sa}$ gene present on the pSa240 insert DNA.

In the eventuality of the *E. coli* catabolic β-ketothiolase not participating in PHB synthesis, intermediates of fatty acid β-oxidation or fatty acid synthesis may be channelized to PHB biosynthesis. *E. coli* strain harboring *phaC*1 gene from *Pseudomonas aeruginosa* has been reported to accumulate polyhydroxyalkanoate with fatty acids as carbon source (Langenbach *et al.* 1997).

Table 3.6 β-ketothiolase and acetoacetyl-CoA reductase enzyme assays in untransformed *E. coli* JM109 and recombinant *E. coli* harboring pSa240 for activity

Organism	**Enzyme**	
	B-ketothiolase (U/mg total protein)	**NADPH-dependent Acetoacetyl-CoA reductase** (U/mg total protein)
Escherichia coli ***JM109***	41.05 ± 5.05	-
Recombinant *E. coli*	38.70 ± 6.28	2.1 ± 0.55

3.3.6 ORFs 3 (or 8) and 5 code for *phaC*$_{Sa}$ and *phaB*$_{Sa}$ genes respectively

PCR amplification of different ORFs was attempted to confirm functions assigned to them. But high G+C content hampered the amplification of the fragments. Hence, transposon insertion analysis was performed to confirm functions assigned to ORF 3 (or 8) and 5.

Transposon Tn5 insertion clones were selected such that one or more of the ORFs were disrupted. From among a total of 92 transposon insertion clones, pSa240^{1} to pSa240^{92}, three were selected, since in these ORFs 3 and/or 5 were disrupted (Fig. 3.12). In pSa240^{16} clone, ORFs 1, 3, 5, 8 and 9 were interrupted; pSa240^{34} had ORF 3 interrupted and pSa240^{40} had ORFs 1, 3 and 8 interrupted (Table 3.7). Recombinant *E. coli* cells harboring these plasmid DNA clones were checked for accumulation of PHB and NADPH-dependent acetoacetyl-CoA reductase activity. Recombinant *E. coli* containing Tn5 insertion clone pSa240^{34} in which only ORF 3, the putative *phaC*$_{Sa}$ gene coding for PHA synthase, was disrupted did not accumulate PHB. In this clone Tn5 insertion was 113 nucleotides downstream of the translation initiation codon ^{275}GTG. This clone, however, showed NADPH dependent acetoacetyl-CoA reductase activity (1.89 ± 0.21 U/mg protein). Recombinant *E. coli* harboring transposon insertion clone pSa240^{40} in which ORFs 1, 3, and 8 were disrupted, did not synthesize and accumulate PHB. In this clone, however, acetoacetyl-CoA reductase activity was detected (1.56 ± 0.33 U/mg protein). Recombinant *E. coli* harboring transposon insertion clone pSa240^{16} in which ORFs 1, 3, 5, 8 and 9 were disrupted neither accumulated PHB nor did it show any acetoacetyl-CoA reductase activity. (ORF 5 is the putative NADPH dependent acetoacetyl-CoA reductase as discussed earlier, see section 3.3.3).

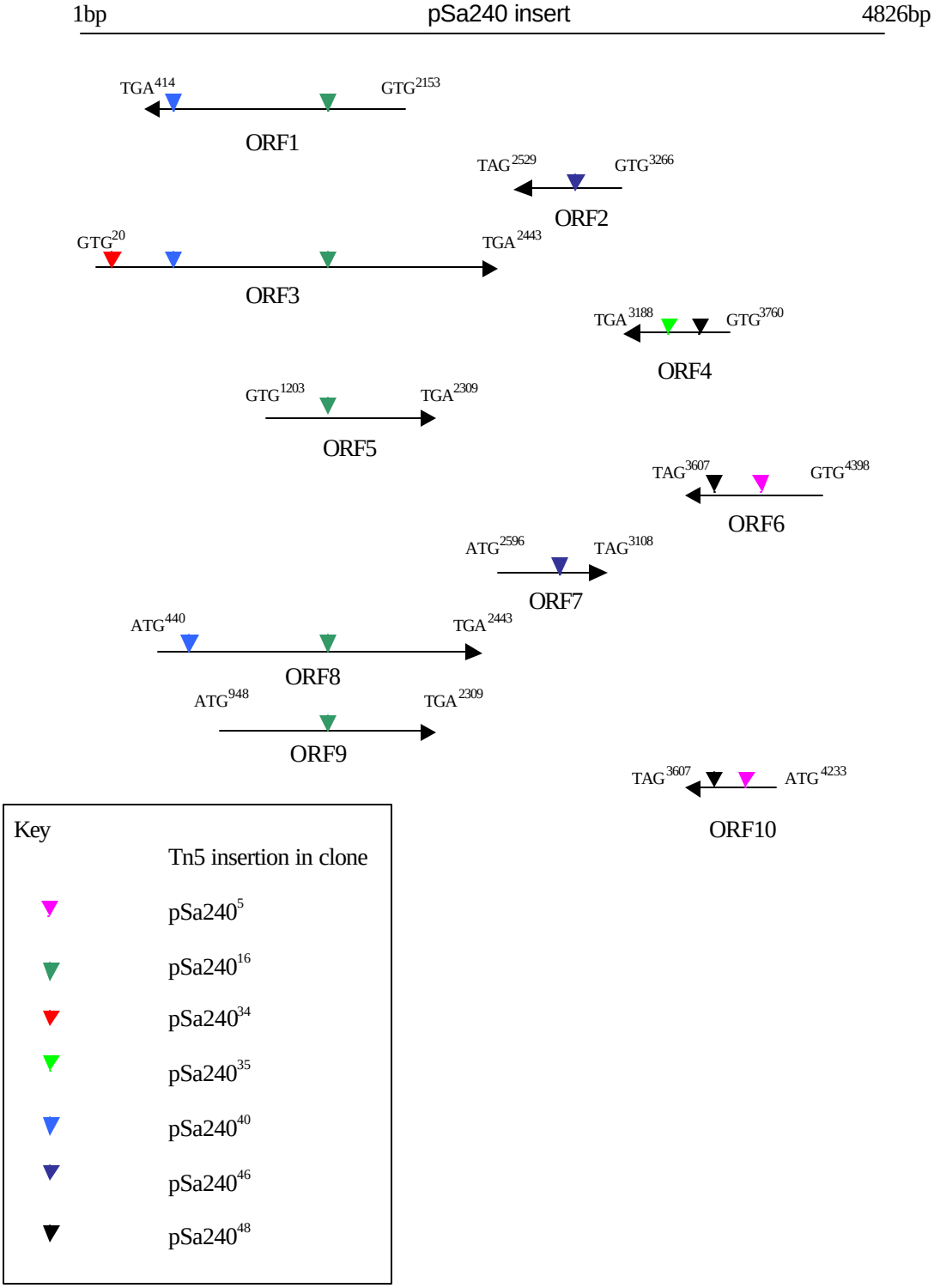

Fig. 3.12 Tn5 transposon insertion clones.

From the foregoing transposon Tn5 insertion studies it became obvious that ORFs 3 and 5 are both required for PHB synthesis. It also became apparent that:

(a) interruption of ORF 3 results in loss of PHB synthesis and accumulation but not in the loss of NADPH-dependent acetoacetyl-CoA reductase activity;

(b) interruption of ORFs 1 and 8 does not result in the loss of NADPH-dependent acetoacetyl-CoA reductase activity;

(c) interruption of ORF 5 results in loss of NADPH-dependent acetoacetyl-CoA reductase activity.

Hence, it can be concluded that ORF 5 represents the *phaB*$_{Sa}$ gene. However, in pSa240[34] the Tn5 insertion resulted in distancing the in frame ^{440}ATG from the upstream promoter region, and probably resulting in loss of activity. The ORF 3 spanning from ^{275}GTG to ^{2443}TGA contains the *phaC*$_{Sa}$ gene could have its translation start point at either ^{275}GTG or ^{440}ATG.

Table 3.7 Analysis of ORFs 3 and 5 using recombinant *E. coli* JM109 cells harboring transposon insertion clones

Transposon insertion clone	**ORFs disrupted**	**P(3HB) % cell dry weight**	**NADPH dependent acetoactyl-CoA reductase activity**
pSa240[16]	1, 3, 5, 8, 9	-	-
pSa240[34]	3	-	1.89 ± 0.21
pSa240[40]	1, 3, 8	-	1.56 ± 0.33

3.3.7 Role of other ORFs in PHB synthesis

Transposon Tn5 insertion clones pSa240[5, 35, 46, 48] were further selected to study the role of ORFs 2, 4, 6, 7 and 10 in PHB synthesis. pSa240[5] had ORFs 6 and 10 interrupted; pSa240[35] had ORF 4 interrupted; in pSa240[46] ORFs 2 and 7 were interrupted and in pSa240[48] ORFs 4, 6 and 10 were interrupted (Fig. 3.12). Recombinant *E. coli* cells harboring these plasmid DNA clones were checked for accumulation of PHB. Recombinant *E. coli* cells containing any of these transposon insertion clones continued to synthesize PHB (Table 3.8), but the levels of PHB accumulated had

dropped to approximately 1% of cell dry weight as against approximately 60% of cell dry weight in the *E. coli* cells harboring pSa240. Thus, ORFs 2, 4, 6, 7 and 10 certainly play a role in PHB accumulation by recombinant *E. coli*. Phasins, encoded by *phaP*, have been reported to positively affect the PHB synthesis in *Ralstonia eutropha* (York *et al.* 2001). As discussed earlier, phasins are low molecular weight proteins, which differ in amino acid sequence even in closely related bacteria (McCool and Cannon 1999). Upstream of ORF 4, only -35 box was identified. However, putative promoter sequences and RBS were identified upstream of ORF 7 and 10. Deduced translational products of ORF 7 and 10 are of approximate M_r. 18000 and 21000 respectively. These observations make the ORFs 7 and 10, the probable candidates for phasin protein encoding genes. ORFs 2 and 6 encode for relatively high molecular weight proteins to be phasins, but are also required for high levels of accumulation of PHB.

Table. 3.8 Analysis of ORFs 2, 4, 6, 7, and 10 using recombinant *E. coli* JM109 cells harboring transposon insertion clones

Transposon insertion clone	**ORFs disrupted**	**P(3HB) % cell dry weight**
pSa240[5]	6, 10	0.79
pSa240[35]	4	0.98
pSa240[46]	2, 7	0.85
pSa240[48]	4, 6, 10	0.97

The biosynthesis of polyketides, which is well studied in *Streptomyces*, is mechanistically related to formation of long-chain fatty acids. However, polyketides, in contrast to fatty acid synthases (FAS) retain ketone, hydroxyl, or olefenic functions and contain methyl or ethyl side groups interspersed along an acyl chain comparable in length to that of common fatty acids. This asymmetry in structure implies that the polyketide synthase (PKS), the enzyme system responsible for formation of these molecules, although mechanistically related to FAS, results in an end product that is structurally very different from long chain fatty acids. The intermediates of these pathways can serve as precursors for the biosynthesis of novel PHAs (Sherman *et al.*

2001). This would also be an economical method for the production of defined PHAs. Currently PHA synthase gene from *R. eutropha* is used for this purpose. *Streptomyces* sp. has G+C rich genome and biased codon usage with strong preference for G and/or C. Hence to design PHA biosynthetic pathway in *Streptomyces* sp., use of *S. aureofaciens* PHA biosynthetic genes would increase the efficiency of the recombinant system. Also cloning of polyhydroxyalkanoate synthase and the polyketide synthase genes from *Streptomyces* sp. and deletion or inactivation of specific genes allows the biosynthesis of novel macrolides (Sherman *et al*. 2001). These applications underline the significance of isolation and characterization of PHA biosynthetic genes from an actinomycete, *Streptomyces aureofaciens*.

CHAPTER 4

PHB SYNTHESIZING GENES FROM *STREPTOMYCES AUREOFACIENS* NRRL 2209: HETEROLOGOUS EXPRESSION IN *ESCHERICHIA COLI*

4.1 INTRODUCTION

Commercialization of PHAs as substitutes for conventional petrochemical-based polymers is hampered by the high production cost of these compounds (Choi and Lee 1997). Much effort has been consequently been devoted towards production cost reduction of PHAs. This has necessisated developing of bacterial strains and, efficient fermentations and recovery processes.

Poly(3-hydroxybutyrate) has been detected in the cytoplasmic membrane of *Escherichia coli* (Reusch 1992). These cells incorporate PHB into their plasma membranes under growth-limiting conditions and during competence development (Huang and Reusch 1996). However, no intracellular PHB granules have been observed in *E. coli.*

The physiology, biochemistry and genetics of *E. coli* have been studied in great detail. This makes it the perfect host for heterologous expression of foreign proteins. The advantages associated with the system are that:

(i) many useful plasmids and mutant strains are available for genetic manipulation and improvement,

(ii) the recombinant methodologies and high cell density cultivation strategies are well established (Yee and Blanch 1992, Lee 1996b, Aristidou *et al.* 1999),

(iii) *E. coli* grows fast and offers a well-defined physiological environment for the construction and manipulation of various metabolic pathways to produce a wide range of PHAs from cost-effective carbon sources (Fang *et al.* 1998, Wong and Lee 1998, Ahn *et al.* 2000, Kim 2000) and that

(iv) *E. coli* cells while accumulating large amounts of PHB become fragile, which is advantageous for polymer isolation.

Moreover *E. coli* does not accumulate PHA inclusion bodies and lacks the PHA depolymerase. In the natural producer *R. eutropha*, PHB is in a mobile amorphous state (Ellar *et al.* 1968, Dunlop and Robards 1973, Barnard and Sanders 1989, Lauzier *et al.* 1992), while in recombinant *E. coli* nascent PHB is in a crystalline form (Hahn *et al.* 1995). Microbial PHB is often recovered by a simple and efficient method using alkaline hypochlorite solution, a reagent that degrades most of the other cellular macromolecules. However, this method also causes severe degradation of amorphous PHB molecules. PHB in the recombinant *E. coli*, however, is protected from hypochlorite digestion by its crystalline morphology (Hahn *et al.* 1995). In addition to

the stability of native PHB from recombinant *E. coli* in sodium hypochlorite solution, non-PHB cell material of the recombinant *E. coli* seemed to be more easily digested by the hypochlorite treatment, since the purity of PHB recovered from recombinant *E. coli* is generally higher than the purity of PHB from *R. eutropha* (Hahn *et al.* 1995). Also, the molecular mass and polydispersity of PHB produced by fermentation of *E. coli* can be controlled by modulating the activity of the PHA synthase (Lee 1997, Sim *et al.* 1997). Based on these observations, *Escherichia coli* holds promise as a source of economical PHA production.

P(3HB) production in recombinant *E. coli* harboring PHA synthesizing polycistron from *R. eutropha* is well studied (Lee *et al.* 1994a, b, Zhang *et al.*1994, Kidwell *et al.* 1995, Lee and Lee 1996, Lee *et al.* 1996). Introduction of the *phaC* gene from *Rhodobacter sphaeroides* into *E. coli* does not result in PHB synthesis (Ji-Hoe and Lee 1997). Since no PHA synthase activity was detected in the recombinant it was proposed that PHA synthase was probably degraded rapidly in *E. coli* in the absence of its substrate, 3-hydroxybutyryl CoA. PHB accumulation also failed in recombinant *E. coli* cells harboring $phaC_{Cv}$ from *Chromobacterium violaceum*, and $phaB_{Re}$ and $phaA_{Re}$ genes from *R. eutropha* (Kolibachuk *et al.* 1999). While the PHB synthesizing genes from *R. eutropha* have been shown to function in *E. coli*, the *phaC* gene from other microbes like the *R. sphaeroides* and *C. violaceum* apparently do not function in the alien *E. coli* environment. However, in the preceding chapters we have seen that the PHB biosynthetic genes from *S. aureofaciens* do express and direct the synthesis of PHB in the alien *E. coli* environment. Considering these facts, the present chapter describes attempts directed at optimization of PHB production by recombinant *E. coli* (ATCC:PTA-1579) harboring PHA synthesizing genes $phaC_{Sa}$ and $phaB_{Sa}$ from *Streptomyces aureofaciens* NRRL 2209.

[A] Effect of different carbon and nitrogen sources on PHB accumulation by *pha*$_{Sa}^{+}$ recombinant *Escherichia coli*

The PHB production cost is determined by PHB productivity, content and yield by the microbial cells, cost of the carbon substrate and the recovery method (s) used. About 40% of the total production cost is for raw material (Choi and Lee 1999a). In the present study the effect of different carbon and nitrogen sources on PHB production by *pha*$_{Sa}^{+}$ recombinant *E. coli* (ATCC:PTA-1579) was studied.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Ampicillin, benzoic acid and poly-β-hydroxybutyrate were purchased from Sigma-Aldrich, USA. All other chemicals and bacteriological media components were of analytical grade and obtained from HIMEDIA, Qualigens Fine Chemicals and E. Merck Laboratories, India.

4.2.2 Media studies

4.2.2.1 Recombinant organism

The recombinant *pha*$_{Sa}^{+}$ *E. coli* JM109 harboring the pSa240 plasmid was used in the study. The recombinant *E. coli* is deposited with ATCC, USA (ATCC-PTA-1579).

4.2.2.2 Growth conditions

Basal medium used for carbon source studies was:

	g/l
Yeast extract	5
Peptone	5
Na_2HPO_4	1
$MgSO_4$	0.2
pH 7.2	

The basal medium was supplemented with different carbon sources, which include glycerol, glucose, molasses, sucrose, ethanol and palm oil. Each carbon source was used at a final concentration of 1% in the medium.

Media used for nitrogen source studies include:

(1) Yeast extract and peptone	g/l	**(2) Yeast extract**	g/l
Yeast extract	5	Yeast extract	10
Peptone	5	Na_2HPO_4	1
Na_2HPO_4	1	$MgSO_4$	0.2
$MgSO_4$	0.2		
pH 7.2		PH 7.2	
Glycerol	10	Glycerol	10

(3) Peptone	g/l	**(4) Corn steep liquor**	g/l
Peptone	10	corn steep liquor	10
Na_2HPO_4	1	Na_2HPO_4	1
$MgSO_4$	0.2	$MgSO_4$	0.2
pH 7.2		pH 7.2	
Glycerol	10	Glycerol	10

In all media ampicillin was added to the final concentration of 100 µg/ml. For each medium, 1% inoculum of recombinant *E. coli* was used. Cells were cultivated at 37°C with continuous shaking (200 rpm). Growth was monitored by measuring cell dry weight (g/l).

4.2.3 Gas chromatography analysis

GC analysis of the freeze dried *E. coli* cells was performed as described earlier using a GC 17-A Shimadzu make GC (see chapter 2, section 2.2.4.2).

4.3 RESULTS AND DISCUSSION

4.3.1 Effect of different carbon sources on PHB accumulation by *pha*$_{Sa}^{+}$ recombinant *Escherichia coli*

A number of carbon sources such as glycerol, glucose, palm oil, sucrose, molasses and ethanol were tested for their ability to support P(3HB) production by *pha*$_{Sa}^{+}$ recombinant *E. coli.*

An overview of *pha*$_{Sa}^{+}$ recombinant *E.coli* cell growth, PHB accumulation and pH of the culture medium is presented in Figs. 4.1a, b, c. Maximum cell growth was observed in basal medium with glycerol as the carbon source (8.01 ± 0.04 g/l). Cell growth in basal medium supplemented with glycerol, palm oil, sucrose or molasses entered stationary phase after 30 h of incubation. In glucose supplemented medium cell growth reached its peak value (7.50 ± 0.17 g/l) after 40 h of incubation. Incorporation of ethanol and molasses inhibited cell growth. The maximum cell growth in presence of ethanol and molasses was 4.26 ± 0.28 g/l and 4.26 ± 0.04 g/l, respectively as against a maximum cell growth of 5.37 ± 0.31 g/l achieved in the basal medium. In ethanol supplemented medium, cell density decreases beyond 20 h of incubation probably due to cell lysis. While ethanol is known to inhibit cell growth of *E. coli* K-12 derivatives (Ingram 1976), molasses probably contains components which inhibit growth. Amongst the six carbon sources used, maximum growth was obtained in basal medium supplemented with glycerol (1%) as a carbon source. It has been reported in prior investigations that glucose, sucrose, molasses and whey support growth and PHB accumulation by recombinant *E. coli* harboring PHA synthesizing genes from *R. eutropha* (Lee *et al.* 1994a, Zhang *et al.* 1994, Kim 2000).

Glycerol as a carbon source also supported maximal P(3HB) accumulation. PHB up to 60% of cell dry weight was accumulated in the recombinant *E. coli* cells (Fig. 4.1b). Approximate 38% P(3HB) of cell dry weight was accumulated in recombinant *E. coli* in the presence of glucose. About 28% P(3HB) of cell dry weight was obtained with palm oil. Plant oils such as olive oil, corn oil and palm oil are reported to support PHB biosynthesis and accumulation by *R. eutropha* (Fukui and Doi 1998). This result is encouraging since for the first time plant oil has been shown to support PHB accumulation in recombinant *pha*$^{+}$ *E. coli*. About 10% PHB of cell dry weight was

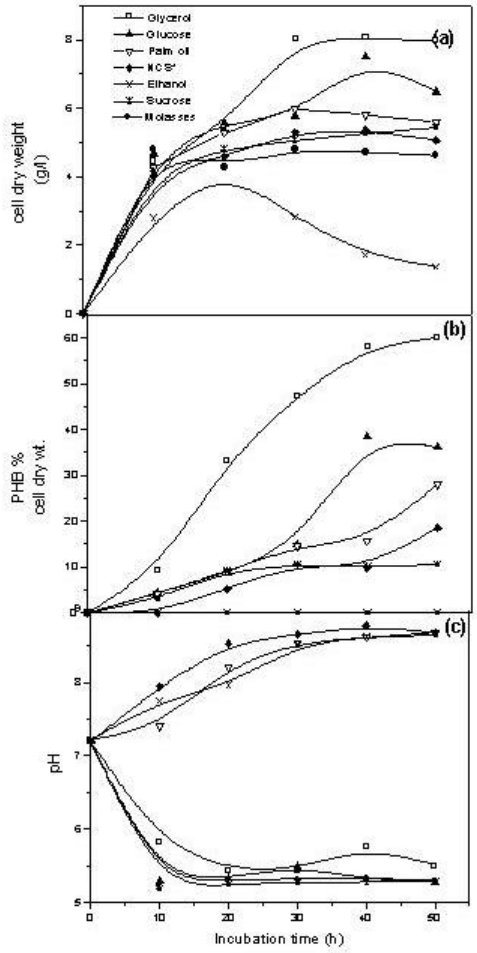

Table 4.2 The effect of different carbon sources on growth (a), PHB content (b) and pH of culture medium (c) during flask culture of recombinant pha_{Sa}^{+} *Escherichia coli*.
(NCS*: Basal medium with no added carbon source)

accumulated in the presence of ethanol which was less than the control medium. In the absence of molecular oxygen or other exogenous electron acceptors, *E. coli* carries out mixed acid fermentation during anaerobic growth in order to achieve metabolic redox balance. Ethanol is one of the fermentation products, which is formed from acetyl- CoA by two sequential NADH-dependent reductions catalyzed by the multifunctional ethanol oxidoreductase (the *adhE* gene product). Despite the fact that both AdhE-catalyzed reactions are reversible, *E. coli* fails to grow on ethanol as a sole carbon and energy source apparently for two main reasons. First, the *adhE* gene is inefficiently expressed under aerobic conditions (Clark and Cronan 1980, Chen and Lin 1991, Leonardo *et al.* 1993). Second, the catalytic half-life of the AdhE protein is shortened during aerobic metabolism by a metal-catalyzed oxidation cycle (Membrillo-Hernadez *et al.* 2000). The medium used in the present study being complex, probably supported growth and PHB accumulation to a small extent even with ethanol as the carbon source. There was, however, no PHB accumulation with sucrose or molasses as the carbon source. Sucrose and molasses seem to negatively regulate PHA synthesizing genes. Carbon source responsive promoter elements have been reported earlier in *Saccharomyces cerevisiae* and *Ustilago maydis* (Scholar and Schuller 1994, Bottin *et al.* 1996). *phaC* gene encoding the PHA synthase of *C. violaceum* and *R. ruber* irrespective of carbon source used were not expressed in *E. coli* at all (Pieper and Steinbüchel 1992, Kolibachuk *et al.* 1999). And now we have a set of genes that probably are regulated by carbon source. Understanding the regulatory mechanisms of these genes, and the ones from *C. violaceum* and *R. ruber* will add to our knowledge of regulation of these biotechnologically important set of genes and aid further in their commercial exploitation.

The recombinant pha_{Sa}^{+} *E. coli* growing in basal medium supplemented with glycerol or palm oil entered stationary state after about 30 h of incubation. However, these cells continue to accumulate PHB till 50 h of incubation. In glucose supplemented medium, the maximum cell growth was reached at 40 h of incubation. Correspondingly maximum PHB accumulation was also observed at 40 h of incubation (Fig. 4.1a, b). In medium with ethanol as carbon source PHB content follows the growth pattern for first twenty hours and then remains steady (Figs. 4.1a, b).

With glycerol, glucose, sucrose and molasses as carbon sources, pH of the medium dropped to became acidic. The medium pH dropped from 7.2 to 5.51, 5.28, 5.29 and 5.30 respectively after 50 h of incubation (Fig. 4.1c). In control and in basal

medium supplemented with palm oil or ethanol as carbon source pH became alkaline (Fig. 4.1c) due to utilization of complex nitrogenous substances present in the medium. In basal medium with no added carbon source and with palm oil or ethanol as carbon source, pH of the medium increased to 8.68, 8.68 and 8.64 respectively. No correlation could, however, be established between the pH change of the culture medium and PHB production by *pha*$_{Sa}^{+}$ *E. coli*.

From the preceding, glycerol, a by-product from the production of biofuels from oilseeds and palm oil, a plant oil emerge as possible candidates for PHB production by recombinant *pha*$_{Sa}$+ *E. coli*. Glycerol, produced as a byproduct in the growing oleochemical industries, is a cheap substrate for PHB production. Besides, several environment friendly processes based on microbial fermentation have been proposed for glycerol utilization. Some other useful chemicals produced from glycerol by fermentation are: β-hydroxypropionaldehyde (reuterin) (EI-Ziney *et al*. 1998, Talarico *et al*. 1998), 2,3-butanediol (Biebl *et al*. 1998), 1,3-propandiol (Menzel *et al*. 1997), and succinic acid (Lee *et al*. 2000a). Although glycerol utilization for PHB synthesis has earlier been demonstrated for *R. eutropha* (Taidi *et al*. 1994) production of PHB from glycerol by recombinant *E. coli* has not been reported thus far. This is the first report where we have shown that *E. coli* expressing a set of heterologous genes for PHB synthesis from *S. aureofaciens* uses glycerol for PHB synthesis. The PHB content obtained in *R. eutropha* with glucose or glycerol as carbon source are similar (~65%). In the present study glucose supports PHB accumulation of only 40% of cell dry weight, as against 60% of cell dry weight with glycerol as a carbon source.

4.3.2 Effect of different nitrogen sources on PHB accumulation by *pha*$_{Sa}^{+}$ recombinant *Escherichia coli*

The effect of yeast extract, peptone and corn steep liquor as nitrogen sources on PHB production by recombinant *E. coli* was studied.

Glycerol, which supported maximum PHB accumulation in recombinant *pha*$_{Sa}^{+}$ *E. coli* was used as the carbon source. Cell growth, PHB content in the cells and pH of the nutrient medium was followed for upto 50 h of culture.

An overview of *pha*$_{Sa}^{+}$ recombinant *E.coli* cell growth, PHB accumulation and pH of culture medium with different nitrogen sources is presented in Figs. 4.2a, b, c. Inclusion of yeast extract and peptone alone and in combination into the medium

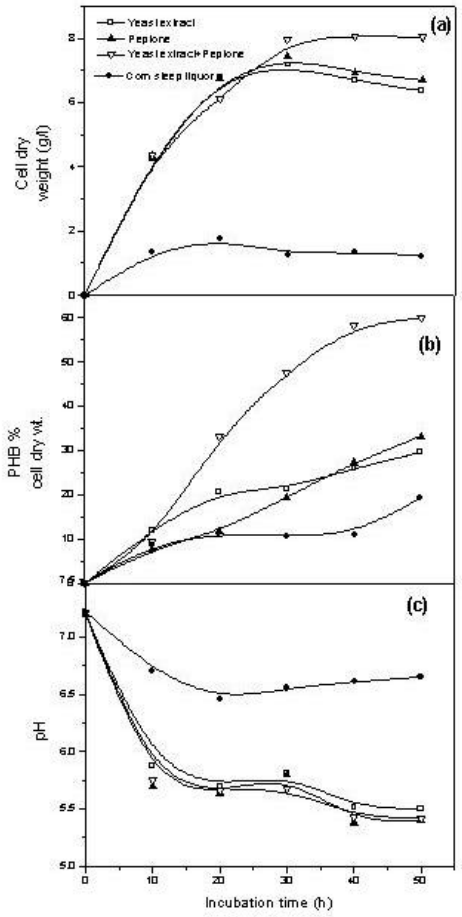

Table 4.2 The effect of different nitrogen sources on growth (a), PHB content (b) and pH of culture medium (c) during flask culture of recombinant pha_{Sa}^{+} *Escherichia coli*

supported growth of recombinant *pha*$_{Sa}$+ *E. coli*. With yeast extract, peptone and yeast extract+peptone in the medium respective cell dry weight of 6.36 g/l, 6.70 g/l and 8.04 g/l was achieved after 50 h of incubation. However, after 50 h of incubation cell dry weight of only 1.23 g/l was obtained with corn steep liquor as the nitrogen source. In the basal medium with yeast extract, peptone and yeast extract+peptone the cell culture entered stationary phase of growth after 30 h of incubation. Maximum cell growth was obtained in the presence of yeast extract+peptone as nitrogen source (Fig. 4.2a).

PHB content of the recombinant *E. coli* was 30% of cell dry weight with yeast extract or peptone as the nitrogen source. But with a combination of yeast extract and peptone in the medium a cumulative effect on PHB accumulation by the recombinant *pha*$_{Sa}^{+}$ *E. coli* cells was observed. The cells accumulated about 60% PHB of cell dry weight. About 20% PHB of cell dry weight was obtained with corn steep liquor (Fig. 4.2b). The PHB content obtained in the presence of corn steep liquor as a nitrogen source was low as compared to the levels obtained for recombinant *E. coli* harboring PHA synthesizing genes from *R. eutropha* (~ 40%) (Lee and Chang 1995). Interestingly, the cells grown in presence of corn steep liquor showed biphasic PHB accumulation pattern. PHB accumulation during the first 20 h was concomitant with cell growth. Thereafter the cell growth and PHB accumulation remained stationary for upto 40 h of incubation. While the cell growth beyond 40 h continued to be stationary PHB accumulation shot up from 10.89±0.10% to 19.28±0.55% of cell dry weight between the period intervening 40 h and 50 h of growth. With yeast extract, peptone, yeast extract+peptone and corn steep liquor as nitrogen sources, pH of the medium dropped to the acidic side (Fig. 4.2c). The respective pH dropped from 7.2 to 5.50, 5.40, 5.42 and 6.65 after 50 h of incubation. No correlation could be established between the pH change of the culture medium and PHB accumulation by *pha*$_{Sa}^{+}$ *E. coli.* Yeast extract and yeast extract+peptone have been implicated in enhancing glycerol utilization by *Anaerobiospirillum succiniproducens* and *Phaffia rhodozyma* respectively (Kusdiyantini *et al.* 1998, Lee *et al.* 2000a). Apparently a similar enhancing effect on glycerol utilization and PHB production by *pha*$_{Sa}^{+}$ recombinant *E. coli* (Figs. 4.1 and 4.2) is observed in the present instance.

From the above studies it transpires that maximum PHB accumulation (approximately 60% PHB of cell dry weight) by recombinant *pha*$_{Sa}^{+}$ *E. coli* occurs on a medium composed of:

	g/l
Yeast extract	5
Peptone	5
Na_2HPO_4	1
$MgSO_4$	0.2
pH 7.2	
Glycerol	10

These results are significant considering the fact that recombinant *pha*$_{Sa}^{+}$ *E. coli* accumulates ~ 60% PHB of cell dry weight as against only 2.4% PHB of cell dry weight (CDW) accumulated by *S. aureofaciens*. In different strains of recombinant *E. coli* harboring PHA synthesizing genes, PHB content of 2 to 85% of CDW has been reported (Liebergesell and Steinbüchel 1992, Liebergesell and Steinbüchel 1993, Lee and Chang 1995, Schubert *et al*. 1988, Slater *et al*. 1988, Taroncher-oldenberg *et al*. 2000). But the remarkable increase from 2.4% in the natural PHB producer *S. aureofaciens* to 60% PHB of cell dry weight in recombinant *pha*$_{Sa}^{+}$ *E. coli* was observed for the first time. The levels of PHB accumulated are not directly comparable as the nutritional requirements of the two species is not likely to be same. Allowing for that, it is still remarkable that recombinant *E. coli* accumulated such high levels of PHB. This finding is important especially for commercial production of PHB involving recombinant *E. coli*.

[B] Effect of amino acids supplementation on the synthesis of poly(3-hydroxybutyrate) by *pha*$_{Sa}^{+}$ recombinant *Escherichia coli*

E. coli cells harboring *Ralstonia eutropha* PHA biosynthesis genes for PHB accumulation (Peoples and Sinskey 1989b) and cultivated in defined media accumulate less PHB than those cultivated in complex media (Lee and Lee 1996). The biosynthesis of amino acids, which is energy intensive can be a burden to the cell during the overproduction of proteins for PHB synthesis (Lee *et al*. 1995b), is suggested as one major reason for low PHB accumulation. This problem is alleviated by growth of cells in complex media which contain rich amino acid sources such as tryptone, peptone, yeast extract etc. The fermentation of recombinant cells in defined media could be improved by the addition of amino acids into it (Mizutani *et al*. 1986, Ramirez and Bentley 1993, Paul *et al*. 1998).

Lee *et al*. (1995b) have shown that cysteine, methionine, isoleucine and proline promote PHB synthesis by recombinant *E. coli* harboring *Ralstonia eutropha* PHA biosynthesis genes. In the present study, recombinant *E. coli* (ATCC: PTA – 1579) harboring the *S. aureofaciens* NRRL 2209 PHB biosynthesis genes was studied for PHB synthesis when cultivated in a defined medium supplemented with various amino acids.

4.4 MATERIALS AND METHODS

4.4.1 Materials

Ampicillin, benzoic acid, amino acids and poly-β-hydroxybutyrate were purchased from Sigma-Aldrich, USA. All other chemicals and bacteriological media components used were of analytical grade and were obtained from HIMEDIA, Qualigens Fine Chemicals and E. Merck Laboratories, India.

4.4.2 Organism and growth

R medium of Lee and Chang (1993) was used as the basal defined medium for cultivation of recombinant *pha*$_{Sa}^{+}$ *E. coli* (ATCC: PTA – 1579) harboring PHA synthesizing genes from *Streptomyces aureofaciens* NRRL 2209. The medium was further supplemented with 10 mg/l thiamine and 100 μg/ml ampicillin.

R Medium

	g/l
KH_2PO_4	13.5
$(NH_4)_2HPO_4$	4.0
$MgSO_4.7H_2O$	1.4 (Sterilized and added separately)
Citric acid	1.7
Trace metal solution	10.0 ml/l

The trace metal solution consisted of the following (per litre of 5 M HCl): $FeSO_4.7H_2O$, 10.0 g; $CaCl_2.2H_2O$, 2.0 g; $ZnSO_4.7H_2O$, 2.2 g; $MnSO_4.4H_2O$, 0.5 g, $CuSO_4.5H_2O$, 1.0 g; $(NH_4)_6Mo_7O_2.4H_2O$, 0.1 g; $Na_2B_4O_7.10H_2O$, 0.02 g. Glycerol (10 g/l) was used as the carbon source. $MgSO_4.7H_2O$ solution was sterilized and added separately.

The medium was supplemented with individual amino acids at a concentration of 50 mg/l (Table 4.1). Cysteine, methionine and isoleucine were also used independently at 50 and 150 mg/l concentrations (Table 4.2). These three amino acids were also incorporated in the medium as eight assorted combinations (Table 4.3). The cells were cultivated at 37°C with shaking at 200 rpm for 48 h, harvested, freeze dried and analyzed for PHB content.

4.4.3 Gas chromatography analysis

GC analysis of the freeze dried *E. coli* cells was performed as described earlier using a GC 17-A Shimadzu make GC (see chapter 2, section 2.2.4.2).

4.4.4 2^3 factorial design

In the 2^3 factorial design (Bailey 1995) three amino acids *viz*. Cysteine, methionine and isoleucine were studied at two different levels each (2^3 factorial). A total of 8 combinations as shown in Table 4.3 were used in medium for cultivation of recombinant *E. coli*. The response was assessed by determining the PHB content of the cells. The effect of changing cysteine concentration from 50 to 150 mg/l was calculated by subtracting the PHB content of the cells at the former concentration of the amino acid from the PHB content at latter concentration with various combinations of methionine (M) and isoleucine (I). The average of four values obtained was the main effect of cysteine (C). Similarly, the main effects of methionine and isoleucine were calculated.

Two factor interactions were calculated at two fixed levels of one factor. The average concentration effect of the other factor was calculated by averaging the

individual measure of the effect of changing concentration of the factor from 50 to 150 mg/l at a fixed concentration of the first factor. The average of the two values obtained will give the two factor interaction such as (C X M), (C X I), and (M X I).

The three-factor interaction is the average difference between the cysteine X methionine interaction and the two levels of isoleucine concentration.

The 2^3 factorial design has eight factor level combinations. Geometrically the design is shown in Fig. 4.3, with eight runs forming the corners of the cube (1:1, 2:a, 3:b, 4:ab, 5:c, 6:ac, 7:bc, 8:abc). This design allows three main effects to be estimated (A, B, and C) along with three two-factor interactions (AB, AC and BC) and the three-factor interactions (ABC). The main effect of A is estimated by averaging the four runs on the right side of the cube where A is at high level and subtracting from that quantity the average of the four runs on the left side of the cube where A is at the low level. Similarly main effects of B and C are calculated. For estimation of two-factor interaction AB; when C is at low level, AB is the average difference in the A effect at the two levels of B. Similarly, when C is at high level, the AB interaction is the average difference in the B effect at the two levels of A. The AB interaction is the average of these two components. The ABC interaction effect is the average difference between the AB interaction and the two levels of C.

4.5 RESULTS AND DISCUSSION

4.5.1 Effect of individual amino acids on PHB accumulation

The effect of amino acid supplements into the nutrient medium used for fermentation of recombinant *E. coli* was investigated. The results obtained are depicted in Table 4.1.

Table 4.1 Effect of amino acid supplementation on PHB synthesis by recombinant *pha*$_{Sa}^{+}$ *Escherichia coli* (ATCC: PTA – 1579) in a defined medium

Amino acid	**PHB % cell dry weight**
Control	16.28 ± 1.17
P	23.25 ± 1.70
C	26.17 ± 0.11
I	23.53 ± 0.37
M	26.20 ± 0.31
G	8.86 ± 1.64
A	16.75 ± 1.39
R	20.70 ± 0.82
N	20.11 ± 1.28
D	20.32 ± 0.37
Q	16.80 ± 0.47
E	18.26 ± 0.57
H	20.37 ± 1.57
L	19.47 ± 1.26
K	19.98 ± 0.78
F	17.39 ± 0.03
S	16.64 ± 0.41
T	21.14 ± 0.78
W	18.80 ± 0.74
Y	22.11 ± 0.30
V	13.20 ± 1.65

Of the twenty amino acids incorporated individually in the medium, alanine, glutamine and serine had no influence on PHB accumulation by recombinant *E. coli* cells. The respective PHB content in the cell was 16.75 ± 1.39%, 16.80 ± 0.47% and 16.64 ± 0.41% of cell dry weight. PHB content of recombinant *E. coli* cells in the control medium was 16.28 ± 1.17% of cell dry weight. Incorporation of valine into the medium resulted in an about 20% less PHB accumulation by the cells (13.20 ± 1.65 as against 16.28 ± 1.17% PHB of cell dry weight in control cells). Addition of glycine to the medium decreased PHB accumulation by the cells by as much as 46% (8.86 ± 1.64% as against 16.28 ± 1.17% PHB of cell dry weight in control cells) of cell dry weight as compared to control. All other amino acids enhanced PHB synthesis when supplemented to the defined medium. Three amino acids, cysteine, methionine and isoleucine promoted PHB synthesis significantly. Addition of cysteine, isoleucine and

methionine increased PHB accumulation by 60%, 45% and 61% respectively when compared to the control in the recombinant *pha*$_{Sa}^{+}$ *E. coli*. These observations are consistent with earlier observations made by Lee *et. al.* (1995b) for recombinant *E. coli* harboring *Ralstonia eutropha* PHA biosynthesis genes. The channelization of intermediates of carbon source metabolism and TCA cycle which serve as precursors for amino acid biosynthesis, may result in depletion of acetyl-CoA pool available for PHB synthesis. Also, the biosynthesis of cysteine, methionine and isoleucine requires large quantities of NADPH (Lee *et al*. 1995b). Thus, PHB synthesis may be limited by the availability of acetyl-CoA and NADPH. In a situation where the endogenous cysteine, methionine and isoleucine pool is supplemented exogenously, it is likely that the flux from carbon source metabolism favors acetyl-CoA pool and also the demand for biosynthesis for these amino acids may be reduced. This should create a favorable condition for enhanced PHB synthesis since acetyl-CoA, the substrate for β-ketothiolase, and NADPH, the cofactor for acetoacetyl-CoA reductase (Peoples and Sinskey 1989a) would not become rate limiting. Addition of phenylalanine in the medium has been shown to enhance the production of chloramphenicol-acetyl-transferase that is rich in phenylalanine (Ramirez and Bentley 1993). In the present instance the possibility of high demand for incorporation of cysteine, methionine and isoleucine into PHA biosynthetic enzyme proteins is ruled out, since *S. aureofaciens* as *R. eutropha* PHA biosynthesis enzymes do not have high contents of cysteine, methionine and isoleucine (Peoples and Sinskey 1989b, also see Chapter 3: Table 3.4a, 3.4b). Thus, availability of acetyl-CoA and NADPH seems to be the major deciding factors in enhancement of PHB synthesis than the amino acid composition of PHA synthesizing enzymes. This is consistently observed in recombinant *E. coli* irrespective of the origin of PHA biosynthesis genes (Lee *et al.* 1995b).

4.5.2 2^3 factorial design method

Two concentrations of cysteine, methionine and isoleucine, 50 mg/l and 150 mg/l resulted in increased PHB synthesis (Table 4.2) with respect to the control (Table 4.1).

Table 4.2 Effect of addition of cysteine, methionine and isoleucine on PHB synthesis by recombinant *pha*$_{Sa}^{+}$ *Escherichia coli* (ATCC: PTA – 1579) in a defined medium

Amino acid concentration mg/l	**PHB % cell dry weight**
Cysteine	
50	26.51 ± 0.18
150	30.82 ± 0.52
Methionine	
50	25.99 ± 0.37
150	19.15 ± 0.27
Isoleucine	
50	23.13 ± 0.54
150	16.58 ± 0.30

In R medium supplemented with 50 mg/l and 150 mg/l cysteine, recombinant *E. coli* cells accumulated 26.51 ± 0.18% and 30.82 ± 0.52% PHB of cell dry weight, respectively. Similarly in presence of 50 and 150 mg/l of methionine cells accumulated 25.99 ± 0.37% and 19.15 ± 0.27% PHB of cell dry weight respectively. While, addition of 50 and 150 mg/l of isoleucine resulted in respective accumulation of 23.13 ± 0.54 and 16.58 ± 0.30% PHB of cell dry weight. Therefore, effect of assorted combinations of different concentrations of these three amino acids on PHB synthesis was studied (Table 4.3). The effects were analyzed using a 2^3 factorial design method. This method offers the advantages of: (a) obtaining a broad picture of the effect of each factor in different conditions furnished by variations in the other factors, (b) the use of a wide range of factor combinations provides a reliable basis for making practical recommendations that will be valid in variable circumstances, (c) allowing estimates of the main effect of three variables with the same precision as the one-factor-at-a-time method requiring 24 runs. An additional advantage of 2^3 factorial design method is the interpretation of the observations produced by the design can proceed largely by using common sense and elementary arithmetic (Bailey 1995, Paul *et al.* 1998).

Table 4.3 Effect of addition of assorted combinations of amino acids on PHB synthesis by recombinant *pha*$_{Sa}^{+}$ *Escherichia coli* (ATCC: PTA – 1579) in a defined medium

Test condition	Cysteine Conc. mg/l	Methionine conc. Mg/l	Isoleucine conc. mg/l	PHB % cell dry weight
1	50	50	50	36.15 ± 1.40
2	150	50	50	40.68 ± 0.78
3	50	150	50	29.87 ± 1.36
4	150	150	50	32.43 ± 0.42
5	50	50	150	26.29 ± 0.65
6	150	50	150	30.02 ± 1.48
7	50	150	150	34.84 ± 0.78
8	150	150	150	51.92 ± 3.97

Considering above advantages, 2^3 factorial design method was exercised to study the effect of assorted combinations of cysteine, methionine and isoleucine on PHB synthesis by recombinant *E. coli.*

The effect of supplementation of different concentrations of the three amino acids during the fermentation of *E. coli* (ATCC: PTA – 1579) is shown in Table 4.3. The results were analyzed using 2^3 factorial design (Fig. 4.1). The data can be interpreted in the following manner:

(i) The interaction effect of cysteine and methionine (C X M) is 2.845 with individual main effects due to cysteine and methionine being 6.975 and 3.98 respectively. Thus, the interaction between the cysteine and methionine is independent of cysteine concentration but is affected by the increasing concentrations of methionine.

(ii) The interaction effect of cysteine and isoleucine (C X I) is 3.43 while the individual main effects due to cysteine and isoleucine are 6.975 and 0.985 respectively. It can be concluded that the effect of cysteine concentration is more than that of isoleucine.

(iii) The interaction effect of methionine and isoleucine (M X I) is 11.245; the individual main effects due to methionine and isoleucine being 3.98 and 0.985 respectively. There seems to be synergistic effect during the interaction of

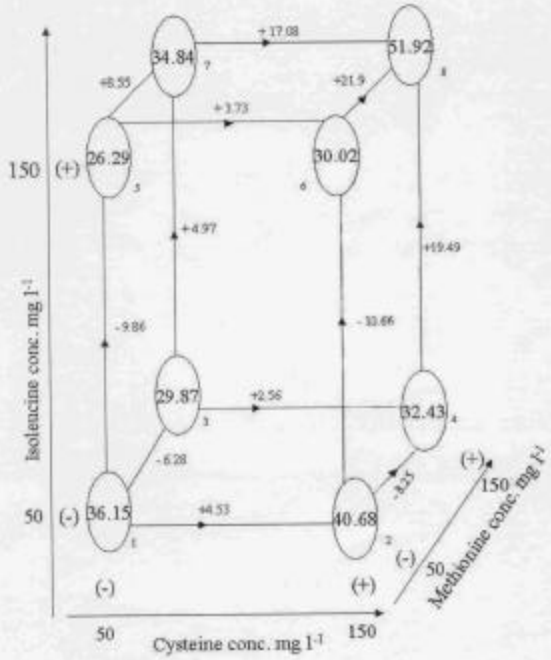

Fig. 4.3 Interaction effects of cysteine, methionine and isoleucine on PHB accumulation in the concentration range of 50 to 150 mg/l of each amino acid: Use of 2^3 factorial design

Effects		Estimates
Average		35.275
Main effects:		
Cysteine	C	6.975
Methionine	M	3.98
Isoleucine	I	0.985
Two-factor interaction		
C X M		2.845
C X I		3.43
M X I		11.245
Three-factor interaction		
C X M X I		3.83

methionine and isoleucine with the effect of methionine being more prominent than that of isoleucine.

(iv) The three factor interaction effect is 3.83 with the main effects due to cysteine, methionine and isoleucine being 6.975, 3.98 and 0.985 respectively. These values indicate that the effect of the three amino acids is in the order of cysteine > methionine > isoleucine.

Exogenous supplementation of amino acid to the medium seems to relieve the metabolic burden for recombinant *E. coli* to a certain extent, resulting in increased PHB production. This phenomenon was observed for most of the amino acids. These results also emphasize the utility of 2^3 factorial design in assessing the effect of three variables at two different concentrations by performing a reasonable number of experiments. The effect of the three amino acids on PHB synthesis as observed by 2^3 factorial design was in the order of cysteine > methionine > isoleucine. Cysteine alone at concentration of 150 mg/l in the R medium resulted in PHB content of 30.82 ± 0.52% of cell dry weight (Table 4.2). Further addition of 50 mg/l each of methionine and isoleucine increased the PHB yield to 40.68 ± 0.78% cell dry weight (Table 4.3). However, the highest PHB content of 51.92 ± 3.97% cell dry weight was obtained in presence of 150 mg/l each of cysteine, methionine and isoleucine (Table 4.3).

Amino acid biosynthetic enzyme activities of several pathways are repressed by end product supplementation (Umbarger 1978). End product inhibition in cysteine biosynthetic pathway controls the carbon flow. This is achieved by the sensitivity of serine transacetylase to cysteine (Kredich and Tompkins 1966). Cysteine supplementation to the medium will hence ensure: (a) a change in carbon flux and (b) elimination of the demand for acetyl-CoA for serine transacetylation reaction. Thus, more of carbon source and acetyl-CoA will be available for PHB synthesis. Methionine and isoleucine degradation yields succinyl-CoA, an intermediate of TCA cycle. This should allow more of acetyl-CoA to enter PHB biosynthetic pathway. Acetate formation during *E. coli* fermentation with glycerol as the carbon source (Lee 1996b) exerts negative effect not only on cell growth but also on recombinant protein production (Yee and Blanch 1992). Methionine and isoleucine have been reported to alleviate the acetate inhibition, with methionine being more effective (Han *et al.* 1993). This combined effect of methionine and isoleucine is prominent as is evident by its cumulative effect on PHB accumulation. These observations would aid in media optimization for PHB production

by recombinant *E. coli*. 2^3 factorial design results affirm its use in media optimization studies.

[C] Characterization of PHB

The polymer formed in recombinant *pha*$_{Sa}^{+}$ *E. coli*, was extracted and subjected to ^{1}H NMR analysis. Gel permeation chromatography and scanning electron microscopy were carried out to determine molecular mass and size distribution of the PHB granules respectively. The mechanical properties of PHB depend on molecular mass. The size of PHB granules also in turn reflects the molecular mass of the polymer formed i. e. higher the molecular mass, larger is the size of PHB granules. The molecular mass of PHB produced by bacteria is influenced both by the producing organism and other environmental conditions (Taidi *et al.* 1995). Determination of physical parameters will help to get comparative account of the PHB formed by *pha*$_{Sa}^{+}$ *E.coli* with those of PHB isolated from different microorganisms.

4.6 MATERIALS AND METHODS

4.6.1 Materials

Ampicillin, poly-β-hydroxybutyrate, $CDCl_3$ and tetramethylsilane were purchased from Sigma-Aldrich, USA. All other chemicals and bacteriological media components used were of analytical grade and obtained from HIMEDIA, Qualigens Fine Chemicals and E. Merck Laboratories, India.

4.6.2 Recombinant organism and growth conditions

Recombinant *pha*$_{Sa}^{+}$ *E. coli* JM109 (ATCC-PTA-1579) (Chapter 2) grown in basal medium (section 4.2.2.2) supplemented with 1% glycerol and ampicillin (100 µg/ml) was used in the study.

4.6.3 PHB isolation

PHB was recovered by using dispersions of sodium hypochlorite and chloroform. PHB was extracted from recombinant *E. coli* cells using Hahn *et al.* (1994) method with slight modification. 8.0 g of freeze dried cell mass was treated with a dispersion of 100 ml chloroform and 100 ml of 30% sodium hypochlorite. The cell powder was treated at 37°C for 90 min and the mixture centrifuged at 8,000 x *g* for 20 min at 30°C. Three phases were obtained. The upper phase was hypochlorite solution, the middle phase contained NPCM (non-PHB cell

materials) and undisrupted cells and the bottom chloroform phase contained solubilized PHB. First the hypochlorite solution was removed with a pipette and then the chloroform phase was obtained after filtration. Later the PHB was recovered from the chloroform phase by nonsolvent precipitation and filtration. The nonsolvent used was methanol (4-6 volume).

4.6.4 ^{1}H-NMR study of the molecular structure of PHB

The ^{1}H NMR analysis of the polyester samples was carried out on Bruker-200 spectrometer (USA). The 200 MHz ^{1}H NMR spectra were recorded at 24°C in $CDCl_3$ solution of polyester (50 mg/ml) with a acquisition time of 2.0480 seconds, sweep width of 4000 Hz. Tetramethylsilane was used as an internal chemical shift standard. The spectra was recorded for commercial PHB (Sigma-Aldrich, USA) and for the polymer extracted from recombinant *pha*$_{Sa}^{+}$ *E. coli*.

4.6.5 Determination of molecular weight by gel permeation chromatography (GPC)

Gel permeation chromatography (GPC) measurements were recorded at 25°C using Waters GPC 150C (USA) equipped with integrated solvent sample management unit and refractive index detector containing thermally shielded flow cell and optics with countercurrent heat exchanger for better baseline stability. A set of GPC columns consisting 100, 500, 10^3, 10^4, 10^5 A° (30cm x 7.8mm id) polystyrene crosslinked with divinyl benzene was used as stationary phase and HPLC and spectroscopic grade chloroform was used at flow rate of 1ml/min as the mobile phase. PHB was used at concentration of 1.0 mg/ml. Weight average molecular weight (Mw), number average molecular weight (Mn) and polydispersity index (PI) of the polymer were determined using polystyrene standard as the calibration standard and polymer standard service (PSS, Germany) software.

4.6.6 Scanning electron microscopy

PHB extracted from recombinant *E. coli* cells was suspended in distilled water. PHB suspension obtained was used for scanning electron microscopy. Gold sputtering was done to attain 25 nm thick layer. The PHB granules were observed using a Cambridge Instruments Stereoscan 120.

4.7 RESULTS AND DISCUSSION

4.7.1 PHB Isolation

PHB was extracted from recombinant *pha*$_{Sa}$+ *E. coli* using chloroform and sodium hypochlorite dispersion. The PHB in chloroform was precipitated with 4 to 6 volume of methanol and filtered. The dried polymer was further characterized by NMR (nuclear magnetic resonance), GPC (gel permeation chromatography) and SEM (scanning electron microscopy).

4.7.2 ^{1}H-NMR study of the molecular structure of PHB

The polymer was dissolved in 1 ml $CDCl_3$ at a concentration of 50 mg/ml. Tetramethylsilane was used as an internal chemical shift standard for ^{1}H-NMR study. The spectrum given in Fig. 4.4a shows presence of three groups of signals characteristic of the PHB homopolymer. A doublet at 1.30 ppm which is attributed to the methyl group coupled to one proton, a doublet of quadruplet at 2.57 ppm which is attributed to a methylene group adjacent to an asymmetric carbon atom bearing a single proton and a multiplet at 5.28 ppm characteristic of the methyne group were seen. Thus, identity of the PHA formed by recombinant *pha*$_{Sa}$+ was further confirmed to be P(3HB). The signal obtained at 7.28 ppm is attributed to chloroform. The NMR spectra of commercial PHB (Aldrich, USA) is also shown in Fig. 4.4b.

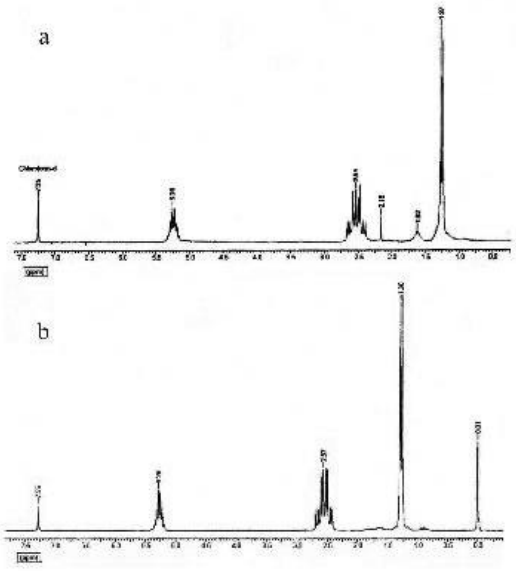

Fig. 4.4 ^{1}H NMR spectrum of PHB obtained from (a) Aldrich, USA and (b) recombinant *E. coli* cells harboring pSa240.

4.7.3 Determination of molecular weight of P(3HB) by gel permeation chromatography (GPC)

Number average molecular weight, *Mn*, and weight average molecular weight, *Mw*, the parameters generally used to represent the molecular weight distribution of polymers, are defined as,

$$Mn = \frac{\Sigma\ m\ Nm}{\Sigma\ Nm}$$

and

$$Mw = \frac{\Sigma\ m^2\ Nm}{\Sigma\ m\ Nm}$$

Where *m* and *N*m are the molecular weight of PHB polymer and the number of PHB polymers with a molecular weight *m*.

Most of the thermodynamic properties such as density, specific heat capacity, refractive index etc. are dependent on the number-average molecular mass. Bulk properties connected with large deformations such as melt and solution viscosity, are largely determined by the weight – average molecular mass, i.e. by the mass to be transferred (Van Krevelen 1972). Another parameter used to characterize polymers include polydispersity index,

$$\text{Polydispersity index (Q)} = \frac{Mw}{Mn}$$

Gel permeation chromatography analysis of PHB isolated from recombinant pha_{Sa}^{+} *E. coli* showed weight average molecular weight (Mw) of the polymer to be 2.85 x 10^5 and number average molecular weight (Mn) to be 1.065 x 10^5. The polydispersity index (PI) was observed to be 2.64 (Fig. 4.5). The number average molecular weights of the PHB recovered by chloroform extraction from recombinant *E. coli* strain harboring PHA synthesizing genes from *R. eutropha* reported earlier varied from 1.15 – 1.53 x 10^6 (Hahn *et al.* 1995, Kidwell *et al.* 1995). In *Methylobacterium extorquens* about 4 to 8%

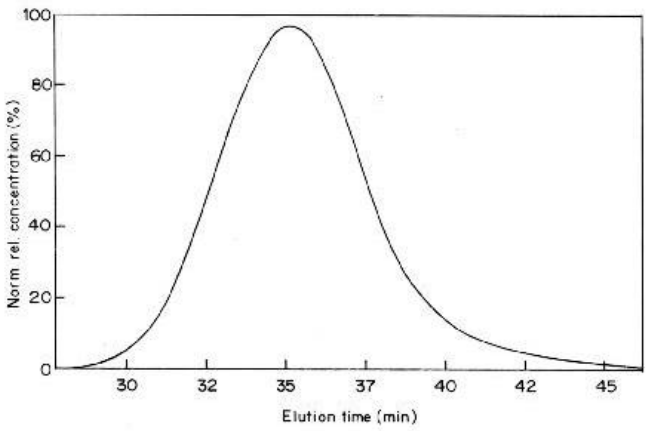

Fig. 4.5 The GPC (Gel permeation chromatography) curve of PHB sample obtained from recombinant pha_{Sa}^{+} *E. coli* cells harboring pSa240.

(w/w) PHB of low molecular weight was accumulated with glycerol as a sole carbon source. In *R. eutropha* PHB with low molecular weight was obtained when glycerol as carbon source (Taidi *et al*. 1994). Different bacteria consistently produce polymers of different molecular weights (Anderson and Dawes 1990). Azotobacters, for example, accumulate PHB in the range 8 x 10^5 to 2 x 10^6 while *Methylobacterium* sp. strain B3-Bp accumulates PHB in the range of 2.5 x 10^5 to 3 x 10^5 (Anderson and Dawes 1990). Molecular weight of the PHB from *Streptomyces* has not been reported till date. Hence, low molecular weight obtained in recombinant pha_{Sa}+ *E. coli* cells could be (i) inherent property of the genes in question, (ii) it could be the effect of carbon source used as observed in case of *M. extorquens* and *R. eutropha* or (iii) high levels of PHA synthase enzyme might have resulted in high levels of low molecular weight PHA as earlier observed in recombinant *E. coli* harboring $phaCAB_{Re}$ genes (Sim *et al.* 1997).

4.7.4 Scanning electron microscopic observation of P(3HB)

The PHB granules were spherical in shape as observed by SEM (Fig. 4.6). The size distribution of PHB granules ranged from 0.11 to 0.35 μm with the mean value of 0.23 ± 0.06 μm. The PHB granules synthesized in *Bacillus megaterium* were reported to be spherical with a diameter of 0.1 to 0.8 μm (Ellar *et al*. 1968). Later, Kofronova *et al.* (1994) showed that the size distribution of PHB granules in *B. megaterium* ranged from 0.88 to 1.56 μm with the mean value of 1.15 ± 0.14 μm as observed by SEM. In recombinant *E. coli* harboring $phaCAB_{Re}$ operon, the size of the PHB granules ranges from 1.13 to 1.25 μm (Middleberg *et al*. 1995). However, granules synthesized in *R. eutropha* are typically spherical with a diameter of 0.1 to 0.8 μm (Middleberg *et al*. 1995). The size of the PHB granules observed in recombinant pha_{Sa}^+ *E. coli* is comparable with that observed in natural producers. But it is smaller than in recombinant *E. coli* harboring $phaCAB_{Re}$ operon from *R. eutropha.* Larger granule of PHB would possess a greater *Mn*. In agreement with this the P(3HB) isolated from recombinant pha_{Sa}^+ *E. coli* has lower *Mn* (1.065 x 10^5) and smaller granule size (0.11 to 0.35 μm) as compared to that of recombinant pha_{Re}^+ *E. coli* having *Mn* (1.15 – 1.53 x 10^6) and granule size (1.13 to 1.25 μm). This could be result of (i) inherent property of the genes in question, (ii) carbon source used as observed in case of *M. extorquens* and *R. eutropha* or (iii) high levels of PHA synthase enzyme might have resulted in high

Fig. 4.6 Scanning electron micrograph of PHB obtained from recombinant *pha*$_{Sa}^{+}$ *Escherichia coli*.

levels of low molecular weight PHA with smaller sized granules as earlier observed in recombinant *E. coli* harboring *phaCAB*$_{Re}$ genes (Sim *et al.* 1997).

The larger granule size and high molecular mass are desirable characteristics for PHB. After release, the PHB granules may be collected by centrifugation. The efficiency of this collection is dictated by the size and density of the PHB granules, hence larger size of PHB granules is preferable from a downstream processing point of view.

CONCLUSIONS

CONCLUSIONS

- *Streptomyces aureofaciens* NRRL 2209 accumulated a maximum of 2.4% poly(3-hydroxybutyrate) in 16 h of incubation in Kannan-Rehacek medium.
- Plasmid pSa240 which contains an approximate 5.0 kb *Sau*3A I genomic DNA fragment from *S. aureofaciens* NRRL 2209, apparently carries all the necessary genetic information to order and direct poly(3-hydroxybutyrate) synthesis in recombinant *Escerichia coli* harboring it.
- The identity of PHB was further confirmed by NMR analysis of the polymer extracted from recombinant pha_{Sa}^{+} *E. coli.*
- Gel permeation chromatography studies of polymer extracted from recombinant pha_{Sa}^{+} *E. coli* revealed weight average molecular weight (Mw), number average molecular weight (Mn) and polydispersity index (PI) of the polymer to be 2.85 x 10^5, 1.065 x 10^5 and 2.64 respectively.
- The PHB granules extracted from recombinant pha_{Sa}^{+} *E. coli* were spherical in shape as observed by SEM. The size distribution of PHB granules ranged from 0.11 to 0.35 μm with the mean value of 0.23 ± 0.06 μm.
- The 4826 bp sequence of pSa240 DNA insert from *S. aureofaciens* obtained was deposited with the GenBank under accesssion number AY032926.
- The 4826 bp pSa240 DNA fragment had a G+C content of 74.6 mol%.
- Nucleotide sequence analysis of both DNA strands, using FramePlot method for predicting protein coding region of bacterial DNA with high G+C content (Ishikawa and Hotta 1999), revealed six open reading frames (ORFs) with GTG as start codon and four ORFs with ATG as start codon.
- Genes encoding for PHA synthase (*phaC*$_{Sa}$) and NADPH dependent acetoacetyl-CoA reductase (*phaB*$_{Sa}$) were identified by sequence analysis and were subsequently confirmed by performing transposon insertion studies.
- The pSa240 DNA fragment from *S. aureofaciens* probably does not carry a *phaA* gene and the host *E. coli* provides this function. In the eventuality of the *E. coli* catabolic β-ketothiolase not participating in PHB synthesis, intermediates of fatty acid β-oxidation or fatty acid synthesis might be channelized to PHB biosynthesis in recombinant *E. coli*.

- ORFs 2, 4, 6, 7, and 10 (Other than the ones encoding for PHA synthase and NADPH dependent acetoacetyl-CoA reductase) certainly play a role in PHB accumulation by recombinant *E. coli*.
- Glycerol, a by-product from the production of biofuel from oilseeds, as a carbon source in complex medium, supported maximal accumulation of P(3HB) of upto 60% of cell dry weight in recombinant pha_{Sa}^{+} *E. coli* harboring pSa240.
- For the first time plant oil, palm oil as a carbon source in a complex medium has been shown to support PHB accumulation in recombinant pha^{+} *E. coli*.
- Yeast extract and peptone as nitrogen sources in the nutrient medium result in levels of up to 60% PHB of cell dry weight in recombinant pha_{Sa}^{+} *E. coli* harboring pSa240.
- The complex medium composition that gives maximum (approximately 60% PHB of cell dry weight) in recombinant pha_{Sa}^{+} *E. coli* harboring pSa240 is

	g/l
Yeast extract	5
Peptone	5
Na_2HPO_4	1
$MgSO_4$	0.2
Glycerol	10
pH 7.2	

- Defined R medium supplemented with cysteine, methionine and isoleucine at 150 mg/l each supported maximum P(3HB) accumulation of ~ 52%.
- 2^3 factorial analysis indicated that the effect of the three amino acids on P(3HB) accumulation by recombinant pha_{Sa}^{+} *E. coli* was in the order of cysteine > methionine > isoleucine.
- This is the first report on isolation and characterization of PHA synthesizing genes from streptomycetes.
- A remarkable increase from 2.4% in the natural PHB producer, *S. aureofaciens* to 60% PHB of cell dry weight in recombinant pha_{Sa}^{+} *E. coli* is observed for the first time.

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AUTHOR'S PUBLICATIONS

PUBLICATIONS, CONFERENCES/WORKSHOPS

- Effect of amino acids supplementation on the synthesis of poly(3-hydroxybutyrate) by recombinant *Escherichia coli*
  L. H. Mahishi and S. K. Rawal
  Manuscript submitted to Enzyme Microb. Technol. (Current status: Manuscript under revision)

- *pha*$_{Sa}^{+}$ recombinant *Escherichia coli* accumulates twenty times more poly(3-hydroxybutyrate) than the natural producer *Streptomyces aureofaciens* NRRL 2209
  L. H. Mahishi, G. Tripathi, T. V. N. Ramachander and S. K. Rawal
  Communicated.

- Effect of various carbon and nitrogen sources on poly(3-hydroxybutyrate) synthesis by recombinant *Escherichia coli*
  L. H. Mahishi and S. K. Rawal
  Communicated.

- Construction of a *Streptomyces sp.* - *Escherichia coli* conjugative shuttle vectors and its application for recombinant biosynthesis of poly(3-hydroxyalkanoic acid)
  G. Tripathi, **L. H. Mahishi,** T. V. N. Ramachander, O. G. B. Nambiyar, S. H. Phadnis and S. K. Rawal
  Manuscript submitted to Biotechnol. Lett. (Accepted for publication).

- Molecular cloning, characterization and tissue specific expression of an elongation factor 1A gene in sugarcane
  D. Vijaykumar, T. V. N. Ramachnader, **L. H. Mahishi,** R. Kaul, P. Pyati and S. K. Rawal
  In press (Plant Science).

- Poster presented at national symposium 'Biotechnology in Agriculture and Environment' held at Punjab State Council for Science & Technology, Chandigarh (March 25-26, 1998) IInd Prize.

- Poster presented at national seminar on 'Emerging frontiers in Plant Biotechnology' held at NCL, Pune (October 28-29, 1999)

- Attended workshop on 'Computer aided Drug Design' held at Centre for Bioinformatics, University of Pune, Pune (January 3-5, 2001)

PATENTS

- Novel *Escherichia coli* having accession number PTA 1579 and its use to produce polyhydroxybutyrate
  L. H. Mahishi, G. Tripathi, T. V. N. Ramachander, S. K. Rawal.
  (**US patent filed**- 09/772,304)

- A process for the production of polyhydroxyoctanoate by *Streptomyces lividans*
  G. Tripathi, **L. H. Mahishi,** T. V. N. Ramachander and S. K. Rawal
  (**US patent filed**- 09/754,112)