CHARACTERIZATION OF A 5.0 Kb GENOMIC DNA FRAGMENT FROM *STREPTOMYCES AUREOFACIENS* NRRL 2209 INVOVELD IN POLY-β-HYDOXYBUTYRATE (PHB) SYNTHESIS

BY
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APRIL 2003

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A THESIS

SUBMITTED TO THE

UNIVERSITY OF PUNE

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

BIOTECHNOLOGY

BY

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APRIL 2003

Dedicated to my Parents

CERTIFICATE

This is to certify that the work incorporated in the thesis entitled "**Characterization of a 5.0kb genomic DNA fragment from *Streptomyces aureofaciens* NRRL 2209 involved in poly-3-hydroxybutyrate (PHB) synthesis**" submitted by ***T. V. Naga Ramachander*** was carried out under my supervision at the Plant Tissue Culture Division, National Chemical Laboratory, Pune. Materials obtained from other sources have been duly acknowledged in the thesis.

Dr. S. K. Rawal

(Research Guide)

ACKNOWLEDGEMENTS

It is my pleasure to express my appreciation to my research guide Dr. S. K. Rawal for his guidance, help and freedom to perform. On a personal level, I would like to thank him for his support in testing times.

I thank Dr. Nagaraja Rao and Dr. Y. Souche for their constant help and support during my entire work period. I thank Dr. B. M. Khan and Mrs. Ulka Potdar for their help and encouragement.

Thanks are due to Dr. Aditi Pant and Dr. M. V. Deshpande for the usage of GC and Fluorescence microscopy facilities. I am indebted to Dr. Bhale Rao and the NMR facility group for providing the NMR faciilty. I take this opportunity to thank Dr. Jan Kormanec for his valuable personal communication and Dr. Terumi Saito for providing me with the intracellular depolymerase gene. Thanks are due to all members of the scientific and supporting staff of the Plant Tissue Culture Division, Biochemical Sciences Division and NCL Library.

Many thanks are due to my labmates Rohini, Manish, Sucheta and Raman for the cordial atmosphere in the lab. I thank my seniors Jawahar, Vijay, GT and Lata for their encouragement and help during the course of this work.

I take this opportunity to thank my friends Rama Krishna, Gururaj Joshi, Satyanarayana, ASN Reddy, Nagesh, Sarang, Suresh, Hutoxy sarkary, Sravanti, Janaki, Rajasekhara Reddy, Pavan and Badri for their help, support and best wishes through out my work period. I also thank Neelam, Aparna, CV, Jui, Rangu, Rajashree, Solanky, Manisha, Nagaprasad, Sreedhar, Mahesh, Ekambaram, DPS, Praveen, KK, RK, Bhoja and Gyanenswar for their constant support, help and lighter moments shared together. I also thank Manish, Anish, LS, Babu, Suresh, Bhushan, Mukund, Ajay, Nilesh, Nitin, Ajit, Sharmili, Aditya and Neeli for incidental help during the course of this work.

Thanks are due to my colleagues at PTC Division Jayeeta, Neelima, Mohan and Laxmiji, Satish, Anjan, Vandana, Swati, Madhumita di, Vijay Joshi, Seema, Bharati, Nooka Raju, Sujata, Shaila, Raman, Sucheta and Girija for their help at all times.

I owe my deepest gratitude to my parents and to my sister who have constantly

encouraged me to excel. Without their enduring support this journey would not have been possible.

I am grateful to Dr. S. Sivaram, Director, NCL and Dr. S. K. Rawal, Head, Plant Tissue Culture Division for providing necessary facilities and permitting me to submit my findings in the form a thesis.

The fellowship awarded by the Council of Scientific and Industrial Research is duly acknowledged.

T. V. NAGA RAMACHANDER

ABBREVIATIONS

BSA	Bovine serum albumin
bp	Base pairs
Ci $mmol^{-1}$	Curie per milli mole
CTAB	Cetyltrimethylammonium Bromide
EDTA	Ethylene diamine tetra acetic acid disodium salt
g	grams
xg	Relative centrifugal force
g L^{-1}	grams per litre
h	hour(s)
IPTG	Isopropyl β-D-thiogalactoside
kb	Kilobase pairs
μ L^{-1}	Micrograms per liter
μCi	Microcurie(s)
mg	milligrams
mM	millimolar
MCS	Multiple cloning sites
min	Minute(s)
O/N	Over night
PEG	Polyethylene glycol
PHB/P(3HB)	Poly-3-hydroxybutyrate
rpm	Rotations per minute
SDS	Sodium dodecyl sulphate (sodium lauryl sulphate)
s	Seconds
U μL^{-1}	Units per microlitre
v/v	volume / volume
wv./vol.	weight / volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

CONTENTS

ABSTRACT

Conventional plastics derived from the fossil fuels pose a threat to the global environment due to their non-degradable nature. To satisfy the environmental imperatives, novel non-petroleum plastics are being developed that are biodegradable besides retaining the desired properties of conventional plastics.

Polyhydroxyalkanoates (PHAs) are the polyesters that accumulate as granular inclusion bodies in a large variety of bacteria usually under biotic stress. Approximately 300 different types of bacteria synthesize and accumulate PHAs. Because of their inherent degradability, PHAs are regarded as an attractive source of non-polluting plastics and elastomers that can be used for specialty and commodity products. Of all the PHAs, Poly (3-hydroxybutyarate) (PHB) has attracted considerable interest as a candidate for the biodegradable and biocompatible plastics.

Streptomyces species are Gram positive soil bacteria. *Streptomyces* DNA is known to have the highest G+C content (>70%) and displays a complex life cycle, which often culminates in the spore formation. They are known to produce > 50 % of the presently known naturally occurring antibiotics as secondary metabolites besides other products. Because of their well studied down stream processing they are considered to be the model organisms both at academic and industrial levels.

In our laboratory at National Chemical Laboratory, Pune, India, a sub-genomic library of the *Streptomyces aureofaciens* NRRL 2209 partial Sau3A I digested g-DNA was constructed in pGEM-3Z plasmid vector for screening PHB biosynthesis genes.

The main features of the present thesis are:

1. *Isolation and cloning of PHA synthesizing genes and characterization of PHA.*
2. *Sequence analysis of a 5.0 kb genomic DNA fragment from S. aureofaciens NRRL 2209.*
3. *Characterization of pha 'C' gene involved in PHB biosynthesis of S. aureofaciens.*
4. *Screening for the depolymerase gene in S. aureofaciens NRRL 2209.*

1. *Isolation and cloning of PHA synthesizing genes and characterization of PHA*

S. aureofaciens Sau3A I sub-genomic library in *E. coli* JM109 was screened for the PHB biosynthesis genes using *R. eutropha pha*C, *pha*B and *pha*A genes. Three positve clones were selected. The signals obtained using $phaA_{Re}$ probe were of low intensity. Based on the intensity of the signals for *pha*C and *pha*B ga clone designated as pSa240 harboring a 5.0 kb g-DNA fragment of *S. aureofaciens* was identified.

pSa240 was grown in basal medium supplemented with different carbon sources. Irrespective of the carbon source used all the cells accumulated maximum amounts of PHA at ~ 40-42 h. Maximum PHA accumulation of 60-66% of dry cell mass was obtained in basal medium supplemented with 1% glycerol. This was 20-25 times higher than the PHA accumulation obtained in the native *S. aureofaciens* which accumulated ~ 2.45. The possibility of methylglyoxal pathway involved in the PHB biosynthesis in recombinant *E. coli* has been shown.

The clone pSa240 was stained with Nile Blue A and observed under fluorescent microscope. The cells showed a characteristic orange fluorescence at 460 nm. Physical characterization of PHA by GC and FTIR revealed the Pha to be PHB. ^{1}H NMR of the purified PHB showed peaks typical to the isotactic PHB homopolymer. TGA ad DSC analysis showed the polymer to be degrade rapidly between 250 and 340°C. Molecular weight of the PHB was found to be 3×10^5. PHB granules were found to be of uniform spherical shape with an average diameter of 1.1 μm.

2. *Sequence analysis of a 5.0 kb genomic DNA fragment from S. aureofaciens NRRL 2209.*

The 5.0 kb insert in pSa240 was subjected to automatic sequencing and the complete sequence was found to be 4826 bp. This was deposited with Genbank under the accession No. AY032926. The sequence was found to be 74.6% G+C rich. The restriction analysis of the sequence was in conformity with the one generated earlier. A total of 23 ORFs coding for 10 putative genes were identified using Frame Plot ver. 2.3.2.

None of the ORFs except ORFs 5, 13, 17 and 23 showed no sequence similarity to any of the well characterized genes in the databanks. ORF 1, however, did possess a lipase box found in all the *pha*C genes. The **Cysteine** residue present in the active site was replaced by **Serine** in ORF 1. D^{518} and H^{546} forming a catalytic triad were also identified. This ORF had all the attributes of *pha*C gene. This showed a sequence similarity of 52.3% to the 1.0 kb Nco I / Stu I fragment of *R. eutropha*. ORF 2 showed a NADP binding site at its 5' end indicating it to be putative $phaB_{Sa}$. This showed a sequence similarity of 50.9% to the 0.74 kb Xcm I / Nde I fragment of *R. eutropha*. Putative promoters regions, RBS sites and transcription termination sites have been identifiifed for both the ORFs. No ORF could be assigned β-ketothiolase function. Interestingly ORF2 is found to be within ORF 1 but in a different frame. The presence of a rare tRNA for leucine implied it be temporally regulated.

ORF 13 encoding 18 kDa protein showed 99.37% sequence similarity to the *ssg*A protein sequence. A promoter region, RBS site and a transcription termination region were found for this ORF. *ssg*A is shown to play an important role in the sporulation and cell division.

3. Characterization of pha 'C' gene involved in PHB biosynthesis of S. aureofaciens.

To assign a possible role to the various ORFs present on the 5.0 kb insert of pSa240, twelve deletion clones were constructed. They were grown in basal medium for PHB accumulation. Their crude cell free extract was assayed for β-ketothiolase, acetoacetylCoA reductase and synthase enzyme activities. A protein gel of the crude extracts was run. From all the above experiments it became evident that ORF(s) present downstream of ^{2705}Pvu II site was playing an important role in PHB biosynthesis. Furthermore, the experimental results confirmed the *ssg*A gene product to play a key role in the high PHB accumulation.

Complementation of putative *pha*C_{Sa} in a clone harboring the *pha*AB_{Re} accumulated PHB thus, confirming the 2.7 kb EcoR I-Pvu II fragment of the 5.0 kb insert to harbor the *pha*C_{Sa} gene. However, minimal amount of PHB was accumulated despite the presence of genes coding for all the three enzymes. This substantiated the view a trans acting element like SsgA present downstream of the 2.7 kb fragment took an active part in the PHB synthesis. Gene reconstruction studies showed that S. aureofaciens has only a single copy of the *pha*C gene.

4. Screening for the depolymerase gene in S. aureofaciens NRRL 2209.

In *S. aureofaciens* NRRL 2209 accumulation of PHB aoccurs at the late log phase and the percentage of PHB accumulated decreases with onset of stationary phase. Based on the above fact it was assumed to possess an intracellular PHA depolymerase. Also, considering the soil habitat it is likely to possess an extracellular depolymerase.

Attempts made to identify and isolate extracellular PHA depolymerase gene(s) by various methods yielded no results. Hybridization of the *S. aureofaciens* g-DNA with an **oligoZ** designed on the basis of consensus depolymerases gene sequences gave no positive signals and ruled out the possibility of *S. aureofaciens* to possess an extracellulat depolymerase.

Hybridization of the *S. aureofaciens* g-DNA slot blot with a 1.0 kb fragment of *R. eutropha* intracellular depolymerase gene gave a positive signal indicating the presence of an intracellular depolymerase (i-depolymerase). Genome reconstruction studies revealed *S. aureofaciens* to possess a single copy the i-depolymerase homologous to r. eutropha i-depolymerase gene.

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CHAPTER 1

GENERAL INTRODUCTION

Plastics and polymers are an integral part of our daily existence. However, because of their properties of durability and resistance to degradation (Lee 1996), these accumulate in the environment at the rate of about 25 million tons per year (Lee *et al.*1991). These form 8% by weight and 20% by volume of the landfills (Richard 1992). Being highly hydrophobic in nature, these inhibit enzyme activities. The low surface area of plastics with inherent high molecular weight further compounds the problem (Robert 1994). Environmental problems associated with plastics have stimulated the formulation of legislations regulating polymer use. With increasing public and political awareness, and to satisfy the environmental imperatives research is directed towards finding suitable substitutes that are biodegradable besides retaining the desired properties of the conventional plastics. Of particular interest are biodegradable polymers from renewable sources, which fit into the ecological cycle (Daniel and Friedrich 1994). Of the many biopolymers available polylactides, aliphatic polyesters, polyhydroxyalkanoates (PHAs), polysaccharides and the copolymers and blends of these (Table 1.1) are under development as biodegradable plastics (Leaversuch 1987; Dawes 1990; Chum 1991; Steinbüchel 1991; Swift 1993; Chang 1994). Since the potential for utilizing biological systems as a source of biodegradable materials is becoming increasingly attractive, polyhydroxyalkanoates (PHAs) have gained importance, as they possess properties close to polypropylene.

1.1 History of Polyhydroxyalkanoates (PHAs)

The presence of sudanophilic lipid like inclusions (Meyer 1903) soluble in chloroform (Stapp 1924) was initially observed in *Azotobacter chroococcum* a century ago. It was Lemoigne (1926, 1927), who while studying *Bacillus megaterium*, established the chemical nature of these inclusion bodies as poly-3-hydroxy-butyricacid P(3HB). Due to studies on several *Bacillus* strains (Macrae and Wilkinson 1958) and phototropic bacteria (Doudroff and Stanier 1959) P(3HB) became more widely known. Wallen and Rohwedder (1974) reported the identification of polyhydroxyalkanoates other than P(3HB) especially poly-3-hydroxyvalerate P(3HV) and poly-3-hydroxyhexanoate P(3HHx). In 1983, Frindley and White reported accumulation of 95% P(3HB), 3% poly-3-hydroxyheptanoate P(3HHp), 2% of an 8-

carbon hydroxyalkanoate (HA) and trace amounts of 3-other HA compounds by batch grown *Bacillus megaterium* using capillary gas chromatography. Around same time Witholt (1983) and coworkers found *Pseudomonas oleovorans* to synthesize and accumulate poly-3-hydroxyoctanoate P(3HO) units and small amounts of 3HHx units when cultivated on n-Octane (De Smet *et al.* 1983, Lageveen *et al.* 1988)

Major impact on the research and commercial sectors was seen with the identification of HA units other than 3HB. While P(3HB) has limited applications due to its brittle nature, incorporation of other 3HA significantly enhanced the biopolymer properties which signified the beginning of the second stage of research on PHAs. Production of P(3HB-co-3HV), a co-polymer of industrial importance, took place during this stage. The emphasis now shifted to identification of all the 3HAs that could be associated with the bacterial polyesters. During this period it became clear that not only Gram negative but also a wide range of Gram positive bacteria, cyanobacteria (aerobic photosynthetic), non-sulfur and sulfur purple bacteria (anaerobic photosynthetic), archaea can synthesize and accumulate these 3HAs. As an astounding number of approximately 150 different HAs are known to occur (Steinbüchel and Valentin 1995; Rehm and Steinbüchel 1999), a more general name comprising of all the constituents namely polyhydroxyalkanoates (PHAs) has been used to designate this family of bacterial reserve polymers.

The third stage of development in this field was the cloning and characterization of genes involved in PHA biosynthesis. Late 1970s, provided tools to successfully decipher genetic information and a molecular biology revolution ensued. By late 1980s, PHA biosynthesis genes of *Ralstonia eutropha* (earlier *Alcaligenes eutrophus*) were cloned and shown to be functionally active in *E. coli* (Slater *et al.* 1988; Schubert *et al.* 1988; Peoples and Sinskey 1989a, b). Detailed studies on *R. eutropha* revealed three enzymes *viz*. β-ketothiolase, acetoacetyl-CoA reductase and PHA synthase to be involved in the biosynthesis of P(3HB) from acetyl-CoA and that the biosynthesis to be regulated at the enzyme level. Of the three enzymes PHA synthase was identified to play a key role in polymerization of HA monomers. To date 40 PHA synthase genes from more than 35 different bacteria have been cloned (Sudesh *et al.* 2000).

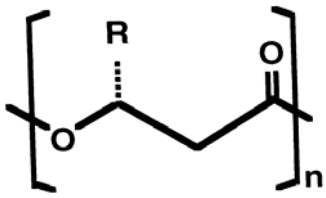

Fig 1.1 General formula showing the monomer repeat unit of PHA. The pendant 'R' could be substituted to get a desired PHA. For example in PHB, R=methyl group; PHV, R=ethyl group and so on.

Table 1.1: A Snapshot of the Biopolymer Family

Polyesters	**Polysaccharides (plant / algal)**
Polyhydroxyalkanoates	Starch (amylose / amylopectin)
Polylactic acid	Cellulose
	Pectin
Proteins	Agar
Silks	Alginate
Collagen/gelatin	Carrageenan
Elastin	Konjac
Resilin	Various gums (e.g., guar)
Adhesives	
Serum albumin	**Polysaccharides (animal)**
Polyamino acids	Hyaluronic acid
Soy, zein, wheat gluten**,** casein,	Chitin / chitosan
Polysaccharides (bacterial)	**Lipids/ surfactants**
Gellan	Acetoglycerides, waxes
Dextran	Emulsan
Xanthan	
Curd Ian	**Polyphenols**
Levan	Lignin
Polygalactosamine	Tannin
Cellulose (bacterial)	Humic acid
Polysaccharides (fungal)	**Speciality polymers**
Yeast glucans	Shellac
Pullulan	Poly-gamma-glutamic acid
Elsinan	Natural rubber
	Synthetic polymers from natural fats and oils (e.g., nylon from castor oil)

SOURCE David L Kaplan *et al*., "Naturally Occurring Biodegradable Polymers," G Swift and R Narayan (eds), Polymer Systems-Synthesis and Utility (NY Hanser Publishing, New York 1994)

A sudanophilic bacterial inclusion identified at the beginning of the twentieth century is going into fourth stage of development, i.e. protein engineering, by which manipulation of PHA synthase and other enzymes would enable us to make environment friendly polymers in an efficient manner (Sudesh *et al*. 2000). Thus, genetic engineering combined with modern molecular microbiology provides a framework for studying plastic properties as a function of genetic and metabolic blueprints (Madison and Huisman 1999).

1.2 Polyhydroxyalkanoates (PHAs)

Polyhydroxyalkanoic acids (PHAs) are polyesters synthesized from optically active thiol esters that accumulate as granular inclusions in the cytoplasm of various bacteria (Anderson and Dawes 1990; Steinbüchel 1991). Their general formula is shown in Fig.1.1 (Steinbüchel 1991; Byrom 1994). The 3-hydroxyalkanoic acids are all in R-configuration due to the stereospecificity of the polymerizing enzyme PHA synthase. Only in the case of *Rhodococcus* sp. S-monomers were detected (Haywood *et al*. 1991). The "oil crisis" of 1970s, prompted the development of polymers from non-petroleum sources. PHAs having properties similar to isotactic polypropylene gained importance (Marchessault 1996). But, their wide spread use has been impeded by high production costs. With the isolation of the genes responsible for PHAs biosynthesis and *in vivo / in vitro* metabolic engineering of the microorganisms, an enhanced and cost effective production of PHAs should be possible in not so a distant future.

1.2.1 Classification of PHAs

Approximately 150 different types of HAs have been detected as constituents of biosynthetic polyhydroxyalkanoates. A terminology now widely used to refer to the occurrence of homopolyesters, copolymers and polymer blends has been proposed by Steinbüchel *et al*. (1992). PHAs consisting of three to five carbon fatty acids (3-hydroxy or 4-hydroxy) are referred to as short chain length polymers or HA_{SCLs}. P(3HB) being the prototype of this group, with a 3-hydroxybutyric acid as the monomer unit. Other monomer units that are oxidized at positions other than the third

carbon such as 4-hydroxybutyrate (Kunioka *et al*. 1988; Doi 1990), 4-hydroxyvalerate (Valentin *et al*. 1992) and 5-hydroxyvalerate (Doi *et al*. 1987) also fall into this group. Medium chain length hydroxyalkanoic acids (HA_{MCLs}) represent hydoxyalkanoic acids with six to fourteen carbon atoms. These include poly-3-hydroxyhexanoic acid P(3HHx), poly-3-hydroxyheptaonoic acid P(3HHp), poly-3-hydroxyoctanoic acid P(3HO), poly-4-hydroxyhexanoate P(4HHx) etc. (Valentin *et al*. 1994). Any HA with sixteen or more carbons is referred to as long chain length PHAs (HA_{LCLs}).

1.2.2 Properties of PHAs

Chemical structure and monomer composition are the most important factors in determining the physical and material properties of a polymer. Most of the studies on physical and thermal properties of bacterial PHAs have been carried out with PHB and PHV. Since the presence of functional groups, epoxy-, hydroxy-, aromatic- side chains, opens up a wide range of possibilities for further chemical modification of the polymer structure, their incorporation to polymers influences the physical and material properties of PHA's (Lageveen *et al*. 1988; Fritzsche *et al*. 1990; Hori *et al*. 1994; Eggink *et al*. 1995; Casini *et al*. 1997).

The most important property of PHAs is their complete biodegradability. PHAs like PHB and PHBV are moldable thermoplastic polymers. PHB has a melt temperature of 175°C and a glass transition temperature (Tg) of 4°C (Byrom 1987; Doi 1990; Marchessault 1990; Holmes 1998). The decomposition temperature of ~200°C is rather too close to the melting temperature. However, Hahn *et al*. (1995) have shown the decomposition temperature of PHB recovered from recombinant *E. coli* to be between 250-300°C. Incorporation of 3HV units into PHB decreases the melting temperature of the co-polymer without affecting the thermal stability, thus making processing easy.

PHB, a short chain length polymer, is optically pure i.e. it is 100% stereo-specific with D(-) configuration (Doi 1990; Steinbüchel 1991) and isotactic in nature. It is relatively stiff (Barham *et al*. 1984; Doi 1990; Holmes 1998) and highly crystalline with crystallanity ranging between 55 to 88% (Lee 1996).

The mechanical properties of P(3HB) like Young's modulus (3.5 GPA) and

the tensile strength (40Mpa) are similar to those of polypropylene. Molded PHB samples do indeed show ductile behavior, but over a period of time at ambient conditions, they slowly become more brittle (Lee 1996). However, the elongation to break for PHB is 5%, which is significantly lower than polypropylene (400%). This unfavorable aging process and poor shatter strength are major drawbacks for the commercial exploitation of the PHB homopolymer. The co-polymer PHB-co-HV has much improved mechanical properties than the homopolymer P(3HB).

In comparison PHA_{MCLs} are generally elastomers with low melting point (42°-58°C). These are also highly amorphous with Tg of -62°C to 26°C. (Preusting *et al*. 1990; Eggink *et al*. 1992; Casini *et al*. 1997; Van der Walle 1999). Crystallinity is hampered with the incorporation of side chains into PHA_{MCL} although saturated PHA_{MCLs} are able to crystallize due to their isotactic configuration. In general PHA_{MCLs} are semi crystalline elastomers with low melting point, low tensile strength and high elongation to break (Gross *et al*. 1989; Preusting *et al*. 1990; Gagnon *et al*. 1992) and can be used as a biodegradable rubber after cross-linking by electron beam irradiation (De Koning *et al*. 1994). Elongation to break of 250 - 350% and a Young's modulus up to 17 Mpa has been reported (Marchessualt 1990). A comparison of PHB properties with that of other polymers is summarized in Table 1.2. The general properties and features of PHB are listed in Table 1.3.

1.2.3 Applications

Owing to a number of novel features, P(3HB) and P(3HB-co-3HV) have drawn considerable interest for a wide range of applications. Initially these were used for packaging items such as bottles, cosmetic containers, pens and golf tees (Baptist 1963a, b; Webb 1990) and also as mulch films for agriculture (Hocking and Marchessault 1994). PHAs latex may be applied to paper and cardboard to form a water resistant layer and to produce a completely biodegradable compound unlike those made out of polyethylene or aluminium (Steinbüchel and Rehm 1998). These can be used in personal hygiene articles, diapers etc. (Shiotani and Kobayashi 1994; Noda 1996a, b). These have been described as hot-melt adhesives (Kauffman *et al*. 1992). PHAs can be used to replace petrochemical polymers in toner and developer

Table 1.2 Properties of some PHAs and synthetic polymers (Poirier *et al.* 1995; Lee 1996; 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	-	~350	≤17
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

Table 1.3 Summary of the properties and features of PHAs

Properties and Features of PHB
• Thermoplastic
• Biodegradability
• Biocompatible
• Non-toxic
• Optically pure
• Isotactic
• Insoluble in water
• Highly crystalline
• Piezoelectric
• Can be produced from renewable Sources

compositions or as ion-conducting polymers (Fuller *et al*. 1991; Reusch and Reusch 1993, 1996). PHAs have been used as matrix in retardant materials for slow release of drugs, hormones, herbicides, insecticides, flavors and fragrances in medicine, pharmacy, agriculture and food industry. Recently these have been used as a source for the synthesis of enantiomerically pure chemicals and as raw materials for the production of paints (Steinbüchel and Rehm 1998).

Their applications have been extended to medical field as Osteo-synthetic materials, bone plates, surgical sutures etc. P(4HB) also called as gamma-HB (GHB) has been found to have a potential therapeutic value as an anaesthetic agent since it can cross the brain barriers and induce sleep like state with cardiovascular stability (Laborit 1964; Vickers 1968; Hunter *et al*.1971). GHB is relatively cheaper than PHB, is produced by various organisms (Nelson *et al*. 1981) and is found to be present in the brain tissues of rats, pigeons, humans etc. A co-polymer of P(3HB-co-4HB) is biocompatible, biodegradable and being used in medical implants (Doi US patent 230461). Summary of the applications is given in Table 1.4.

1.2.4 Detection and physical state of the *in vivo* inclusions

PHA inclusions, typically of 0.2 to 0.5 μm in diameter, in cytoplasm can be viewed with a phase-contrast light microscope due to their high refractivity (Dawes and Senior 1973). Native PHB inclusions can be stained using Sudanblack B (Burdon 1946) and oxazine dyes like Nile blue A exhibiting a strong fluorescence at an excitation wave length of 460 nm (Ostle and Holt 1982). Nile red can also be used to detect PHB in growing cultures (Spiekermann *et al*. 1999). However, purified polymer cannot be stained by these stains due to the loss of a membrane surrounding these PHA granules during purification (Dawes and Senior 1973).

The early X-ray diffraction studies of solid PHB by Alper *et al.* (1963) and Lundgren (1964) led to the conclusion that PHB granules inside the cytoplasm are crystalline in nature. Barham (1984) in order to resolve questions like polymer chain elongation by PHA synthase within a crystal and the mechanism of the internal depolymerase, used physical techniques and showed that the granules *in vivo* are in an amorphous state. Barnard and Sanders (1988, 1989) using solution state NMR

Table 1.4: Summary of the applications of PHAs in various fields

Applications of PHAs
• Packaging films, bags and containers • Biodegradable carrier for long term dosage of drugs, medicines, fertilizers etc. • Disposable hygiene products like razors, diapers, female hygiene products etc. • Raw material for chiral compounds that are enantiomerically pure chemicals and paints • Water resistant layers and hot melt adhesives • Ion conducting polymers • Intravenous anesthetic • Osteosynthetic material and bone plates • Surgical pins, sutures, staples and swabs • Blood vessel replacements • Wound dressing • Piezoelectric stimulation of bone growth and healing

technique confirmed the *in vivo* P(3HB) to be a mobile amorphous polymer. This discovery raised questions about the existence of an internal plasticizer or nucleation inhibitor(s) and the reasons for crystallization upon isolation.

PHA inclusions have water as a minor component and it was suggested as the plasticizer (Barnard and Sanders 1989; Harrison *et al*. 1992). Water was also proposed to be the chain transfer agent during the polymerizing mechanism of PHA synthase (Kawaguchi and Doi 1992). The rearrangement of polymer into lamellar crystals was found to be due to the removal of 5-10% water present in the nascent P(3HB). On the basis of the foregoing it was suggested that the water molecules form hydrogen bonds with the carbonyl group of the polyester backbone to form pseudo-

cross links between adjacent polymer chains.

1.2.5 Physiological role of PHAs

Physiologically PHAs were first associated with sporulation of bacteria (Slepeckey and Law 1961). These are formed before the onset of sporulation and utilized rapidly preceeding sporulation. However, PHAs are not always associated with sporulation since not all spore formers make polymer(s). Macrae and Williamson (1958) observed that the PHA accumulation in *Bacillus megaterium* increases as the C:N ratio increases. This observation led to the conclusion that the bacteria make and store PHAs when they lack the complete range of nutrients required for cell development but for generous supplies of carbon. A deficiency of magnesium, sulfur, nitrogen, phosphate and / or oxygen can initiate PHA biosynthesis.

Approximately 300 different bacteria encompassing Gram positive and Gram negative have been reported to accumulate various PHAs (Steinbüchel 1991). For many of them, the polymer once accumulated serves both as a carbon and energy source during starvation. It constitutes ideal carbon energy storage due to its low solubility, high molecular weight and inert nature, thus exerting negligible osmotic pressure on the bacterial cell (Dawes and Senior 1973). The presence of PHA in a cell frequently, but not universally, retards the degradation of cellular components such as RNA and proteins during nutrient starvation (Anderson and Dawes 1990).

Senior and Dawes (1971) proposed that PHA also serves as a sink for reducing power and could be regarded as a redox regulator within the cell. In members of Azotobacteriaceae, it is reported to provide an oxidizable substrate giving respiratory protection to nitrogenase under environmental conditions when an exogenous substrate is not immediately available for oxidation (Senior and Dawes 1973). PHA accumulation has been implicated both as an energy source and a regulator for controlling the viability of reducing power for the operations of nitrogenase in symbiotic nitrogen fixing bacteria (McDermott *et al*. 1989).

PHB has been reported in the membrane fractions of prokaryotes and eukaryotes. It is in association with inorganic phosphate, with highest concentrations in mitochondria and microsomes (Reusch and Sadaoff 1983; Reusch 1989; Reusch *et*

al. 1986, 1997) and possibly plays a role in regulation of intracellular calcium ion concentrations and in calcium signalling in eukaryotes (Reusch and Sadoff 1988; Reusch 1989). It is also proposed to be a prerequisite for the development of competence in *A. vinelandii* and *B. subtilis* (Reusch and Sadoff 1988) and to play an important role in the transmembrane transport of DNA in *E. coli* (Reusch *et al*. 1986). The monomer, 3HB-CoA has been detected in relatively large amounts in human blood plasma and as a common intermediate metabolite present in all higher animals (Reusch *et al*. 1992). Therefore, it was concluded that any implants using P(3HB) in mammalian tissues would be non-toxic.

1.3 Enzymes involved in PHA biosynthesis

P(3HB) biosynthesis pathway was the first to be deciphered paving way to understand the natures role which subsequently led to discovery of other PHA biosynthesis pathways. The P(3HB) biosynthesis, otherwise called as the three step biosynthesis pathway, mainly consists of three enzymatic reactions catalyzed by three distinct enzymes. The enzymes are β-ketothiolase, acetoacetyl-CoA reductase and PHA synthase.

1.3.1 β-ketothiolase

β-ketothiolase catalyzes the first step in P(3HB) formation. It is a member of the family of enzymes involved in the thiolytic cleavage of substrate into acyl-CoA and acetyl-CoA. This is the most thermodynamically favored reaction. But under the availability of reducing equivalents in the form of NADPH, it acts against the thermodynamically favored direction and aids in the P(3HB) biosynthesis (Masamune *et al*. 1989). These are found through out nature from higher eukaryotes to yeasts to prokaryotes and are found to be in soluble form in *in vivo*.

Based on the substrate specificity thiolases are divided into two groups. First group of thiolases fall into the enzyme Class EC.2.3.1.16 and have a broad specificity for β–ketoacyl-CoAs ranging from C_4-C_{16}. While the second group, belonging to enzyme Class EC.2.3.1.9. has a narrow range of chain length specificity from C_3-C_5. The first group is involved mainly in the degradation of fatty acids and is located in

the cytoplasm of prokaryotes and in the mitochondria and peroxisomes of mammalian and plant cells. The second type is considered to take part in biosynthesis of ketone bodies, steroid and isoprenoid biosyntheis, and P(3HB) biosynthesis (Madison and Huisman 1999).

In *Ralstonia eutropha* (earlier referred to as *Alcaligenes eutrophus*) two β-ketothiolases, enzyme A and enzyme B, have been discovered to take part in biosynthesis of PHA. The major difference between the two enzymes is substrate specificity. Enzyme A is a homo-tetramer of 44-kDa subunits and converts acetoacetyl-CoA and 3-ketopentanoyl-CoA to P(3HB) and P(3HV) respectively. On the contrary enzyme B, a homo-tetramer of 46 kDa subunits, has broad substrate specificity and cleaves acetoacetyl-CoA, 3-ketoheptanoyl-CoA, 3-ketooctanoyl-CoA, 3-ketodecanoyl-CoA (Madison and Huisman 1999). Though it was earlier thought that enzyme B is associated with the fatty acid degradation while enzyme A actively participated in P(3HB) biosynthesis (Haywood 1998a), Slater *et al*. (1998) have shown that enzyme B is the primary catalyzer for the P(3HB-3HV) formation.

The enzymatic mechanism of β-ketothiolase consists of biological Claisen condensation of two acetyl-CoA-moieties wherein the formation of carbon-carbon bond is catalyzed by β-ketothiolase (EC.2.3.1.9). In this enzymatically deprotonated anion derived from acetyl-CoA reacts as a nucleophile with an electrophilic acetyl-s-enzyme intermediate derived from the binding of acetyl-CoA to the active cysteine of thiolase (Masamune *et al*. 1989). Two cysteines are supposed to take part in the above reaction. With the cloning, overexpression and purification of thiolase genes of *Z. ramigera*, definitive roles of the cysteines (**Cys 89** and **Cys 378**) were determined (Davis *et al*. 1987; Masamune *et al*. 1989; Thompson 1989) and all the thiolases reported till date seem to contain the two cysteines.

1.3.2 Acetoacetyl-CoA reductase

This is a (R)-3-hydroxyacyl-CoA dehydrogenase. Like ketothiolase, it is also a soluble protein. It catalyzes second step in the P(3HB) biosynthetic pathway by stereo-selective reduction of acetoacetyl-CoA formed by β-ketothiolase to 3-hydroxybutyryl-CoA. Two types of reductases are found in organisms (Haywood

et al. 1988a, 1988b). First type is a NADH dependant reductase, EC.1.1.1.35, while the second type, EC.1.1.1.36, is a NADPH dependant. The former is a tetramer with identical subunits of 30 kDa and plays role in the β-oxidation of fatty-acids, and in association with thiolase (EC.2.3.1.16) participates in the conversion of butyrate, crotonoate and β-HB to acetyl-CoA. The latter is a homo-tetramer of 25 kDa subunits and participates in the biosynthesis of P(3HB) by reduction of acetoacetyl-CoA to 3-HBCoA (Fukui *et al*. 1987; Ploux *et al*. 1988; Schembri *et al*. 1994). In contrast to NADPH-dependant acetoacetyl-CoA reductase which uses exclusively D(-) isomer of β-HA-CoA, the NADH-dependant enzyme uses preferentially but not exclusively L(+) isomers as substrates for the oxidative direction of catalysis (Steinbüchel and Schlegel 1991). The only known NADH-dependant acetoacetyl-CoA reductase aiding in PHB synthesis has been reported from *A. vinosum* (Leibergesell and Steinbüchel 1992) and *S. coelicolor* (Packter and Flatman 1983).

1.3.3 P(3HB) synthase

The last reaction in the polymer formation is catalyzed by the enzyme PHB synthase which links D(-)-β-hydroxybutyryl moiety to an existing polyester molecule by an ester bond. This key enzyme determines the type of PHA synthesized. PHB synthase is soluble only as long as no PHB synthesis and accumulation occurs in an organism. It, however, becomes granule associated under storage conditions (Fukui *et al*. 1976, Haywood 1989a). The first synthase gene isolated, cloned and expressed was from *R. eutropha* (Peoples and Sinskey 1989a). It prefers C_4 substrates leading to the formation of poly-β-hydroxybutyrate.

P(3HB) polymerase is a member of the PHA polymerases with approximate molecular mass of 64 kDa except for the polymerases of *A. vinosum* (Liebergesell *et al*. 1994), *T. violacea* (Liebergesell and Steinbüchel 1993) and *Synechococcus* sp. (Kaneko *et al*. 1996; Hein *et al*. 1998) (introduced later in the section). To date almost 50 PHA synthase structural genes from various Gram positive, Gram negative as well as cyanobacteria have been cloned and the nucleotide sequence of 36 genes has been obtained (Rehm and Steinbüchel 1999; Steinbüchel and Hein 2001). Three different types of PHA synthases have been identified.

1.3.3.1 Types of PHA synthases

Based on the primary structure and substrate specificities PHA synthases have been Classified into three different Classes (Fig. 1.2 and Steinbüchel *et al*. 1992). With respect to size and structure, type I and type II are distinguished from type III synthases. While type I and type II synthases are homodimeric, type III synthases are heterodimers (Steinbüchel and Hein 2001).

The type I synthases include those that are active towards HA_{SCL} monomers. The HA_{SCL} monomers are defined as the (R) enantiomers of 3-, 4-, and 5- HA containing 3-5 carbon atoms. The prototype synthase of this group is the PHB synthase from *R. eutropha* (*pha*C_{Re}). It has a MW of 64 kDa and exists as a monomer in equilibrium with its dimer in solution. It occurs in the phototrophic nonsulfur purple bacteria and in most heterotrophic bacteria except the Pseudomonads belonging to the rRNA homology group I (Steinbüchel and Hein 2001). Recently, it has been shown that PHA synthase of *R. eutropha* can also incorporate small amounts of 3HHx, 3HO and 3HDD units (Dennis *et al*. 1998; Antonio *et al*. 2000). PHA synthases of *A. cavie* (Fukui and Doi 1997), *Rhodospirillum rubrum* (Brandl *et al*. 1989), *Rhodococcus ruber* (Haywood *et al*. 1991), *Rhodocyclus gelatinosus* (Matsusaki *et al*. 2000) apparently prefer HA_{SCL} but can also incorporate 3HHx monomers. *R. gelatinosus* PHA synthase can also incorporate 3HHp when grown on heptanoic acid (Liebergesell *et al*. 1991).

Class II or type II synthases are represented by the two PHA synthases of *Pseudomonas oleovorans*. Like their Class I counterparts these are composed of only one type of subunit with an apparent molecular mass of 69 kDa and are shown to exist in equilibrium of monomer and dimer. Each of these synthases can function independently and exhibit similar properties resulting in similar extent of PHA accumulation (Qi *et al*. 1997). These incorporate efficiently larger (R)-3HA monomers containing 6-14 carbon atoms (HA_{MCL} monomers). *In vivo* these substrates are derived mainly from intermediates of fatty acid oxidation (Huijberts *et al*. 1994; Langenbach *et al*. 1997; Qi *et al*. 1998) or from fatty acid *de novo* biosynthesis (Rehm *et al*. 1998; Fiedler *et al*. 2000). It has been shown that the synthases of *Pseudomonas* sp. 61-3 incorporate HA_{SCL} (3HB units) when expressed in

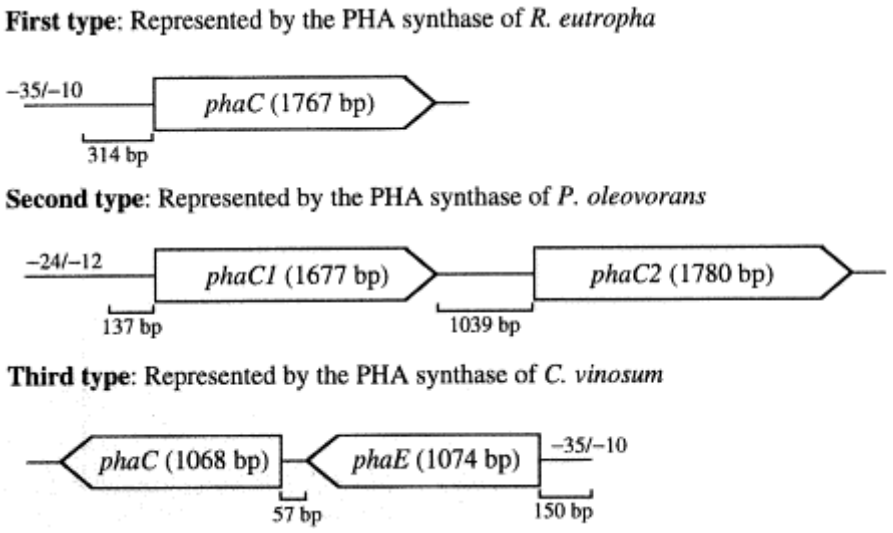

Fig. 1.2: Types of PHA synthases (Sudesh *et al*. 2000)

R. eutropha PHA negative mutants (Matsusaki *et al*. 1998, 2000). Recently Class II PHA synthases have been purified by metal chelate affinity chromatography and *in vitro* activity has been achieved (Qi *et al*. 2000).

The Class III PHA synthases represented by *Allochromatium vinosum* (earlier *Chromatium vinosum*) are composed of two subunits of 40 kDa each. While one of the subunits (*pha*C subunit) shows an amino acid similarity of 21-28% to Class I and Class II synthases, the other subunit (*pha*E) does not show any sequence similarity. The substrate specificity is similar to that of Class I synthases except for *T. pfennigii* synthase which shows substrate specificity to HA_{MCLs} and shows 85% (both the subunits) similarity to type I PHA synthase (Liebergesell *et al*. 1993). Both the subunits have been purified and found to form a complex of 80 kDa which further aggregates to form trimers and hexamers in solution. Other synthases of this group are from *Thiocystis violacea* (Liebergesell and Steinbüchel 1993) and *Synechocystis* sp. (Hein *et al*. 1998). Both Class I and Class III enzymes show a biphasic kinetics. Class I synthases show a lag phase followed by a more rapid phase unlike Class III enzymes wherein a rapid phase is followed by a slower phase.

The PHA synthases show no significant homologies to the other proteins in

data banks. Extensive comparison of the 36 PHA synthases reveals 21-28% amino acids identity among themselves. Interestingly, there are only 8 fully conserved amino acid residues among the known PHA polymerases (Madison and Huisman 1999; Steinbüchel and Hein 2001). This is remarkable, as this does not even form 3% of the total amino acids in these enzymes. This might be due to the vast spectrum of intracellular conditions to which the synthases would have adapted. It has also been found that N-terminal region (about 100 AA relative to type I PHA synthases) is highly variable. It is also dispensable as revealed in *R. eutropha* wherein synthase lacking 36 to100 amino acids did not show loss of active function (Schubert *et al.* 1991).

Based on the substrate specificities it became evident that PHA synthases represent a class of highly homologous enzymes (Steinbüchel *et al*. 1992). The possibilities of limitations imposed by metabolic pathways that supply monomer units in a particular environment cannot be overlooked. A good example for this is *T. pfennigii* PHA synthase, which shows an unusual substrate range when heterologously expressed in a PHA (–) ve mutant of *P. putida* (Liebergesell *et al*. 1991, 1993; Valentin *et al*. 1994; Steinbüchel and Valentin 1995). *T. pfennigii* PHA synthase conferred upon *P. putida* the capability to synthesize equimolar amounts of P(3HB) and P(3HHx) plus a small amount of P(3HO). Also, the establishment of a functionally active PHA biosynthesis pathway in *R. eutropha* $PHB^{-}4$ mutant, *P. oleovorans* and *P. putida* suggesting alternative routes for 3-HB-CoA. In contrast, PHA biosynthesis pathway was not established in *E. coli* probably due to the lack of β-ketothiolase and acetoacetyl-CoA reductase (Liebergesell *et al*. 1993).

To date the main hindrance in the study of PHA synthase has been its non-availability in pure state as also due to limited availability of substrate monomers in the form of Coenzyme A thioesters (Haywood *et al*. 1989). Recent cloning, over-expression and purification of PHA synthases from *R. eutropha* (Gerngross *et al*. 1994) and *Allochromatium vinosum* (Liebergesell *et al*. 1994) have facilitated detailed examination of their substrates *in vivo* as well as *in vitro*. Comparison between three Classes of PHA synthases has been summarized in Table 1.5.

Table 1.5: Comparison between three Classes of PHA synthases.

	Class I	Class II	Class III
Prototype organism	*R. eutropha*	*P. oleovorans*	*A. vinosum*
Substrate specificity	HA $_{SCL}$	HA $_{MCL}$	HA $_{SCL}$
Subunits	One subunit	One subunit	Two subunits
Active form	Dimer	Dimer	Trimer or Hexamer
Kinetics	Biphasic	Not available	Biphasic
Mode of action	Lag phase followed by a rapid phase	Not available	Rapid phase followed By a slow phase

1.3.3.2 Mode of Action

PHA synthase studies involving various sulfahydryl inhibitors have suggested that these are sulfahydryl enzymes (Griebel *et al*. 1968). Ballard and coworkers (1987) proposed a model with two thiol groups being involved in locating HA monomers. It was later modified by Kawaguchi and Doi (1992) stating that water may act as a chain transfer agent. The modified model was contradicted based on the assumption that the PHA synthase is not capable of transferring to a new chain (Gerngross and Martin 1995; Su *et al*. 2000). However, the proposed model was purely hypothetical.

The nucleotide sequences of PHA synthases show a conserved lipase box with (**G-X-[S/C]-X-G**) sequence (Steinbüchel *et al*. 1992) in which the essential active **serine** of the lipasebox is replaced with cysteine. The successful purification of *R. eutropha* (Gerngross *et al*. 1994) and *Allochromatium vinosum* (Liebergesell *et al*. 1994) PHA synthase enhanced biochemical and mechanistic studies. Studies involving *A. vinosum* PHA synthase using protein homology modeling and site-directed mutagenesis revealed this enzyme to be a member of the prokaryotic lipase superfamily (Jia *et al*. 2000). Which in turn is a member of the α/β hydrolase family (Ollis *et al*. 1992; Heikinheimo *et al*. 1999) and not a β-ketosynthase family member (fatty acid synthase) as previously proposed (Bohm *et al*. 1992; Berdichewsky *et al*. 1999).

Lipases have been proposed to require the catalytic triad, **Ser-His-Asp**, for triacylglycerol hydrolysis (Wodzinska *et al*. 1996; Rogl *et al*. 1998; Rehm *et al*. 2001) though the actual role of **Asp** has not been established. Based upon the above findings and the mutagenesis studies conducted in *A. vinosum* catalytic triad involving **C149**, **H331** and **D302** has been proposed. **C149** forms the nucleophilic center for covalent catalysis wherein it is acylated. **H331** is shown to act as the general base catalyst that activates the nucleophile **C149** for covalent catalysis. Studies with **D302** mutants show that it could act as a general base catalyst by deprotonating the 3-hydroxyl of HBCoA or that of the bound 3-hydroxybutyrate to render possible nucleophilic attack on the covalently linked thiol ester intermediate (Jia *et al*. 2000). However, the exact function of **D302** is yet to be established.

A model has been proposed based on fatty acid synthases for *R. eutropha* (Rehm and Steinbüchel 1999) wherein two thiol groups, **C149** and **S260** have been shown to take part in catalytic cycle of polymerization involving posttranslational modification at **S260**. However, post-translational modifications have been ruled out as the PHA synthases have been expressed in various organisms from different kingdoms. Recent mechanistic studies on *R. eutropha* synthase by Jia *et al*. (2001) show PHA synthase to possess a similar catalytic mechanism as enumerated above for *A. vinosum* synthase. Here, the amino acids **C319**, **H508** and **D480** have been shown to form the catalytic triad. Mutagenic studies by Rehm *et al*. (2002) has shown that a highly conserved **W425** may play a role in protein–protein interaction *i.e*. in the dimerization of the synthase sub-unit by generating a hydrophobic surface. Mutations to **E267**, **Y445** and **L446** have shown reduced activity of the synthase without changing the substrate specificity thus, indicating a functional role of these residues.

Taguchi *et al*. (2002), applied *in vitro* evolution by mutations to get a highly active mutants of *R. eutropha* PHB synthase. Mutation of **S80** to **Proline** resulted in reduced activity while the mutant with a **Phenylalanine** at position **420** to **Serine** had substantially higher activity. When the gene with **F240** converted to **Serine** was over-expressed the PHA synthase enzyme and the specific activity was found to have increased 2.4 folds towards 3HB-CoA compared to the wild type. However, in *pha*C_{Re} and *pha*C_{Av} exchange of active **Cysteine** nucleophile in the lipase box with any other

residue including **Serine** resulted in the loss of enzyme activity.

It could be thus, concluded that Class I and Class III synthases are mechanistically similar and structurally homologous, despite their physical and kinetic differences. Mechanistic studies involving Class II synthases are yet to be investigated.

1.3.3.3 Structural studies of PHA synthases

With a number of PHA synthase nucleotide sequences available, the secondary structure prediction based on multiple sequence alignment indicated with an expected accuracy of about 72%, shows that PHA synthases are mainly composed of variable loops amounting to 49.7%, while the α-helical and the β-sheets amounting to 39.9% and 10.4% respectively (Rost and Sander 1993, 1994).

As reported these PHA synthases exist in an equilibrium of monomeric and dimeric forms (Class I and II) (Rehm and Steinbüchel 1999; Rehm *et al*. 2001). It was observed that dimerization is significantly induced in the presence of substrate i.e 3HB-CoA and of trimeric CoA analogs of the substrate i.e $(3HB)_3$–CoA (Rost and Sanders 1993, 1994). However, enzymatic lag phase is reduced and the specific activity increased in the presence of trimeric analogs (Wodzinska *et al*. 1996). Thus, it was concluded that the dimeric form is substantially more active than the monomeric form which was confirmed by Wodzinska *et al*. (1996). They observed that labeled trimeric-CoA analogs were covalently bound to the dimeric form of PHA synthase as indicated by size exclusion chromatography. Immunoelectron microscopy studies on *A. vinosum* PHA synthase have shown the presence of PHA synthase complexes comprising of 10 sub-units at the surface of the PHA granule (Liebergesell *et al*. 1994; Meyer *et al*. 1996). Hence apparently *in vitro* PHA synthase consisted of 2 sub-units and is composed of 10 sub-units while associated with PHA granule *in vivo* (in *A. vinosum*).

The most urgent needs for research from a biological point of view and the significance of the studies are given in Table 1.6. With the *in vitro* synthesis of PHAs consisting of 3HB / 3HV monomer units by purified enzymes from *R. eutropha*

Table 1.6: Academic and Technical relevance of research studies (Rehm 1999)

	Academic	Technical
Tertiary and Quaternary structure of PHA synthases	High	Medium
Structure-function relationships	High	High
Analysis of intracellular degradation	High	Low
Structure of cytoplasmic PHA inclusions	Medium	Low
Analysis of the molecular basis for biosynthesis	High	High
Metabolic engineering pathways	Medium	High
Scale up of *in vitro* synthesis	Low	High
Scale up of fermentative production	Low	High
Transformation of plants for PHA production	High	High
Environmentally friendly extraction process	Low	High

(Gerngross and Martin 1995) and *A. vinosum* (Jossek *et al.* 1998; Jossek and Steinbüchel 1998) promising approaches towards obtaining a better PHA could be achieved in association with polymer chemists and engineers.

1.3.4 Phasins

Phasins are proteins (PhaP) often found associated with PHA biosynthesis. These are found attached to the outer membrane of the PHA granule. In analogy to oleosin proteins at the surface of lipid inclusions in plant cell and having a structural function homologous to that of latter these proteins are referred to as phasins (Murphy 1993; Steinbüchel *et al*. 1995). Detailed studies have been done on the phasins of *R. ruber* ($PhaP_{Rr}$) (Pieper *et al*. 1995) and *R. eutropha* (Wieczorek *et al*. 1995; Hanley *et al*. 1999; York *et al*. 2002).

Phasins provide an amphiphilic layer between the hydrophobic core consisting of PHAs and the mostly hydrophilic components of the cytoplasm. All phasins contain atleast one markedly hydrophobic region, which is proposed to take part in direct binding and anchoring of the protein to the PHA granules (Hanley *et al*. 1999).

Recombinant strains of *E. coli* which expressed P(3HB) biosynthesis pathway in the absence of the phasin or in the presence of a truncated phasin protein, accumulated a single large granule of PHA instead of many small granules. This might have a significant effect on the surface area of the catalytically active proteins such as PHA synthases / PHA depolymerase which catalyze the polymerization or depolymerization of PHAs respectively. The accumulation of PhaP protein in *R. eutropha* is dependent on the production of PHB in cells suggesting an evolved regulatory mechanism that can detect the synthesis and presence of PHB in cells, thus acting as a marker (York *et al*. 2001). Whether these contribute to the regulation of PHA production is not yet known (Steinbüchel and Hein 2001).

Phasin genes are arranged in a variety of fashion in various organisms (see Fig. 1.4) (Liebergesell and Steinbüchel 1992; Liebergesell *et al*. 1992; Schembri *et al*. 1994; Maehara *et al*. 1999; McCool and Cannon 1999). In summary, these studies indicate an important, but not essential function of phasins for metabolism of PHA.

1.3.5 PHA depolymerases

As PHA is a storage compound for excess carbon in microbial cells, naturally a mobilization system should exist to recover the stored carbon. Several research groups have investigated the detailed cyclic metabolism of P(3HB) (Doi 1990; Anderson and Dawes 1990; Steinbüchel 1991). In contrast to PHA synthase, the intracellular PHA depolymerase(s) are not very well characterized.

Early studies on enzymatic depolymerization of PHA inclusions involved the utilization of soluble enzymes from *R. rubrum* to degrade native P(3HB) inclusions isolated from *B. megaterium* (Merrick and Doudoroff 1964). In *Z. ramigera* also the PHB depolymerase activity was found in the soluble enzyme fraction when assayed with protease treated P(3HB) inclusions. This led to the suggestion that PHA inclusions may be protected from attack by the depolymerase by proteins on the surface (Saito *et al.* 1992).

The intracellular degradation of PHB in *R. eutropha* is very slow compared to its synthesis (Hippe and Schlegel 1967; Doi *et al*. 1992). During the degradation

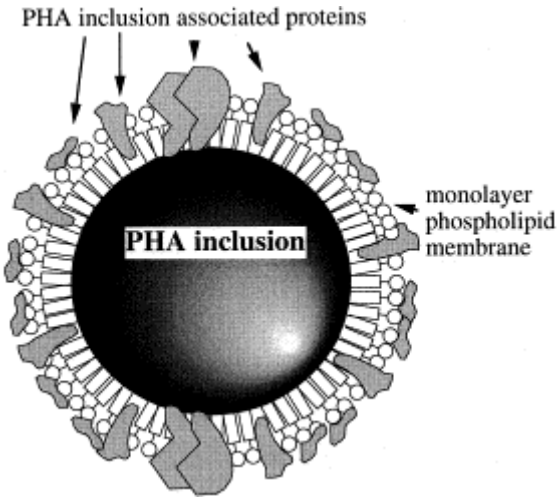

Fig 1.3: Model of a PHA granule (Sudesh *et al*. 2000)

process the number of polymer chains remain almost constant suggesting exo-type hydrolase acting at the carbonyl terminus of the polymer chain (Doi *et al*. 1992). These intracellular depolymerases are characterized by the presence of the **G-X-S-X-G** lipase box. *P. oleovorans* and *P. aeruginosa* are also reported to have PHA depolymerases which are probably intracellular depolymerases (Steinbüchel *et al*. 1995). No concrete mechanism for their action has been established so far.

Extracellular degradation is the utilization of an exogenous polymer by not a necessarily accumulating microorganism that secretes extracellular PHA depolymerase. Many extracellular PHB depolymerases have been isolated and their mode of action have been detailed (Jendrossek and Handrick 2002). The lipase box pentapeptide mentioned above acts as the active site here also. These extracellular depolymerses get adsorbed on to the insoluble PHB first and then hydrolyze the polymer chains. Enzymatic hydrolysis of P(3HB) results in 3HB dimer as the major product besides small amounts of 3HB monomer. These cleave mainly the second and third ester linkages from the hydroxyl terminus (Sudesh *et al*. 2000). These have been discussed in Chapter 6.

1.4 Model for PHA granule

Lundgren *et al*. (1964) proposed that PHA inclusions are associated with proteins on their surfaces. Inclusion bodies of *B. megaterium* were composed of 97.7% P(3HB), 1.87% protein and 0.46% lipids (Griebel *et al*. 1968). It is now established that PHA inclusions consist of a hydrophobic core of amorphous PHA (Barnard and Sanders 1989) surrounded by phospholipid monolayer membrane consisting of various catabolic and non-catabolic proteins (Fig. 1.3) which include a group of proteins designated as phasins (PhaP) (Steinbüchel *et al*. 1995). Electron microscopic analysis of freeze fractured monolayer membranes has revealed the thickness of surface layer to be 3 - 4nm which is half the diameter of the cytoplasmic unit membrane which has two layers of phospholipids (Mayer and Hoppert 1997). A granule with a diameter of 350nm will, for example, contain approximately 40,000 poly-3- hydroxybutyrate molecules if the MW is approximately 3 x 10^6 g mol^{-1} (Steinbüchel *et al*. 1995).

1.5 Essential genes for PHA Biosynthesis

Since the cloning of the PHA operon of *Ralstonia eutropha* approximately 13 years ago, more than 50 PHA synthase genes and other structural genes related to PHA biosynthesis from various bacteria have been cloned and analyzed at molecular level. These genes comprise of structural genes for:

(i) PHA synthase,
(ii) granule associated proteins,
(iii) enzymes which catalyze the formation of hydroxyacyl-CoA thioesters and
(iv) proteins with regulatory functions.

Based on the molecular data available seven different types of arrangements (Fig 1.4) have been observed for PHA synthesizing genes (Madison and Huisman 1999; Steinbüchel and Hein 2001).

1.5.1 Terminology

Genes encoding proteins involved in PHA biosynthesis are referred in alphabetical order as *pha*A (β-ketothiolase), *pha*B (acetoacetyl-CoA reductase), *pha*C (PHA synthase), *pha*G (3-hydroxy-acylcarrier protein Co-enzyme A transeferase), *pha*J (enoyl CoA hydratase) etc. The gene required for degradation of PHA are referred to in the reverse alphabetical order as *pha*Z (PHA depolymerase), *pha*Y, *pha*X, *pha*W etc. The genes for phasins whose expression is dependent upon PHA biosynthesis are termed as *pha*P. Other proteins which are granule-associated or aid in regulation or are of unknown function, are referred to as *pha*F, *pha*I, *pha*Q, *pha*R etc. The origin of a gene or a protein is indicated by the first letter of the genus and the species and added as subscript *e.g*. PHA synthase of *R. eutropha* is written as *pha*C_{Re} (in case of gene) and PhaC_{Re} (in case of the gene product).

1.5.2 Organization of the genes

In *Ralstonia eutropha*, *Burkholderia cepasia*, *Acinetobacter* sp., *Alcaligenes latus* and *Pseudomonas acidophila* the three genes involved in PHA biosynthesis are arranged in tandem although not necessarily in same order (Peoples and Sinskey 1989a, b; Schembri *et al*. 1994; Umeda *et al*. 1998). As already reported, *R. eutropha* genes are arranged as *pha*CAB while in *Acinetobacter* sp. they are arranged as *pha*BCA. In *Z. ramigera*, *Rhizobium melioti* and *Paracoccus denitrificans* the *pha*AB and *pha*C loci are separated (Peoples and Sinskey 1989c; Tombolini *et al*. 1995; Yabutani *et al*. 1995; Lee 1996; Ueda *et al*. 1996).

PHA polymerase in *Allochromatium vinosum*, *Thoicystis violacea* and *Synechocystis* is a two subunit enzyme encoded by *phb*C and *phb*E genes. In these organisms *phb*AB *and phb*EC are in one locus but, divergently oriented (Liebergesell and Steinbüchel 1992, 1993; Park *et al*. 1997; Oldenburg *et al*. 2000). So far in *Methylobacterium extorquens*, *Nocardia corallina*, *Rhizobium etli*, *Rhodococcus ruber* and *Rhodobacter sphaeroides* only PHB polymerase encoding gene has been identified (Pieper and Steinbüchel 1992; Hustede and Steinbüchel 1993; Valentin and Steinbüchel 1993; Cevellos *et al*. 1996; Hall *et al*. 1998). The PHA polymerase in *Aeromonas caviae* is flanked by a unique PHA biosynthesis enzyme encoded by *pha*J

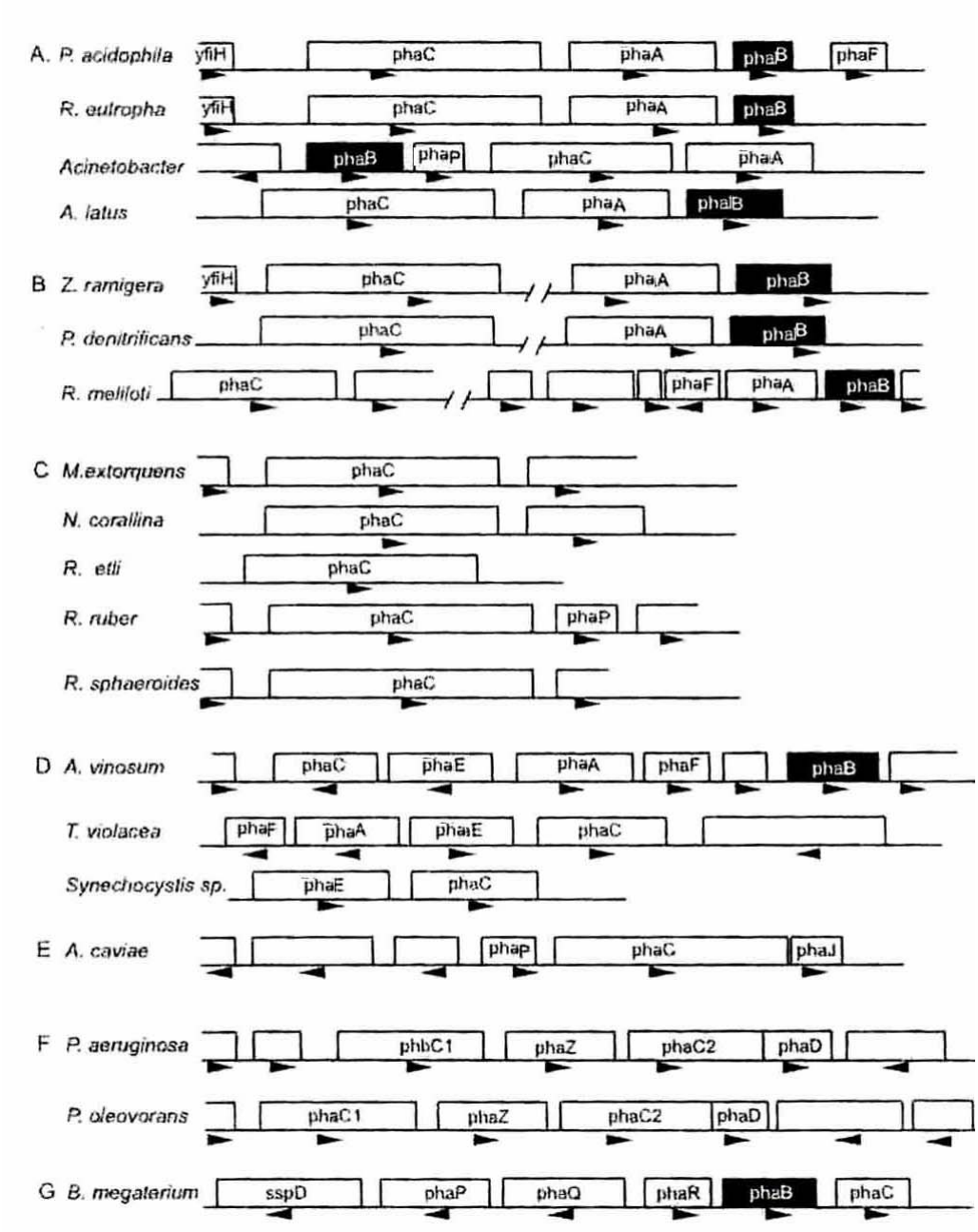

Fig 1.4: Organization of PHA biosynthesis and related genes

(modified from Madison and Huisman 1999)

gene where in (R)-3-hydroxyacyl-CoA monomer units are supplied via the (R)-specific hydration of enoyl-CoA (Fukui and Doi 1997). In MCL-PHA producing *P. oleovorans and P. aeruginosa*, the *pha* loci contain two *pha*C genes (Huisman *et al*. 1991, Timm and Steinbüchel 1992) separated by the *phaZ* gene encoding for an intracellular depolymerase. Recently, Hang *et al*. (2002) have shown *Burkholderia caryophylli* to possess two synthases genes interspersed with *phaZ* gene as in *Pseudomonas*.

In *Bacillus megaterium*, the *pha*C gene is similar to that of *Allochromatium vinosum* in size and sequence. However, unlike *pha*C_{Av}, the *pha*C_{Bm} requires *pha*R_{Bm} for PHA accumulation but not *pha*E (McCool and Cannon 1999, 2001). *pha*R_{Bm} is probably an orthologous replacement for *pha*E_{Bm}.

1.6 PHA Biosynthesis

Since the finding that other hydroxyalkanoate monomers besides 3HB may be integrated into PHA formation, we now know that a wide variety of monomers form the constituents of PHA because of PHA synthases substrate specificity. In the recent years it has been shown that PHA can be synthesized by microorganisms utilizing various carbon sources ranging from inexpensive complex waste effluents like beet or cane molasses (Hangii 1990; Page 1992) to plant oils (Fukui and Doi 1998) and its fatty acids (Eggink *et al*. 1995; Tan *et al*. 1997), alkanes (Lageveen *et al*. 1988) as well as simple carbohydrates. Based on the monomer incorporated, various metabolic pathways have been shown to be involved (Steinbüchel 1991; Madison and Huisman 1999).

Three major biosynthesis pathways have been elucidated as under which are illustrated in Figure 1.5.

(i) Biosynthesis pathway (Pathway I) represented by *R. eutropha* and

(ii) Biosynthesis (Pathways II and III) represented by Pseudomonads.

1.6.1 Biosynthesis pathway represented by *R. eutropha*

The enzymes involved in this biosynthesis pathway have been extensively studied (Anderson and Dawes 1990). The monomer for *pha*C is provided by the

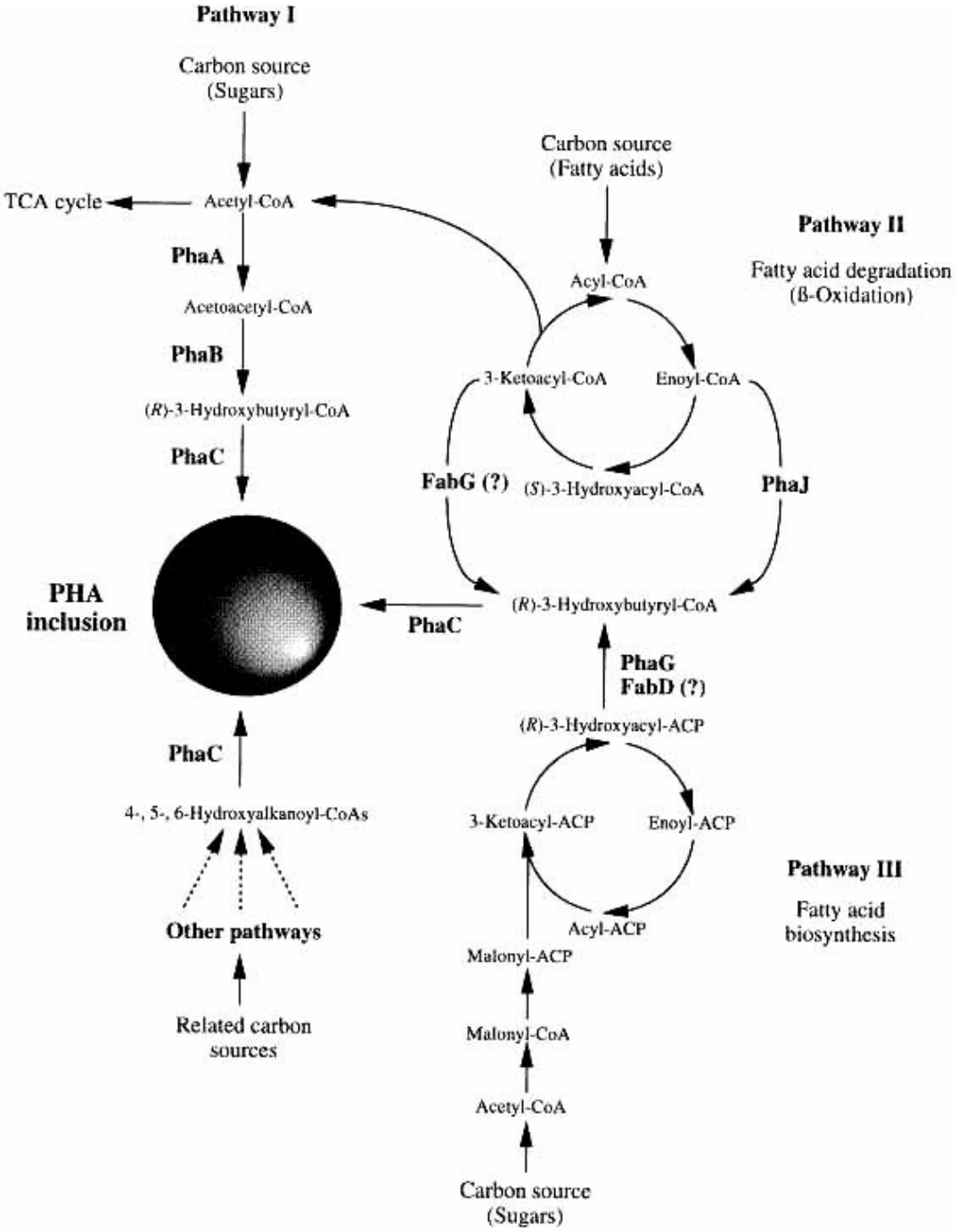

Fig. 1.5 Biosynthesis pathways for PHA production

(Sudesh *et al*. 2000)

action of β-ketothiolase and acetoacetyl-CoA-reductase. On the other hand, *R. rubrum* which shows similar PHA biosynthesis pathway as *R. eutropha*, the reductase gives rise to (S)-isomer of 3-HB-CoA. This is converted to (R)-type by two enoyl-CoA hydratases (Moskowitz and Merrick 1969). The (R)-3HB-CoA is then converted to (R)-P(3HB) by PHA synthase. In *Aeromonas caviae* the (R)-specific enoyl-CoA hydratase (PhaJ) is involved in the production of P(3HB-co-3HHx) (Fukui and Doi 1997; Fukui *et al*. 1998).

Various carbon sources ranging from simple carbohydrates to plant oils act as substrates for the synthesis of PHA in these bacteria. However, it is observed that irrespective of the substrate provided the PHA monomer incorporated was always between C_3-C_5. *R. eutropha* accumulates copolymer of 3HB and 3HH when grown on substrates with odd number of carbon atoms (Akiyama *et al*. 1992). It is also capable of accumulating PHA from 4-HB, γ-butyrolactone and 1,4-butanediol which gives rise to P(3HB-co-4HB) copolymer (Doi *et al*. 1989; Doi *et al*. 1990).

1.6.2 PHA biosynthesis represented by Pseudomonads

This pathway is exhibited in Pseudomonads (*P. oleovorans)* belonging to rRNA homology group I. These synthesize PHA_{MCL} from various alkanes, alkanols or alkanoates (Lageveen *et al.* 1988), and generally do not synthesize PHA_{SCL}. In this group 3-hydroxyacyl CoA substrates of C_6-C_{14} are obtained from intermediates of fatty acid β-oxidation pathway (Fig 1.5 pathway II). Interestingly the monomer composition of the PHA is a reflection of the substrates provided (Lageveen *et al*. 1988). Even unsaturated monomers are incorporated when carbon sources are 1-alkenes (Lageveen *et al*. 1988). Specific enzymes like enoyl-CoA hydartases (PhaJ) (Fukui *et al*. 1999) and 3-ketoacyl-CoA-reductase (FabG) (Taguchi *et al*. 1999) are presumably involved in the conversion of fatty acid β-oxidation intermediates to suitable monomers to be polymerized by PHA synthase.

In contrast all other Pseudomonads belonging to rRNA homology group I except *P. oleovorans*, synthesize PHA containing MCL monomers from unrelated carbon sources, such as gluconate, glycerol, fructose, acetate or lactate (Anderson and Dawes 1990). Mainly PHA containing 3-hydroxydecanoate (3HD) as the predominant

monomer is accumulated. The 3-hydroxyacyl monomers are derived from the *de novo* fatty acid biosynthesis pathway (Huijberts *et al*. 1994) (Fig. 1.5 pathway III). Usually (R)-3-hydroxyacyl-ACP is formed as a fatty acid biosynthesis intermediate. PhaG (3-hydroxyacyl-CoA-ACP transferase) converts it into (R)-3-hydroxyacyl-CoA. *P. aeruginosa*, *P. aureofaciens*, *P. citronellis*, *P. mendocina* and *P. putida* are shown to possess this pathway. Even over expression of transacylating enzyme such as malonyl-CoA-ACP transacylase (FabD) also seems to generate monomers for PHA biosynthesis (Taguchi *et al*. 1999).

1.6.3 Factors effecting Molecular weight of PHAs

As commodity materials, molecular weight of PHA accumulated by bacteria is of significance. Since the PHAs have to be purified from bacterial cells, the molecular mass depends upon the purification procedure. As such determination of true molecular weight of PHA is not possible. But, GPC is usually employed for MW determination. Usually polystyrene with low polydispersity index is used as the standard to construct the calibration curves (Doi 1990).

The molecular weight of the PHAs depends on several factors:

1) the physiological background of the organism with respect to the substrate available for PHA synthase,
2) presence of PHA depolymerases (Jendrossek *et al*. 1996) or unspecific esterases (Mukai *et al*. 1993; Jaeger *et al.* 1995),
3) level of expression of active PHA synthase,
4) pH of the medium and
5) the extraction procedure.

In *Pseudomonas* and recombinant *E. coli* harboring its genes an increase in the activity of PHA synthase resulted in a decrease in the number average molecular weight (Mn) of the PHA produced (Sim *et al*. 1997; Huisman *et al*. 1992). In contrast, no difference was observed in the molecular weight of PHA produced by *C. acidovorans* and the recombinant *E. coli* harboring its PHA biosynthesis genes. However, with the increase in the copy number of PHA synthase gene in *Comamonas*

acidovorans, a concomitant increase in PHA synthase activity was observed with enhanced production of P(4HB) from 4-hydroxy butyric acid (Sudesh *et al*. 1999). Heterologous expression of *R. eutropha* PHA synthase gene resulted in accumulation of higher molecular weight species of PHB. The molecular weight of the P(3HB) produced by recombinant *R. eutropha* was found to be independent of the levels of the synthase activity (Kichise *et al*. 1999).

A low molecular weight, non-storage PHA species referred to as cP(3HB) ('c' for complexed) is also present in microorganisms. This is associated with other macromolecules and is widely distributed in all biological phyla (Reusch 1995; Huang and Reusch 1996). As such the control of MW remains unknown.

1.7 Reglulation of PHB biosynthesis

Although various aspects of PHA production have been studied extensively, knowledge of the regulatory mechanism at the molecular level is relatively limited. Regulation of PHA metabolism can take place at different levels:

(i) activation of *pha* gene expression due to specific environmental signals, such as nutrient stress etc.,

(ii) activation of the PHA synthase enzymes by specific cell components or metabolic intermediates,

(iii) inhibition of metabolic enzymes of competing pathways and therefore enrichment of required intermediates for PHA synthesis and

(iv) a combination of these.

1.7.1 Regulation at enzymatic level

The three key enzymes, which play a central role in PHB synthesis, regulate polymer synthesis (Senior and Dawes 1971; Haywood *et al*. 1988a, b; Mothes *et al*. 1997). High NAD(P)H / NAD(P) ratio *in vivo* reduces the citrate synthase activity which facilitates the metabolic flux of acetyl-CoA to PHB biosynthesis pathway (Lee 1995). Thus citrate synthase activity can control the availability of CoA (Henderson and Jones 1997). Kessler and Witholt (2001) have shown a faster rate of PHB accumulation in a *R. eutropha* isocitrate-dehydrogenase leaky mutant. These findings

underline the importance of redox balance in the cell to accumulate PHB. PHB accumulation rate *in vivo* is controlled by β-ketothiolase condensing acetyl-CoA produced from glycolysis to acetoacetyl-CoA or acetoacetyl-CoA reductase reducing acetoacetyl-CoA to 3-hydroxybutyryl-CoA in recombinant *R. eutropha* (Jung *et al*. 2000). However, the PHB synthase is the most critical enzyme in determining the accumulation of intracellular PHB. In *Synechocystis* sp. acetylphosphate could act as a signal of C / N balance affecting PHB metabolism (Miyake *et al*. 1997)

In *Pseudomonas* Qi *et al*. (1998) have shown that inactivation of β-oxidation process by acrylic acid leads to PHB accumulation. Alternatively, *E. coli* mutant deficient in enzymes of the β-oxidation process but containing PHA polymerase gene is found to accumulate PHAs (Langenbach *et al*. 1997) suggesting that PHA polymerase competes with the enzymes of β-oxidation for substrates.

1.7.2 Regulation at transcription level

It is not known whether any specific protein(s) are involved in the *pha* gene expression. However, regulatory proteins like PhaF (Timm and Steinbüchel 1992; Prieto *et al.* 1999), PhaR (York *et al*. 2001, 2002), PhaS (Madison and Huisman 1999), GacS (Castaneda *et al*. 2000) etc. are suggested to take part in PHB biosynthesis regulation. Miyamoto *et al.* (1998) have shown the LuxR regulatory protein in *Vibrio harvey* to control both bioluminescence and PHB biosynthesis.

1.7.3 Regulation of PHA biodegradation

In general it is assumed that the synthesis and depolymerization of PHA is highly regulated. Most PHA degrading bacteria represses PHA depolymerase gene expression in presence of soluble carbon source and is de-repressed once the carbon source is exhausted (Jendrossek 1993; Schirmer *et al.* 1993; Foster *et al.* 1995). However, Doi *et al.* (1992) have reported that the PHA depolymerase is always active and that PHA is synthesized and degraded simultaneously. However, with inactivation of the genes *phb*I and *phb*H, encoding homologues of *E. coli* PEP-PTS system (Phosphoenolpyruvate-sugar phosphotransferase system), rate of PHB degradation increased suggesting that depolymerases are controlled by

Table 1.7: Heterologous expression of PHA biosynthesis genes (Steinbüchel and Hein 2001)

Host organism	Foreign genes	PHA accumulated
Aim: Identification of PHA synthase genes		
A. caviae PHA-	$phaC_{Ac}$, $phaJ_{Ac}$	Poly(3HB-*co*-3HHx) or PHA
E. coli	$phaCAB_{Re}$	Poly(3HB)
E. coli	$phaC_{Sw}$	Poly(3HB)
P. putida GPp104	$phaC1_{Pp}$, $phaZ_{Pp}$, $phaC2_{Pp}$	Poly($3HA_{MCL}$)
P. putida GPp104	$phaC1_{Po}$, $phaZ_{Po}$, $phaC2_{Po}$	Poly($3HA_{MCL}$)
R. eutropha PHB$^-$4	$phaCAB_{Re}$	Poly(3HB)
R. eutropha PHB$^-$4	$phaC_{Rr}$, $phaP_{Rr}$	Poly(3HB)
R. eutropha PHB$^-$4	$phaC_{Cv}$	Poly(3HB)
R. eutropha PHB$^-$4	$phaC_{RrHa}$	Poly(3HB)
R. eutropha PHB$^-$4	$phaC_{Rs}$	Poly(3HB)
R. eutropha PHB$^-$4	$phaC_{Me}$	Poly(3HB)
R. eutropha PHB$^-$4	$phaC_{Tv}$	Poly(3HB)
R. eutropha PHB$^-$4	$phaC_{Lr}$	Poly(3HB)
R. eutropha PHB$^-$4	$phaC_{As}$	Poly(3HB)
R. eutropha PHB$^-$4	$phaC_{Al}$	Poly(3HB)
R. eutropha PHB$^-$4	$phaC_{Pa}$	Poly(3HB)
R. eutropha PHB$^-$4	$phaC_{Bc}$	Poly(3HB)
R. eutropha PHB$^-$4	$phaC_{RrATCC}$	Poly(3HB)
Aim: Overexpression of PHA synthesis genes for purification		
Trichoplusia ni	$phaC_{Re}$	PHA synthase protein
Aim: Large scale production of PHAs		
E. coli	$phaCAB_{Re}$	Poly(3HB)
E. coli	$phaCAB_{Re}$	Poly(3HB-*co*-3HV)
E. coli	$phaC_{Re}$ + $ORFZ_{Ck}$	Poly(4HB)
Aim: Novel PHAs		
P. putida GPp104	$phaC_{Tp}$	Poly(3HB-*co*-3HHx-*co*-3HO)
P. putida GPp104	$phaC_{Tp}$	Poly(3HB-*co*-3HHx-*co*-4HHx)
P. putida GPp104	$phaC_{Tp}$	PHAs containing 5HHx, 4HHp, 4HO
R. eutropha PHB$^-$4	$phaC_{Tp}$	Poly(3HB-*co*-3HHx-*co*-4HHx)
P. oleovorans	$phaCAB_{Re}$	Poly(3HB) and poly (3HO-*co*-3HHx)
R. eutropha PHB$^-$4	$phaCB_{Re}$	Poly(3HB-*co*-3HHx)
K. aerogenes	$phaCB_{Re}$	Poly(3HB-*co*-3HHx)
P. putida GPp104	$phaCB_{Re}$	Poly(3HB-*co*-3HHx)
E. coli (*fadB*)	$phaCAB_{Re}$	Poly(3HB-*co*-3HD-*co*-3HDD)
R. eutropha PHB$^-$4	$phaCA_{Cviol}$	Poly(3HB-co-3HHx), poly(3HB-*co*-3HV-*co*-3HHp)
K. aerogenes	$phaCA_{Cviol}$	Poly(3HB-co-3HHx)
Aim: Metabolic engineering		
E. coli	$phaC_{Re}$ + $ORFZ_{Ck}$	Poly(4HB)
E. coli (*fadB*)	$phaC1_{Pa}$	various poly($3HA_{MCL}$)
E. coli (*fadB*)	$phaC1_{Pa}$ and/or $phaC2_{Pa}$	various poly($3HA_{MCL}$)
E. coli	$phaC1_{Pa}$ and/or $phaC2_{Pa}$	various poly($3HA_{MCL}$)
E. coli	$phaC_{Ac}$ + $fabG_{Ec}$	Poly(3HB-*co*-3HHx)
E. coli	$phaC_{Ps}$ + $fabG_{Ec}$	Poly(3HHx-*co*-3HO-*co*-3HD)
E. coli	$phaC_{Ac}$ + $fabD_{Ps}$	Poly(3HB)

phosphorylation through metabolic signals involving PEP-PTS system (Pries *et al.* 1991).

1.8 Manipulation of PHA biosynthesis by genetic engineering

PHA production in natural producers is affected mainly due to the mobilizing enzymes in the form of depolymerases and esterases present *in vivo*. Moreover, many native organisms grow slowly. An alternative to increase the PHB content in the native organisms is to use a mutant devoid of PHA degradation pathways or heterologously express the genes in fast growing organisms lacking PHB biosynthesis and degrading genes.

For many purposes, ranging from academic importance to industrial production, PHA biosynthesis genes have been expressed in various heterologous systems, which have been listed in the Table 1.7 (Steinbüchel and Hein 2001). They have been expressed heterologously and the following facts have been unearthed:

1. Genes for PHA synthases or entire PHA biosynthesis pathways were identified during cloning by phenotypic complementation.
2. Expression in *E. coli* will make PHA synthase proteins more readily accessible for purification and therefore available in large quantities for further studies such as detailed biochemical analysis, generation of antibodies and *in vitro* PHA biosynthesis.
3. Heterologous expression allows one to provide the functionality of single enzymes or entire pathways.
4. Metabolic engineering may reveal novel pathways for synthesis and production of PHAs.
5. Other organisms may, for several reasons be much more suitable for the commercial production of PHA than the original host possessing the required gene(s). Therefore, organisms growing at faster rate grow to high cell densities and accumulate high amounts of PHB using cheaper carbon sources and are easy for downstream processing thus making them commercially important.

1.8.1 Expression in bacteria

A number of studies have successfully demonstrated heterologous expression of PHA synthases. Establishment of functionally active PHA biosynthesis pathways in *E. coli* requires not only a PHA synthase but also enzymes for conversion of metabolites to (R)-isomers of hydroxyacyl-CoA thioesters that are used as substrates by respective PHA synthase. Native organisms defective in *pha*C gene such as *R. eutropha* PHB^{-}4 (Schlegel *et al*. 1970) and *P. putida* GPp104 (Huisman *et al*. 1991) were used for physiological studies and cloning experiments. These require only PHA synthase gene for phenotypic complementation so as to accumulate PHA and such strains are easily identified on agar plates by the enhanced opacity of the colonies (Schlegel *et al*. 1970), by staining the colonies with lipophilic dye Sudan black (Schlegel *et al*. 1970) or Nile blue A (Ostle and Holt 1982) or by enhanced fluorescent of colonies in the presence of Nile red (Spiekermann *et al*. 1999).

A variety of heterologous systems have been used for high cell density cultivation and production of bulk amounts of PHAs, but recombinant *E. coli* and *R. eutropha* have been found to be of commercial importance (Lee and Choi 2000).

1.8.2 Expression in eukaryotic microorganisms

Among the eukaryotic organisms only *Saccharomyces cerivisiae* has been transformed to accumulate P(3HB). This was achieved by expressing the *pha*C_{Re} in the *S. cerivisiae* cytoplasm. In contrast to *E. coli* and plants, yeast does not require the expression of β-ketothiolase and acetoacetyl-CoA reductase genes for the PHA accumulation. The authors suggest that these steps are catalyzed by the native Erg10 and Fox2 proteins, respectively which are involved in β-oxidation but functioning as β-ketothiolase and acetoacetyl-Co A reductase (Leaf *et al*. 1996).

1.8.3 Expression in animal cells

Successful expression of PHA biosynthesis genes in animal cells has been achieved in the cells of insect *Spodoptera frugiperda* and *Trichopulsiani*. In *Spodoptera*, P(3HB) was synthesized when a dehydratase domain mutant rat fatty acid synthase cDNA and *pha*C_{Re} were expressed simultaneously thus, creating an

alternative pathway for the biosynthesis of PHAs (Williams *et al*. 1996). A baculovirus, *Autographa californica* nuclear polyhedrosis virus system was used to express $PhaC_{Re}$ in *Trichopulsiani* cells which accumulated PHA synthase in large amounts ranging to 50% of the total cell protein. This was used to purify large amounts of highly pure PHA synthase protein in one single liquid chromatography step for further biochemical characterization (Willams *et al*. 1996).

1.8.4 Expression in plants

Expression of PHA biosynthesis genes and production of PHA by transgenic plants have been investigated extensively. The PHA biosynthesis genes from microorganisms have been successfully expressed in *Arabidopsis thaliana* (Poirier *et al*. 1992; Nawrath *et al*. 1994; Poirier *et al*. 1995), *Brassica napus* (Valentin *et al*. 1999), *Gossypium hirsutum* (John and Keller 1996), *Nicotiana tabacum* (Nakashita *et al*. 1999), *Solanum tuberosum* (Mittendorf *et al*. 1998) and *Zea mays* (Hahn *et al*. 1999). However, the PHAs accumulated in these transgenic plants contributed to only a small percent of the total cellular matter. In many cases stunted growth or impaired functions were observed (Steinbüchel and Hein 2001). To establish PHA biosynthesis in plants, *pha*C_{Re} and *pha*B_{Re} alone or together with *pha*A_{Re} have been used. In many cases *pha*A expression is not required as it is inherently present in plants and is available for PHB synthesis. Replacement of *pha*A_{Re} by *bkt*B_{Re} in plants enabled them to accumulate co-polymers (Slater *et al*. 1998; Valentin *et al*. 1999). PHB biosynthesis in transgenic plants has been established in various compartments of plant cells and tissues. Of all the transgenic plants *B. napus* stands out as a Classic example wherein the PHA accumulation occurred in leucoplasts without affecting the overall growth of the plant (Valentin *et al*. 1999).

1.9 *In vitro* synthesis of PHB

An alternative approach to PHA synthesis is *in vitro* synthesis using isolated enzymes. This is of interest since specific functional groups could be incorporated into PHA. Though the studies in this direction were started by Gerngross and Martin in 1995, not much progress has been made. The main problems encountered are

Table 1.8: ***In vitro*** **synthesis of PHA by various purified PHAs**

PHA synthase of *R. eutropha*	Gerngross and Martin (1995)
PHA synthase of *A. vinosum* with Propionyl CoA transferase of *Clostridium propionicum*	Jossek and Steinbüchel (1998)
PHA synthase of *A. vinosum* with Acetoacetyl-CoA reductase of *S. cerevisiae,* Propionyl-CoA transferase of *Clostridium propionicum*	Jossek and Steinbüchel (1998)
PHA synthase of *T. pfennigii*, butyrate kinase and Phosphotransbutyrylase of *C. acetobutylicum*	Liu and Steinbüchel (2000)
PHA synthase of *P. aeruginosa* and acyl-CoA synthetase of *Pseudomonas* sp.	Qi *et al.* (2000)

the stabilization of the polymerase enzyme, elimination of the lag phase and the high cost of the precursor, CoA thioester. Lenz *et al*. (1999) were successful in stabilizing the polymerase with the help of glycerol and eliminating the lag phase *in vitro*. The list of the organisms where from PHA polymerases were purified and used for *in vitro* synthesis of PHA is listed in Table 1.8.

A system currently under development is the use of nano-technology to immobilize the PHA synthase on various supports including gold glass and anodic alumina etc. such that *in vitro* synthesis of crystalline PHA is facilitated along with the reuse of the synthase (Paik *et al*. 2001). Once this system is developed the production cost of PHB may be reduced considerably.

1.10 Commercial status

Momentum is building up for biopolymers because of their complete biodegradability. Since 1960s when W. R Grace, an American company for the first time commercialized the bacterial PHA, many companies have come up with **Table**

1.9: List of the companies producing biopolymers from various sources.

Company	Commercial Name	Source
Metabolix, USA	BIOPOL (PHBV)	Glucose and Propionic acid
Novamont, Italy	Mater-Bi	Starch
Cargill Dow, USA	Natureworks (PLA)	Corn
Rhodenburg Biopolymers, Holland	Solanyl	Starch (Potato peels)
Procter and Gamble, USA	Nodax (PHBHx)	Low cost feeds
DuPont, USA	Sorona 3GT polyester	Corn Sugar

different forms of biopolymers (Table 1.9). In 1970s ICI Bioproducts, an UK based company started producing PHBV copolymer on a commercial scale from glucose / propionic acid utilizing *R. eutropha* (Steinbüchel 1991). This was marketed under the trade name BIOPOL and used for the manufacturing of biodegradable films and bottles. With the potential applications of these polymers being extended to various fields, the demand for the biopolymer is growing despite its high cost. This firm was taken over by Monsanto, USA and now the rights have been transferred to Metabolix, USA.

The potential for biomedical applications is very promising, since the added value to these special products is remarkably high (Lafferty *et al*. 1988; Hocking and Marchessault *et al*. 1994, Duvernoy *et al*. 1995; Willams *et al*. 1999). Although research in this field is of unique complexity, it is both technologically and economically very compelling to succeed.

After extensive research in the fermentation and down stream processing of PHAs, the production costs have come down. Use of sucrose and other cheap carbon sources have further brought down the costs. In the present day market the cost per kg of PHBV has come down to $3.95 / kg at a production scale of 100,000 tons per year. This cost is supposed to come down eventually with the increase in the demand.

1.11 Streptomycetes

Streptomycetes are Gram-positive bacteria with a unique capacity for the production of a multitude of varied and complex secondary metabolites. Though, the most common *Streptomyces* secondary products are antibiotics, other useful biological molecules such as immuno modulators and enzyme inhibitors are also available (Good Fellow and O'Donnell 1992; Hodgson 2000). They have a complex life cycle including differentiation into at least three distinct cell types (Hodgson 2000).

Streptomycetes inhabit soil, which, because of the high numbers of microbial competitors, is an oligotrophic environment. Their role appears to be as general saprophytes. They are mainly present in soils rich in carbon and poor in nitrogen and phosphorous. There exists a variety and multiplicity of carbohydrates catabolic pathways in Streptomycetes, which led to investment in pathway-specific and global regulatory networks (Hodgson 2000).

Streptomycetes have been shown to contain trehalose, PHB, glycogen and neutral lipids as carbon storage molecules. These are proposed to have different roles in the cell. While trehalose is seen in the vegetative mycelium (Elbein 1967a, b, 1968; McBride and Ensign, 1987), glycogen is present in the spores, which later on get metabolized during germination (Hey-Ferguson *et al*. 1973). Olukoshi and Packter (1994) reported accumulation of neutral lipids in hyphal vacuoles during exponential phase and the late stationary phase. PHB was found to be a minor storage compound in the mycelium and not in spores or hyphae (Kannan and Rehacek 1970). This indicated that PHB was associated with primary metabolism (Olukoshi and Packter 1994).

Scope of the Present Thesis:

Kannan and Rehacek (1970) reported accumulation of PHA in various *Streptomyces* sp., which was later confirmed by Ranade and Vining (1993). However, no attempt was made to isolate the genes responsible for PHA synthesis. In this thesis we report the isolation of a g-DNA fragment from *Streptomyces aureofaciens* NRRL 2209 responsible for the accumulation of PHA.

The objectives of the present thesis are:

1) (a) Isolation and cloning of PHA synthesizing genes
   (b) Characterization of the PHA
2) Sequence analysis of a 5.0 kb genomic DNA fragment from *S. aureofaciens* NRRL 2209
3) Characterization of *pha*C gene involved in PHB synthesis
4) Screening for the depolymerase gene in *Streptomyces aureofaciens* NRRL 2209.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Ampicillin, Kanamycin, Tris, IPTG, X-gal, PEG-8000, SDS, bovine serum albumin, Nile blue-A, benzoic acid, EDTA, Ethidium bromide and poly-β-hydroxybutyrate were purchased from Sigma-Aldrich, USA. Agarose, restriction enzymes, T4 DNA ligase, RNase A, lysozyme, pronase, proteinase K 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 Bhabha Atomic Research Centre (BARC), India. X-ray films were obtained from Konica (Japan) or Kodak (USA). All other chemicals and solvents were of analytical grade and purchased from HIMEDIA, Qualigens Fine Chemicals and E-Merck Laboratories, India. Pipette tips and microcentrifuge tubes were purchased from Axygen (USA).

Table 2.1 Bacterial strains and plasmid vectors used in this study

Strain or Plasmid	**Important features (reference or source)**
S. aureofaciens NRRL 2209	Organism from which PHA synthesizing genes were isolated (NCIM 2417 and ATCC 10762)
E. coli JM109	F'; *lac* for blue/white screening (Promega)
E. coli DH5α	F^-; *lac* for blue/white screening (Gibco-BRL)
pGEM-3Z	Cloning vector (Promega)
pGEM-5Z	Cloning vector (Promega)
pSa240	pGEM-3Z carrying an ~ 5.0 kb g-DNA insert from *S. aureofaciens*
pGT07	pUC18 harboring the *Ralstonia eutropha* PHA synthesizing operon *pha*CAB
Recombinant *E. coli* JM109 harboring pSa240	***ATCC: PTA-1579***

2.2 Bacterial Culture Conditions

E. coli cells were grown at 37°C with shaking at 200 rpm in Luria Bertani (LB) broth and maintained on LB plates with 1.5% agar (Sambrook *et al.* 1989). For medium scale plasmid DNA preparation, recombinant *E. coli* cells were grown in Terrific Broth (TB) (Sambrook *et al.* 1989) supplemented with appropriate antibiotic. *Streptomyces auroefaciens* NRRL 2209 cells were grown at 28°C on a rotary incubator shaker with shaking at 200 rpm in baffled flasks containing MGYP broth (Hopwood *et al.* 1985) and were maintained on MGYP plates with 2.0% agar (Sambrook *et al.* 1989). For genomic DNA isolation *S. aureofaciens* was grown in YEME broth (Hopwood *et al.*1985).

S. aureofaciens was grown in vegetative medium (Kannan and Rehacek 1970) and transferred to production medium (Kannan and Rehacek 1970) for the accumulation of PHA. For PHA accumulation in recombinant *E. coli*, the cells were grown in basal medium.

Luria Bertani Broth (LB) (g L^{-1})

Bacto-tryptone	10
Yeast extract	5
NaCl	10

pH adjusted to 7.0

Terrific Broth (TB) (g L^{-1})

The following were added to 900 mL of deionized water

Bacto-tryptone	12
Yeast extract	24
Glycerol	4 mL

To the above sterilized medium 100 mL of sterilized 0.17 M KH_2PO_4 + 0.72 M K_2HPO_4 was added before inoculation of the bacterial culture (to 90 mL of deionized water (DW) 2.31 g of KH_2PO_4 and 12.54 g of K_2HPO_4 were added and volume made up to 100 mL with DW and sterilized by autoclaving).

MGYP Medium	**(g L^{-1})**	
Malt extract	3	
Glucose	10	
Yeast extract	3	
Peptone	5	pH adjusted to 7.0

YEME Medium	**(g L^{-1})**
Yeast extract	3
Peptone	5
Malt extract	3
Glucose	10
Sucrose	340

After autoclaving, sterilized 2 mL of 2.5 M $MgCl_2$. $6H_2O$ and 25 mL of 20% glycine solutions were added.

Vegetative Medium	**(g L^{-1})**	
Glucose	10	
Corn steep liquor	2	
Soybean meal	2	
Peptone	10	
Soybean oil	2	pH adjusted to 7.2

Production Medium	**(g L^{-1})**	
Glucose	20	
$(NH_4)_2SO_4$	5	
Yeast extract	2.5	
KCl	3	
$CaCO_3$	4	
Soybean meal	100 mL	pH adjusted to 6.8

Soybean meal is prepared by suspending 10 g soybean powder in 1L distilled water at 4°C for 24 h.

Basal Medium	**(g L^{-1})**
Yeast extract	5
Peptone	5
Na_2HPO_4	1
$MgSO_4$	0.2
Carbon Source	1.0%
pH adjusted to 7.2	

Antibiotics

Antibiotic of choice was added to the medium from stock solutions.

Antibiotic	*Stock solution*	*Final Conc. needed in medium*
Ampicillin	100 mg mL^{-1} in water	100 μg mL^{-1}
Kanamycin	100 mg mL^{-1} in water	30 μg mL^{-1}
Chloramphenicol	34 mg mL^{-1} in ethanol	170 μg mL^{-1}

2.3 The Nucleic Acids

2.3.1 Genomic DNA Isolation

Genomic DNA of the *Streptomyces aureofaciens* NRRL 2209 was extracted by using salting out method (Pospiech and Neumann 1995). *S. aureofaciens* NRRL 2209 was grown in YEME medium, pelleted and suspended in 5 mL SET buffer. 100 μL lysozyme (final conc. 1 mg mL^{-1}) was added to the above solution and incubated at 37°C for 30-60 min. 140 μL of proteinase K (final conc. 0.5 mg mL^{-1}) and 600 μL of 10% SDS solutions are added, mixed by inverting the tubes and incubated for 2 h at 55°C with occasional mixing. 2 mL of 5 M NaCl (final conc. 1.25 M) was added and the mix was cooled to 37°C. 5 mL Chloroform was added and mixed by inverting the tubes for 30 min at RT. Centrifuged for 15-20 min, 4,500 x g at RT. The supernatant was transferred to a fresh tube and 0.6 vol. isopropanol added to precipitate DNA, which was spooled out. The DNA was washed with 70% ethanol, air dried and dissolved in 1-2 mL of 10 mM Tris-1mM EDTA, pH 8.0 (TE) buffer at 55°C.

The g-DNA obtained by this method was essentially free of RNA. Alternatively, to make the samples RNA free, 10 μL of RNase A (1 mg mL^{-1}) was added to the restriction enzyme (RE) digestion reaction mix or RNase A treatment was given separately and the g-DNA was purified by extraction with chloroform.

Solutions

SET buffer: 75 mM NaCl, 25 mM EDTA (pH 8.0), 20mM Tris (pH 7.5)

Lysozyme: 50 mg mL^{-1} in water

Proteinase K: 20 mg mL^{-1} in water

SDS: 10% (may be preheated to 55°C)

TE buffer: 10 mM Tris-Cl (pH 8.0), 1 mM EDTA (pH 8.0)

5M *NaCl*; *Chloroform*; *Isopropanol*; *70% Ethanol*

2.3.2 Plasmid DNA Isolation

The alkaline lysis method of Sambrook *et al*. (1989) was improvised upon so that 12-24 samples are processed conveniently for plasmid DNA extraction within 3 h, with yields of 5-30 μg per 1.5 mL culture depending on the host strain and the plasmid vector. Another feature of this protocol was the use of PEG for purification, which resulted in precipitation of high quality super-coiled plasmid DNA free of contamination (PRISM™ 1995). This method is recommended for JM109, XL1 Blue, MV 1190 and highly recommended for DH5α and HB101.

Solutions

TEG Buffer: 25mM Tris-HCl (pH 8.0); 10mM EDTA (pH 8.0); 50mM Glucose.

Sol. II: 0.2 N NaOH, 1% SDS (freshly prepared)

Sol III: 3.0 M Potassium acetate (pH 4.8)

RNase A: 10 mg mL^{-1}

PEG-NaCl Solution: 4 M NaCl, 13% PEG_{8000} (sterilized by autoclaving)

Chloroform; *70% Ethanol*; *Deionized water*

The bacterial cultures were grown overnight (O/N) at 37°C in Terrific Broth (TB), with appropriate antibiotic. 1.5 to 3 mL culture was pelleted for 1 min at 4,000 rpm in a micro-centrifuge. The bacterial pellet was resuspended in 200 µL of TEG buffer by pipetting up and down. 300 µL of Sol II was added, mixed by inversion till the solution becomes clear and incubated on ice for 5 min. The above solution was neutralized by adding 300µL of Sol III, mixed well and incubated on ice for 5 min. The cell debris was removed by centrifuging for 1 min at 12,000 x g at room temperature. The supernatant was transferred to a clean tube, RNase A to a final concentration of 20 µg mL^{-1} (Sambrook *et al.* 1989) was added and incubated at 37°C for 20 min. To the above solution 400 µL of chloroform was added, mixed for 30 s and centrifuged for 1 min at 12,000 x g. The upper aqueous layer was transferred to a clean tube. Equal volume of isopropanol was added with mixing and centrifuged immediately for 10 min, 12,000 x g at room temperature. The pellet was washed with 70% ethanol and dried under vacuum for 3 min. The dried pellet was dissolved in 40 µL of deionized water and to it 40 µL of PEG-NaCl solution was added. The mixture was incubated on ice for 20 min and the plasmid DNA pelleted out by centrifugation for 15 min, 4°C at 12,000 x g. The supernatant was aspirated carefully, the pellet washed with 70% ethanol and air-dried. The dried pellet was resuspended in 20 µL of deionized water and stored at -70°C.

2.3.3 Extraction and purification of DNA from agarose gels

The restriction enzyme digested DNA was run on a 1.2% agarose gel in 1X TAE buffer (see section 2.6.2). The gel was stained with ethidium bromide (0.5 µg mL^{-1}). Stained gel was viewed under UV transilluminator and the fragment of interest was excised from the gel. The fragment was transferred to a 1.5 mL microcentrifuge tube and weighed. To it 2.5 vol. (~250 µL / 100 mg of agarose slice) of the 6M sodium iodide (NaI) solution from the GeneClean kit (Bio 101, USA) was added. The tube was incubated at 55°C for 5 min to facilitate complete dissolution of the agarose slice. To each tube, 5 µL of glass milk (GeneClean kit) for up to 5 µg of DNA was added (1 extra µL glass milk for each additional microgram DNA) and vortexed briefly. The mix was incubated on ice for 5 min with occasional mixing and

centrifuged at 12,000 x g for 3 min (to spin down the glass milk). The supernatant was decanted, 500 µL of chilled NEW WASH solution (GeneClean kit) was added to suspended the glass milk. The tube was centrifuged for 3 min at 12,000 x g. The step was repeated two more times and the tubes left to air dry at room temperature. The pellet was resuspended in 15 µL of TE buffer (10 mM Tris, 1mM EDTA, pH 8.0). Incubated for 5 minutes at 55°C and spun for 3 min at 12,000 x g. The supernatant containing DNA was recovered.

2.4 Sub-Genomic library of *Streptomyces aureofaciens* NRRL 2209

The genomic DNA of *S. aureofaciens* was partially restriction digested with Sau3A I and fractionated on 1.2% low melting point (LMP) agarose gel in 1X TAE running buffer. DNA fragments 2-6 kb in size were isolated (Sambrook *et al.* 1989) and shot gun cloned into BamH I digested pGEM-3Z plasmid vector. The recombinants were used to transform *E. coli* JM109 (Tripathi 1999).

2.5 Transformation of *E. coli* cells and selection of the transformants

Competent *E. coli* cells were made in 100 mM $CaCl_2$. LB medium (50 mL) was inoculated with 1% of the overnight grown culture and allowed to grow till 0.5 OD at 600 nm. The cells were harvested by centrifugation at 5,000 rpm at 4°C for 10 min, suspended in 100 mM ice-cold $CaCl_2$ and kept on ice for 30 min. Cells were centrifuged again and the pellet suspended in 1 mL of 100 mM ice-cold $CaCl_2$ and stored as aliquots of 200 µL at 4°C.

The competent *E. coli* cells, thus formed, were transformed according to Sambrook *et al*. (1989). DNA (~50 ng in a volume of 10 µL or less) was added to competent *E. coli* cells, mixed and kept on ice for 30 min. The cells were then incubated at 42°C for 2 min. To each tube 800 µL of LB broth was added and further incubated at 37°C for 1 h. About 100 µL of the transformed competent cells was spread onto LB plates containing appropriate antibiotic, IPTG and X-gal (Sambrook *et al.* 1989).

Solutions	*Stock*	*Final conc. in medium*
1) IPTG stock solution	200 mg mL^{-1} in sterile distilled water	40 μg mL^{-1}
2) X-gal Stock solution	20 mg mL^{-1} in dimethyl formamide	40 μg mL^{-1}

2.6 Nucleic Acid Blotting

2.6.1 Slot Blot

The apparatus used for the slot blot was soaked overnight in 0.5 % SDS, washed with distilled water and dried. The following procedure was used for slot blotting.

2.6.1.1 Membrane preparation

Two Whatman No. 1 filter papers and the Hybond N^+ membrane were cut to the required size and soaked in 6X SSC solution. Two prewetted Whatman No. 1 filter papers were put onto the filter support plate and care was taken such that no air bubbles were trapped within. This was achieved by rolling a pasteur pipette over the filter papers. Later the membrane was mounted in the same manner as the filter papers and ensured free of any air bubbles. The sample well plate was mounted onto the membrane and sealed tightly.

2.6.1.2 Denaturation of the DNA

To the sample DNA, 0.1 vol. of 3 M NaOH was added and incubated for 1 h at 60 - 70°C. The samples were diluted in the same buffer prior to their application onto the membrane.

2.6.1.3 Sample Application

Individual wells were washed thoroughly with 500 μL of 6X SSC by applying low vacuum. The diluted samples were then applied under low vacuum. Once the solution had seeped down, the apparatus was dismantled, the membrane taken out and air-dried.

2.6.1.4 Immobilization

Air dried membrane was sandwiched between two Whatman No. 1 filter papers, kept between two glass plates and baked at 80°C for 2 h.

2.6.2 Southern Blotting

Solutions

1X TAE:	0.04 M Tris-Acetate (pH 8.0), 0.001 M EDTA (pH 8.0)
20X SSC:	3 M NaCl, 0.3 M Sodium citrate (pH 7.0)
Depurination solution:	0.25 N HCl
Denaturation solution:	1.5 M NaCl, 5 M NaOH
Neutalization solution:	0.5 M Tris-HCl (pH 7.4), 3 M NaCl
Gel loading dye (6X):	0.25% Bromophenol blue in 40% (w/v) sucrose in water

For Southern hybridization (Southern 1975) the DNA samples were electrophoresed on an agarose gel in 1X TAE buffer containing 0.5 μg mL^{-1} ethidium bromide. The gel was rinsed in deionized water (DW) and placed in depurination solution for 10 min. The gel was rinsed in DW and immersed in denaturation solution for 30 min with gentle shaking. The gel was again rinsed in DW and transferred to neutralization solution buffer for 15 min. The gel was next set up for capillary transfer of DNA to solid membrane support.

A tray was filled with the transfer buffer (20X SSC). A platform was made and covered with a wick, made from 2 sheets of Whatman 3MM filter paper saturated with transfer buffer and the gel was placed on it. It was surrounded with a saran wrap to prevent the transfer buffer being absorbed directly by the paper towels stacked above the membrane. A sheet of Hybond-N^+ membrane (Amersham, UK) of the exact gel size was wetted with deionized water followed by transfer buffer (20X SSC) and placed on the top of the gel. A glass rod was rolled on the top of the membrane to remove any air bubbles trapped between the membrane and the gel. Two pieces of Whatman 3 MM paper wetted with 2X SSC were placed on the membrane. A stack of absorbent paper towels was placed on top of the 3 MM Whatman papers. A glass

plate was placed on the top of the paper towels. An ~ 0.5 kg weight was placed on the glass plate. Transfer of DNA was allowed to proceed for about 18 h. The membrane was marked for orientation, removed carefully and washed with 6X SSC. The membrane was air dried and baked for 2 h at 80°C to immobilize DNA onto the nylon membrane. Hybridization and autoradiography were carried out as described in the following sections.

2.7 Probe Preparation

2.7.1 Random primer labelling

Random primer labelling of the probe was done using the Megaprime DNA labelling system supplied by Amesham, UK. Reaction (50 μL) was set up as follows:

25 ng of DNA (used as probe)	5.0 μL
Primer solution (Random hexanucleotides) (3.5 A_{260} units)	5.0 μL

Above mixture was heated in a boiling water bath for 10 min and cooled to room temperature facilitating the primer annealing to the DNA.

10X reaction buffer (500 mM Tris-HCl, pH 8.0; 100 mM $MgCl_2$	5.0 μL
10 mM DTT; 0.5 mg mL^{-1} acetylated BSA)	
0.5 mM of dATP, dGTP, dTTP solutions (333 mM Tris-Cl	12.0 μL
pH 8.0; 33.3 mM $MgCl_2$; 10 mM β-Mercaptoethanol)	(4.0 μL each)
[α-^{32}P-dCTP (Sp. activity 3000 Ci $mmol^{-1}$)	5.0 μL
Sterilized deionized water	16.0 μL
Exonuclease free Klenow fragment (2 U μL^{-1})	2.0 μL
TOTAL VOLUME	50.0 μL

The reaction was carried out at 37°C for 45 min. The reaction was stopped by incubating the reaction mixture in a boiling water bath for 10 min and snap chilled on ice.

2.7.2 5' End Labelling

For 5' end labelling the DNA was dephosphorylated using calf intestinal alkaline phosphorylase (CIAP) before the end labelling reaction (Sambrook *et al*. 1989). The reaction was set up as follows:

Oligo nucleotide (10 pmol μL^{-1})	1.0 µL
10X T_4 PNK buffer	2.0 µL
γ ^{33}P dATP (3000 Ci $mmoles^{-1}$)	5.0 µL
Sterile deionized water	10.0 µL
T_4 polynucelotide kinase (PNK)	2.0 µL

The reaction mixture was incubated at 37°C for 30 min. The labelled probe was used immediately. In case of dsDNA, the probe is heated in boiling water bath and snap chilled on ice.

2.8 Hybridization

Solutions

20X SSC:	See section 2.6.2
Hybridization buffer:	1% BSA; 1.0 mM EDTA, pH 8.0; 0.5 M Sodium phosphate, pH 8.0; 7% SDS
Low stringency wash buffer:	2 X SSC; 0.1% SDS
High stringency wash buffer:	**0.2 X SSC; 1% SDS**

The blots made in section 2.6 were prehybridized at 55°C in 30 mL of hybridization buffer for 6-8 h in a hybridization incubator (Robin Scientific, USA). The buffer was decanted after 6-8 h and fresh buffer along with the denatured radiolabelled probe was added. Hybridization was carried out at 55°C for 14-18 h. The solution was decanted and the membrane washed with low stringency buffer at 55°C for 15 min. A high stringency wash at 65°C for 15 min followed. The moist blot was wrapped in saran wrap and exposed to X-ray film at –70°C in a cassette with intensifying screen.

2.9 PHB isolation from recombinant *E. coli* cells

Modified method of Hahn *et al.* (1994) was used to recover the polymer from the recombinant *E. coli*. Briefly, the cells were harvested by centrifugation at 2000 x g, washed twice with deionized water and freeze-dried under vacuum. The lyophilized cell pellet was shaken for 90 minutes at 37°C with chloroform and 30% sodium hypochlorite (1:1). The dispersion was centrifuged at 8000 x g at room temperature for 10 min. Lower chloroform phase that contained the solubilized polymer was recovered and the polymer precipitated by addition of 4 volumes of methanol.

2.10 GC analysis of PHB

2.10.1 Esterification of the PHA

Acid propanolysis of the PHA was carried out according to the method of Riis and Mai (1988). About 40 mg of the dry cell pellet was taken in a tightly sealable vial. 2 mL of 1,2-Dichloroethane (DCE), 2 mL of acidified propanol (1 vol. concentrated HCl + 4 vol. n-propanol) and 200 μL of the internal standard (2.0 g benzoic acid in 50 mL n-propanol) were added to the pellet and incubated at 85°C for 4 h. The mixture was shaken from time to time. After cooling to room temperature, a mixture of 2 mL DCE and 2 mL acid propanol was added and mixed properly. The solution was centrifuged at 10,000 x g for 5 min allowing the solid and untreated cell debris to settle down. The clear supernatant was injected into the gas chromatograph. The quantitative evaluation was effected by the peak areas of the hydroxybutyl and the benzoyl esters formed.

2.10.2 GC conditions

GC analysis was performed using a Shimadzu GC 17-A gas chromatograph. A 0.32 mm diameter BP 1 capillary column of 25 m length was used (J & W Scientific Co., USA). The analysis started at 80°C for 5 min followed by 7°C min^{-1} rise in temperature to reach the final temperature of 200°C. Nitrogen (5 mL min^{-1}) was used as the carrier gas. Injection: split-less and volume 0.6 μL.

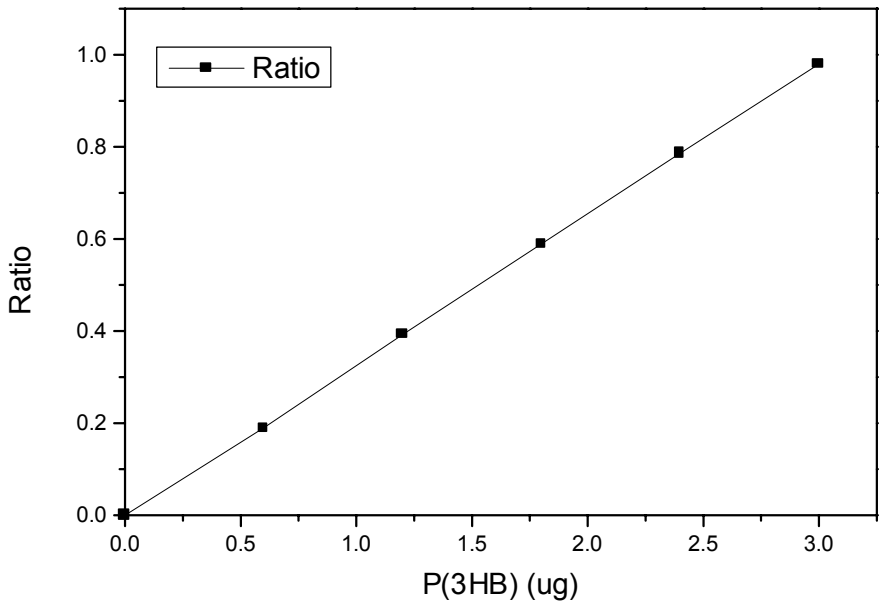

Fig. 2.1 Graph showing the amount of PHB injected per sample plotted against the ratio of the concentrations of 3-hydroxybutyryl propyl ester and benzoic acid propyl ester.

2.10.3 Calibration curve for PHB

In a 10 mL volumetric flask 200 mg PHB was dissolved in DCE by heating. After cooling to room temperature the solution was made up to 10 mL. Volumes of 200, 400, 600, 800 and 1000 μL of the above solution were esterified as described in section 2.10.1 and subjected to GC analysis. The relationship between the peak areas and the amount of PHB was observed to be linear (Fig. 2.1).

2.11 Preparation of the cell free lysate

The cell pellet of 36 h grown recombinant *E. coli* culture was suspended in phosphate buffer (pH 7.0) and sonicated at 160 Hz for 5 cycles of 1 min each on ice. The lysate was centrifuged at 12,000 x g for 15 min at 4°C. The clear supernatant was transferred into a new microfuge tube. This crude cell free extract was stored at - 4°C until further use.

2.12 Sodiumdodecylsulfate - Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Laemmli (1970) SDS-PAGE system is the widely used electrophoretic system for protein separations. The resolution in a Laemmli gel is excellent because the treated peptides are stacked in a stacking gel before entering the separating gel. Following are the stock solutions required for the Laemmli gel electrophoresis.

Solutions

Monomer solution (200 mL)	30% acrylamide and 2.7% bis in water. Stored at 4°C in the dark.
4X Running Gel Buffer (200 mL)	1.5 M Tris-Cl (pH 8.8)
4X Stacking Gel Buffer (200 mL)	0.5 M Tris-Cl (pH 6.8)
10% SDS (200 mL)	20 g dissolved in 200 mL water
Initiator (5 mL)	10% Ammonium persulfate (make fresh)
Tank Buffer (500 mL)	0.025 M Tris; 0.192 M Glycine; 0.1% SDS pH 8.3
Staining Solution (500 mL)	0.025% Coomassie Brilliant blue G in 40% Methanol and 7% acetic acid
Destaining Solution (500 mL)	7% acetic acid; 5% methanol
Water Saturated n-Butanol	5 mL water in 50 mL n-Butanol
2X loading Buffer (10 mL)	0.125 M Tris-Cl (pH 6.8); 4% SDS; 20% glycerol; 0.1% Bromophenol blue; 10% 2-mercapto ethanol

2.12.1 Preparation of the separating gel

A vertical slab gel (Bangalore Genei, India) was assembled as per the instructions provided by the manufacturer using 1.0 mm spacers. In a side-armed vacuum flask, 10% separating gel solution was made according to Table. 2.1, leaving out ammonium persulfate and TEMED. Flask was stoppered and vacuum applied for several minutes while stirring on a magnetic stirrer. TEMED and ammonium persulfate were added and mixed gently without generating bubbles. The solution was poured into the vertical gel slab with the help of a pipette leaving 4 inches from the

top. The slab was overlaid with water saturated n-butanol to remove trapped air bubbles and to layer evenly across the entire surface. When a sharp liquid-gel inter-surface was observed after the gel polymerization, the slab is tilted to pour off the overlay.

2.12.2 Preparation of the stacking gel

After the separating gel is casted and the overlay poured off, stacking gel was prepared according to Table. 2.1 leaving out ammonium persulfate and TEMED. As in the separating gel, this solution was degased. TEMED and ammonium persulfate were added, mixed and overlaid on the separating gel. A comb was inserted without creating air bubbles beneath the teeth of the comb. The gel was left for 1 – 2 h to polymerize.

2.12.3 Preparation of the sample

Equal parts of the sample and the loading buffer were mixed in a microfuge tube and kept in a boiling water bath for 90 seconds. The samples were centrifuged at 10,000 x g for 10 min., supernatant collected and used for electrophoresis.

2.12.4 Loading and running the gel

The comb from the gel was removed carefully. The wells were flushed with distilled water and drained off completely. Wells were filled with tank buffer and the samples were underlaid in each well using a syringe. Lower and upper buffer chambers were filled with tank buffer. Voltage was set between 70 – 80 volts. The run was stopped when the dye reached the bottom of the gel.

2.12.5 Staining and destaining the gel

The gel apparatus was disassembled and the gel was carefully transferred into the staining solution and allowed to stain with shaking for 4-6 h. The gel was removed and transferred into destaining solution until the bands were clearly visualized.

CHAPTER 3

ISOLATION AND CLONING OF PHA SYNTHESIZING GENES AND CHARACTERIZATION OF PHA

3.1 INTRODUCTION

Non-degradability and persistence in the environment of the fossil fuel based plastics has prompted the scientific community to look for degradable alternatives with comparable properties. The discovery of naturally occurring biodegradable polymers like polyhydroxyalkanoates (PHAs), polylactides, polyglycolic acids, polysaccharides and their blends has opened avenues for their utilization (Leaversuch 1987; Dawes 1990; Chum 1991; Steinbuchel 1991; Graham 1993; Chang 1994).

A better understanding of the physical and thermal properties of biopolymers will help us design these with improved properties for commercial purpose. While Medium chain length PHAs have been found unsuitable for applications due to their low melting and decomposition temperature and low glass transition temperatures (Gross *et al.* 1989; He *et al.* 1999), it has been shown that co-polymers of various PHAs possess superior thermal and mechanical properties than the homopolymers (Steinbuchel 1991).

Among actinomycetes, apart from other organisms (Emeruwa 1981; Haywood *et al.* 1991; Piper and Steinbuchel 1992), different *Streptomyces* sp. (Kannan and Rehacek 1970; Ranade and Vining, 1993; Manna *et al.* 1999) are reported to accumulate polyhydroxyalkanoates albeit in minor quantities. PHB (poly-3-hydroxybutyrate), the most studied of the PHAs, by itself cannot be used as the polymer because of its shortcomings (see Chapter 1). Thus, improvement in its properties is desired. Though, many organisms produce PHB, it's properties differ depending upon the organism producing it (Van der Walle 2001). This necessitates physical characterization of the polymer from streptomycetes.

Analytical techniques such as GC, NMR, FTIR and improving upon the protocols from time to time has helped to identify, characterize and quantitate PHAs in relatively quick time (Riis and Mai 1988; Rozsa *et al.* 1996; Hong *et al.* 1999; Misra *et al.* 2000). This chapter presents the characterization data of the biopolymer PHB synthesized by recombinant *Escherichia coli* harboring an approximately 5.0 kb genomic DNA fragment from *Streptomyces aureofaciens* NRRL 2209. Also, 25 – 28 times higher PHB

accumulation by the recombinant *E. coli* than the native organism *S. aureofaciens* using glycerol as the sole carbon source is also reported.

3.2 EXPERIMENTAL PROCEDURES

3.2.1 Organisms and growth

E.coli JM109 harboring PHA synthesizing genes from *Ralstonia eutropha* designated as pGTO7 (Tripathi, 1999) and *Streptomyces aureofaciens* NRRL 2209 designated as pSa240 were grown and maintained as enumerated in Chapter 2.

3.2.2 Sub-genomic Library construction of *Streptomyces aureofaciens* NRRL 2209

The construction of the library is explained in section 2.4 of Chapter 2.

3.2.3 Screening the clones for PHA synthesizing genes.

The library was screened for phaC and phaA genes as described by Tripathi (1999) and Mahishi (2001). The 1.55 kb Nco I / Stu I, 1.0 kb Stu I / Stu I and 0.74 kb Xcm I / Nde I fragments from the clone pGTO7 were used as probes to screen the library for phaC, phaA and phaB genes respectively (Fig. 3.1). Colony blotting and Southern hybridization were performed (Sambrook et al. 1999) and positive clones selected.

3.2.4 Restriction Analysis of the plasmid pSa240

The pSa240 plasmid, was restriction digested with Apa I, EcoR I, EcoR V, Hind III, Kpn I, Nsi I, Not I, Pvu II, Stu I, Sma I, Sal I, Sph I and Xba I. A restriction endonuclease map was subsequently generated. The enzyme reactions were carried out as per the manufacturer's instructions.

3.2.5 Biopolymer synthesis in presence of various carbon sources

The recombinant E. coli harboring pSa240 plasmid was grown in BMG medium (each liter of the medium contained: yeast extract 5.0 g, bacterial peptone 5.0 g, disodium hydrogen phosphate 1.0 g, magnesium sulfate 200 mg, glucose 10.0 g, pH 7.2). Glucose

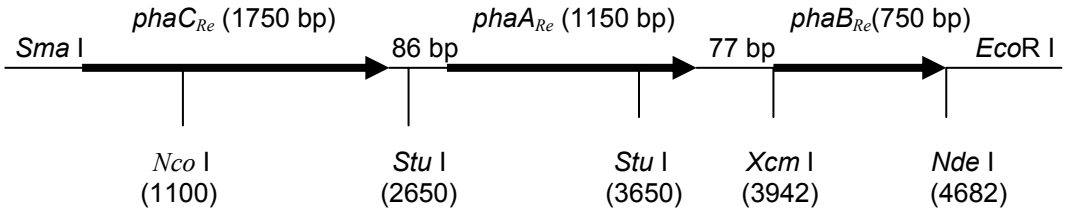

Fig. 3.1: Nco I - Stu I, Stu I - Stu I and Xcm I – Nde I fragments of pGTO7 which were used as probes in hybridization experiments

in BMG medium was replaced either with 1% glycerol (BMGL) or 1% sucrose (BMS)

or 0.5% sodium acetate (BMSA). The cells were also grown in Luria Bertani (LB) broth. Cultures were incubated at 37°C with shaking at 200 rpm for 40 h.

S. aureofaciens NRRL 2209 cells were grown in production medium (Section 2.2, Chapter 2). The cells were grown at 28^{o}C in baffled flasks with continuous shaking at 200 rpm and harvested at different points in time.

3.2.6 Recovery of the Biopolymer from the cells

This is enumerated in section 2.10 of Chapter 2.

3.2.7 In vitro detection of PHB by fluorescent microscopy

Heat fixed bacterial smear was stained with 1.0% aqueous solution of Nile blue A at 55°C for 10 min. The slide was 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 and blotted dry. Before examination, the slide was remoistened with water and a cover slip was placed on the smear (Ostle and Holt 1982). The slides were observed under the oil immersion lens of a Leica fluorescent microscope at an excitation wavelength of 480 nm.

3.2.8 FTIR analysis

Biomass from a 100 mL cell culture was pelleted, resuspended in 10 mL distilled water and lyophilized at –58°C. A sample (2 mg lyophilized cells) was thoroughly mixed with 100 mg KBr (Spectroscopic grade). A 15 mg KBr treated pellet was taken and dried at 100°C for 4 h (Misra *et al.* 2000). FTIR spectrum was taken using a Perkin Elmer (USA) model 1720 Fourier transform IR spectrophotometer.

3.2.9 Gas Chromatography analysis

This has been detailed under section 2.11 of Chapter 2.

3.2.10 ^{1}H NMR analysis of extracted polymer

Standard PHB (1% wt./vol.) solution was prepared in $CDCl_3$. The polymer extracted from the recombinant *E. coli* cells was also solubilized in $CDCl_3$ at a concentration of 5% (wt./vol.). ^{1}H NMR spectra for both the samples were recorded using Bruker Ac200 at 24°C (Rozsa *et al.* 1996).

3.2.11 Determination of molecular weight and molecular weight distribution

A Waters model 150-CV gel permeation chromatograph at 40°C was used for determining the molecular weight and molecular weight distribution of the recovered polymer (0.1% PHA in $CHCl_3$). Monodisperse polystyrene and chloroform were used as the molecular weight standard and the mobile phase respectively.

3.2.12 Thermogravimetric analysis of PHA

Thermogravimetric change of the PHA sample was analyzed using Rheometric TG analyzer (Rheometric Scientific, USA). The analysis was carried out under nitrogen flow rate of 15 mL min^{-1} with a scanning rate of 10°C min^{-1}.

3.2.13 Differential Scanning Calorimetry

To determine the morphological state of the polymer, the melting temperature and the enthalpy of fusion were measured using a Perkin Elmer Differential Scanning Calorimeter (DSC-7). About 10 mg of the purified sample was used.

3.2.14 Scanning and Transmission Electron Microscopy

Sample for scanning electron microscopy was prepared by sonicating a purified suspension of PHB at 20 kHz for 3 cycles of 1 min each. A drop of the suspension was dried on a brass stub under an IR lamp and later coated with gold using a Polaron sputter coater unit. SEM photographs were taken using a LEICA Stereoscan 440 Scanning Electron Microscope equipped with a Phoenix EDAX attachment.

For TEM (Van Krevelen 1972) the bacterial cell pellet was fixed for 2 h in 0.1 M sodium cacodylate (pH 7.2) containing 2% glutaraldehyde. Following fixation, the sample was washed thrice with 0.1 M sodium cacodylate buffer (pH 7.2), mixed with 1% OsO_4 and 1% potassium ferrocyanide in 0.1 M sodium cacodylate buffer (pH 7.2) for 2 h at room temperature. The specimen was washed with water, poststained with 2% uranyl acetate for 1 h, dehydrated in graded concentrations of acetone (70%, 90% and 100% v/v) and embedded in epon resin by incubating overnight at 70°C. Thin section of 50nm were cut, stained with lead citrate and photographed with Tecnai 12 Electron microscope.

3.3 RESULTS AND DISCUSSION

3.3.1 Screening and Isolation of a clone harboring PHA synthesizing genes of *S. aureofaciens* NRRL 2209

Three clones of the *S. aureofaciens* sub-genomic library screened by colony blotting with the heterologous probes Nco I / Stu I, Stu I / Stu I and Xcm I / Nde I from *Ralstonia eutropha* for *pha*C, *pha*A and *pha*B genes gave positive signals for the three genes(Tripathi 1999; Mahishi 2001) (Fig 3.2). However, the signals obtained with Stu I / Stu I probe were of very low intensity. Based on the intensity of the signals obtained for

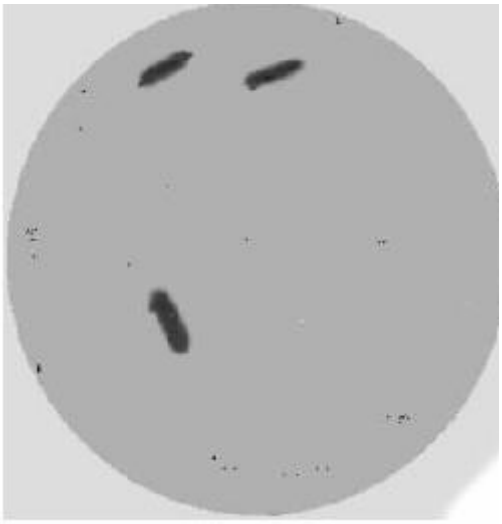

Fig. 3.2. Colony hybridization representative autoradiogram for screening of the partial genomic library of *S. aureofasciens* NRRL 2209 with Nco I/ Stu I and Xcm I / Nde I fragments from pGT07 clone harboring *pha*CAB genes of *R. eutropha*

*pha*C and *pha*B, a clone designated as pSa240 harboring an approximate 5.0 kb DNA fragment was picked up with which further studies were carried out.

3.3.2 Restriction analysis

The pSa240 plasmid DNA was digested with the restriction enzymes mentioned in section 3.2.4 of this Chapter and electrophoresed on 1% agarose gel. In the ~5.0 kb insert only one site each for Stu I, Nsi I, Sph I was found. No sites were found for the restriction enzymes Apa I, EcoR I, EcoR V, Hind III, Kpn I, Not I, Sma I and Xba I. The Stu I - Nsi I fragment from the insert was ligated to Nsi - EcoR V sites of pGEM-5Z vector and later digested with Pvu II to generate three fragments of 0.5, 1.0 kb and 2.7 kb. Accordingly Pvu II restriction sites were mapped on pSa240. Two sites were present for Sal I. All these sites are shown on the restriction endonuclease map in Figure 3.3.

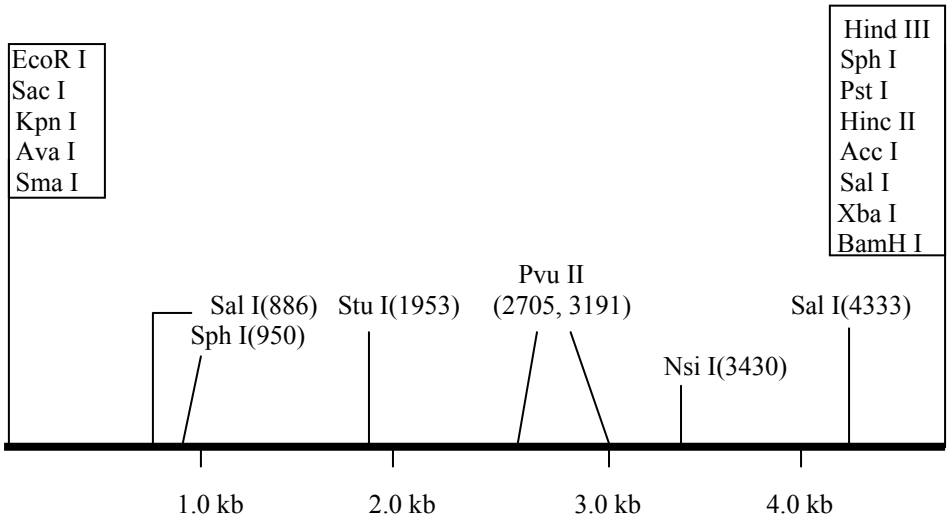

Fig. 3.3 Restriction endonuclease map of the pSa240 insert

3.3.3. PHB synthesis in recombinant E. coli and Streptomyces aureofaciens

PHA accumulation by the recombinant *E. coli* expressing the PHA biosynthesis genes from *S. aureofaciens* was studied by growing these cells in a basal medium supplemented with different carbon sources (Table 3.1). The cells were also grown in LB medium. Irrespective of the carbon source used the cells accumulate PHA through out the culture period reaching peak value after 40h of growth. Although acetate and glucose are the precursors for the synthesis of acetyl-CoA (Anderson *et al*. 1990), each supported a mere 4% accumulation of PHA on cell dry weight basis. This was in total contrast with 70 to 90% PHB accumulation observed for recombinant *E. coli* expressing the PHB biosynthetic genes from *Ralstonia eutropha* (Schubert *et al.* 1988). Sucrose likewise supported only 5% PHA accumulation by the recombinant *E coli* cells.

Maximum PHA accumulation of 60-66% was observed when 1% glycerol was incorporated in the medium as the sole carbon source. This was the medium of choice for all the experiments involving pSa240. The possibility of the ~5 kb genomic DNA fragment from *S. aureofaciens* carrying genes which facilitate the conversion of glycerol

Medium	PHA % [d]
LB broth	1.0 ± 0.5
BMG	4.0 ± 1.2
BMGL	60.0 ± 6.0
BMS	5.0 ± 0.8
BMSA	4.0 ± 1.1

[d] Cell dry mass basis

Table 3.1 PHA accumulation by recombinant *E. coli* in different media.

to acetyl-CoA *via* either glycolysis or methylglyoxal pathway (Fig.3.4) and its subsequent incorporation into PHA cannot be ruled out. The other possibility lies in the glucose repression of gene expression. It is possible that the expression of the PHB biosynthesis genes is repressed due to glucose availability in the medium or due to glucose-kinase gene product which interacts with one of the intermediate compounds in PHA biosynthesis or glucose-kinase itself which might be interacting with the promoter and inhibiting the expression of genes (Hodgson 2000).

In contrast, the native *S. aureofaciens* NRRL 2209 accumulated a peak amount of 2.4% PHB between 16-18 h of growth (Fig. 3.5). Kannan and Rehacek (1970) had earlier reported a maximum accumulation of 1.2% PHB in *S. aureofaciens*. This difference in PHB accumulation may be attributed to the analytical method employed in the present study. While the most sensitive and reliable GC based analytical method was used in this study Kannan and Rehacek (1970) resorted to acid hydrolysis of PHB to crotonic acid and then colorimertric estimation. The PHB content in the cells declined after 18h to undetectable levels by the onset of stationary phase at 30h of culture period. The probable reason for the accumulation of PHB during the log phase of the growth might be

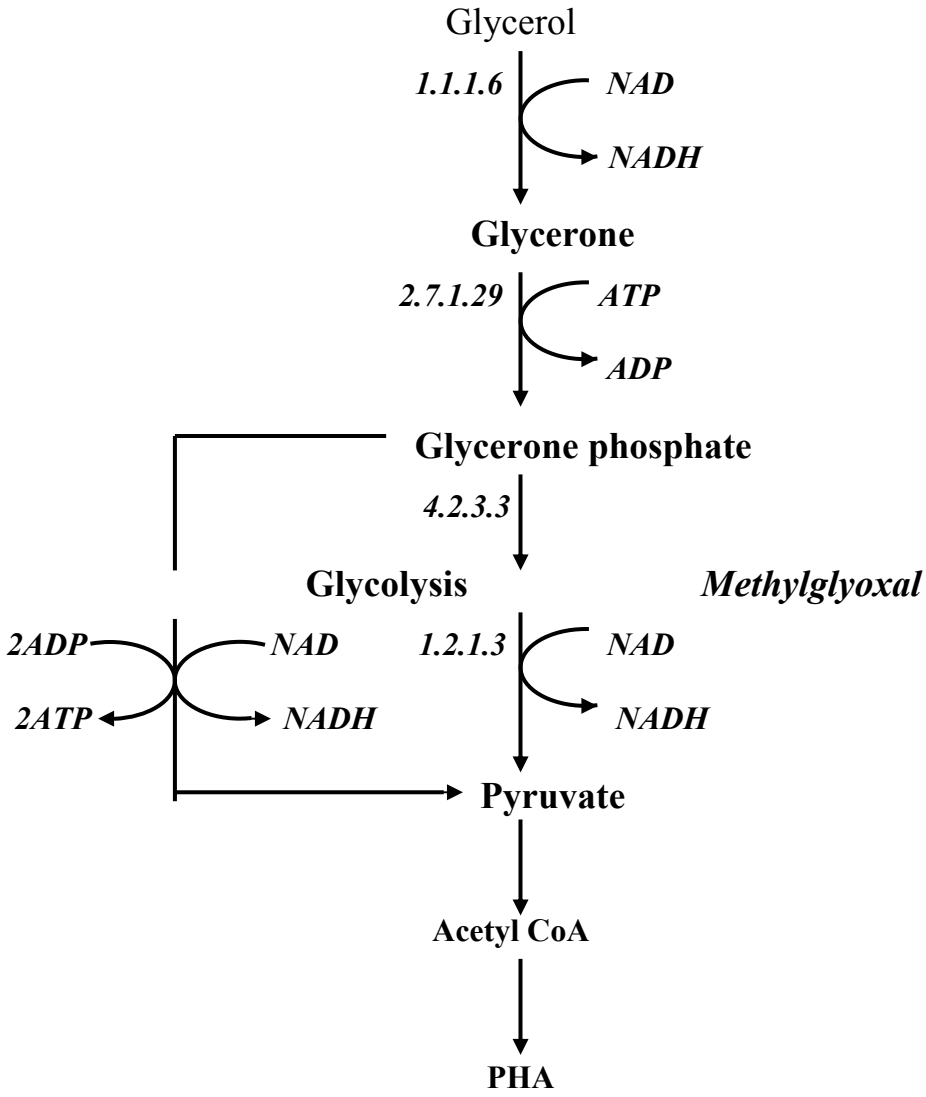

Fig. 3.4 PHA synthesis by Methylglyoxal pathway. 1.1.1.6, 2.7.1.29, 4.2.3.3 (earlier 4.2.99.11) and 1.2.1.3 are the IUBMB nomenclature for the enzymes Glycerol dehydrogenase, Glycerone kinase, Methylglyoxal synthase and Aldehyde dehydrogenase, respectively.

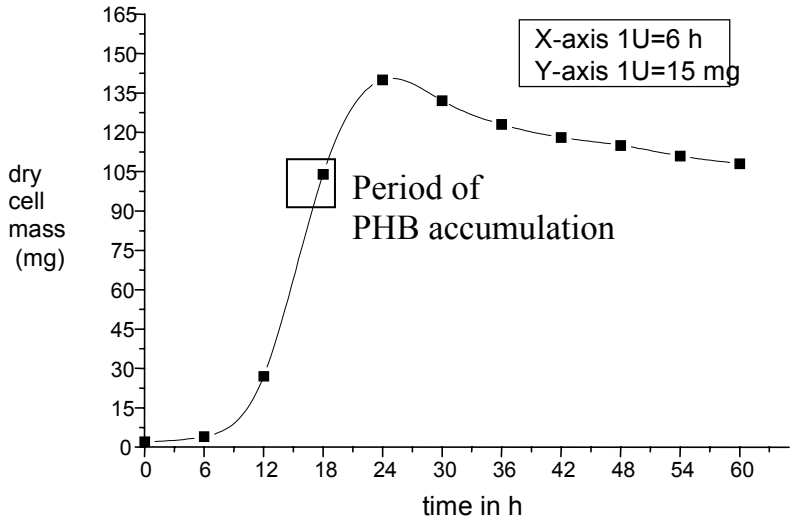

Fig. 3.5 *S. aureofaciens* NRRL 2209 growth curve as a function of time. The box show the period of PHB accumulation (max 2.4%) during the growth period of the cells.

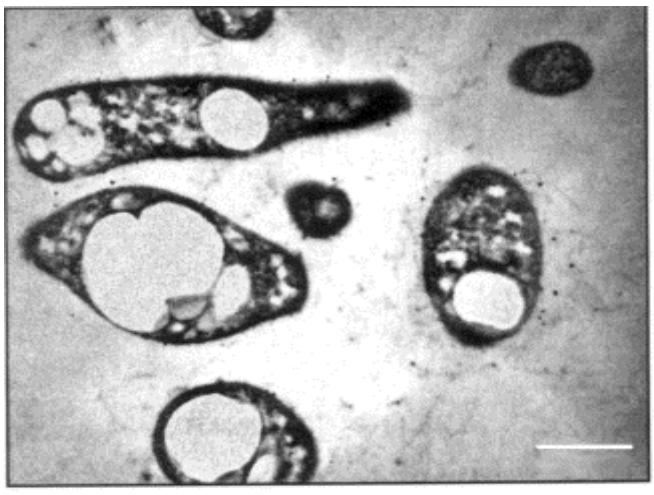

Fig. 3.6 Transmission electron microscopy of the recombinant *E. coli* cells showing PHB as cytoplasmic inclusion bodies. Bar represents 1 μm.

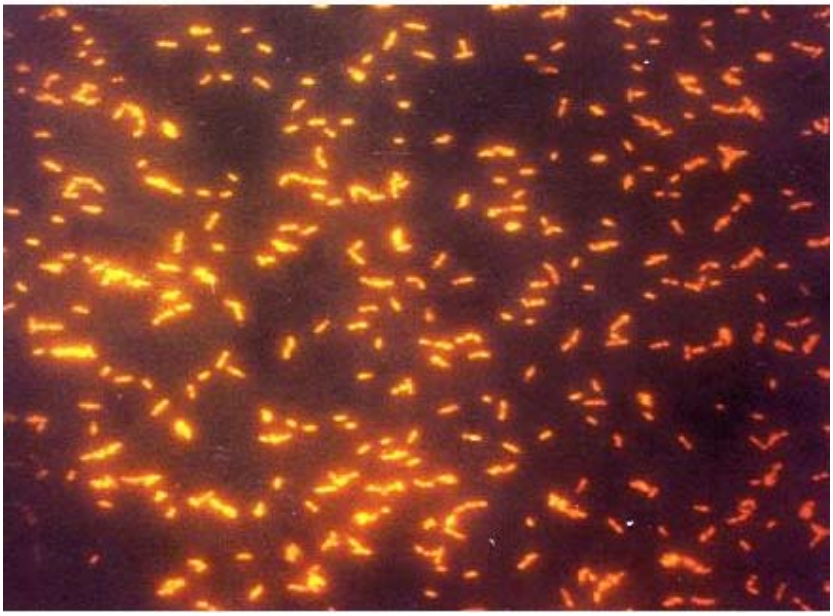

Fig. 3.7 Fluorescent microscopy of the recombinant *E. coli* stained with Nile Blue A, showing fluorescence due to PHA accumulation.

to accumulate the carbon and energy sources in an insoluble but readily mobilizable form required later in the development process (Brana *et al.* 1980). The metabolism of accumulated PHB may be due to the action of unspecific esterases or lipases (Mukai *et al*. 1993; Jaeger *et al*.1995) or depolymerases (Steinbuchel 1991) present within the *S. aureofaciens* cells. Thus, heterologous expression of the *S. aureofaciens* PHA biosynthesis genes in *E. coli* resulted in about 25 to 28 times higher PHA accumulation as also in preferential utilization of glycerol as the carbon source than glucose.

TEM of the 40-42 h recombinant *E. coli* showed presence of large PHA granules in the cell cytoplasm. The polymer granules grew so large that gross deformation of the cell shape occurred (Fig. 3.6), however, most of the cells were found to be intact.

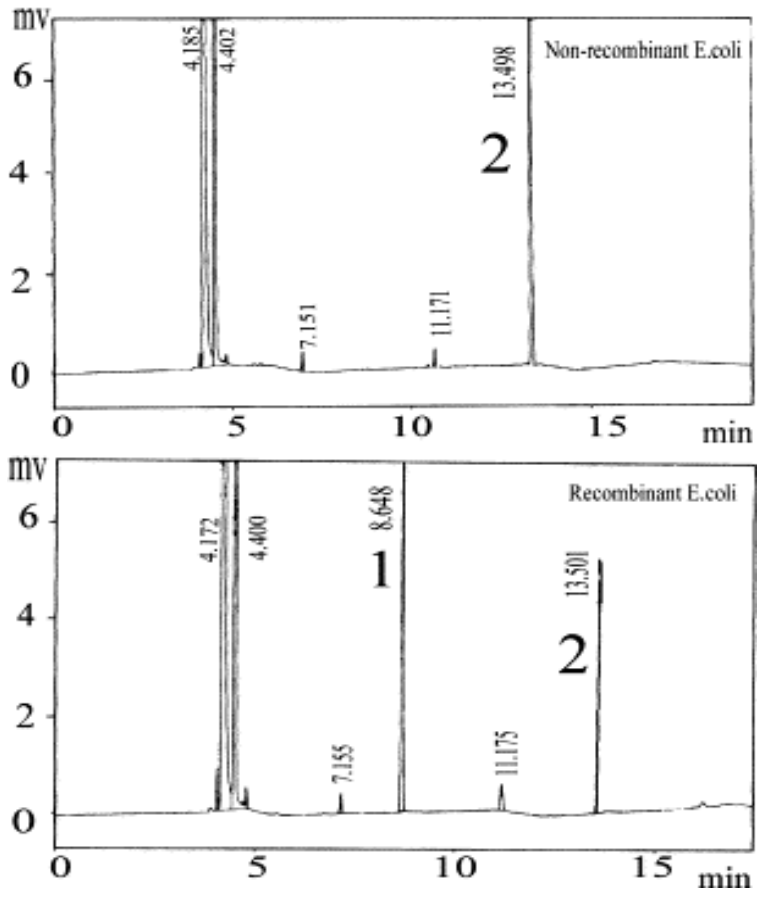

Fig. 3.8 Gas chromatograms of non-recombinant and recombinant *E. coli*. Propyl ester of PHB and benzoic acid are represented by Peaks 1 and 2, respectively.

3.3.4 Characterization of the PHA

Recombinant *E. coli* cells harboring ~5.0 kb genomic DNA fragment from *S. aureofaciens* NRRL 2209 were initially screened for PHA accumulation by fluorescence microscopy. The cells stained with Nile blue A showed characteristic orange fluorescence when observed under fluorescence microscope at an excitation wavelength of 460nm indicating PHA accumulation in these cells (Fig. 3.7).

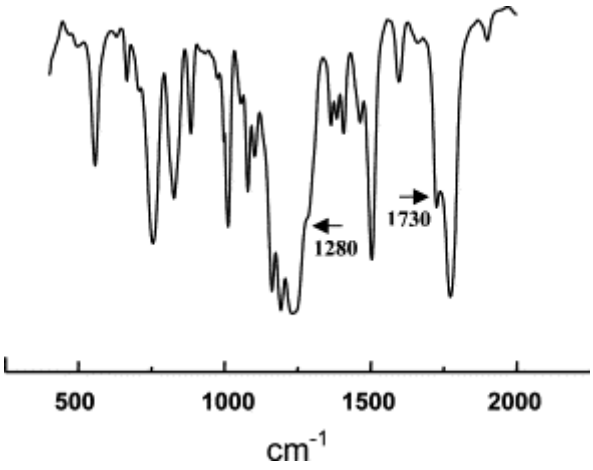

Fig. 3.9 FTIR spectra of recombinant *E. coli* cells containing PHB. The absorption bands at 1280 and 1730 cm^{-1} correspond to C=O and C---O of PHB, respectively.

GC analysis of dried and esterified recombinant and non-recombinant *E. coli* cells grown for 40h, revealed a prominent peak corresponding to the propyl ester of 3-hydroxybutyrate (3-HB) in the recombinant *E. coli* cells. This peak was not observed in the non-recombinant *E. coli* (Fig. 3.8).

FTIR analysis of the lyophilized recombinant *E. coli* cell pellet showed two absorption bands at 1280 cm^{-1} and 1730 cm^{-1} (Misra *et al*. 2000) corresponding to C=O and C-O stretching groups respectively. This confirmed the presence of PHB in the recombinant cells (Fig. 3.9).

The PHB extracted by sodium hypochlorite / $CHCl_3$ dispersion method from the recombinant *E. coli*, upon GC analysis showed that the polymer was of high purity. This purified PHB sample was next subjected to 1D ^1H NMR. Trimethylsilane was used as the internal chemical shift standard. The spectrum (Fig. 3.10) revealed the presence of three groups of signals characteristic of PHB homopolymer. The doublet at 1.3ppm was attributed to the methyl group coupled to one proton; the doublet of quadruplet at 2.57 ppm to the methylene group adjacent to an asymmetric carbon atom bearing a single proton and the multiplet at 5.28 ppm to the methylene group. Chloroform-d gave a

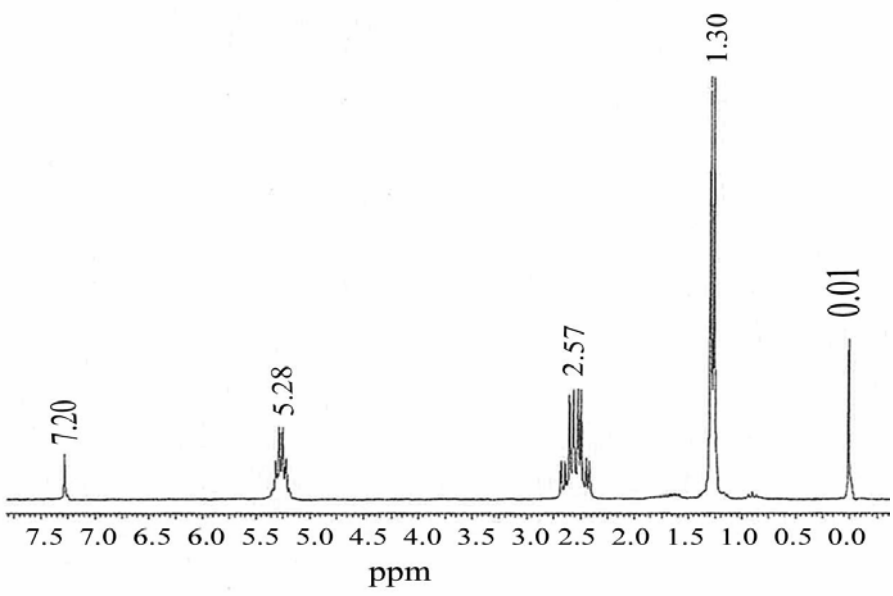

Fig. 3.10. ^{1}H NMR spectra of the PHB recovered from the recombinant *E. coli*. showing a doublet, doublet of quadruplet and a multiplet at 1.3, 2.57 and 5.28 ppm which are due to proton coupling. The signal at 7.25 ppm is characteristic of chloroform-d.

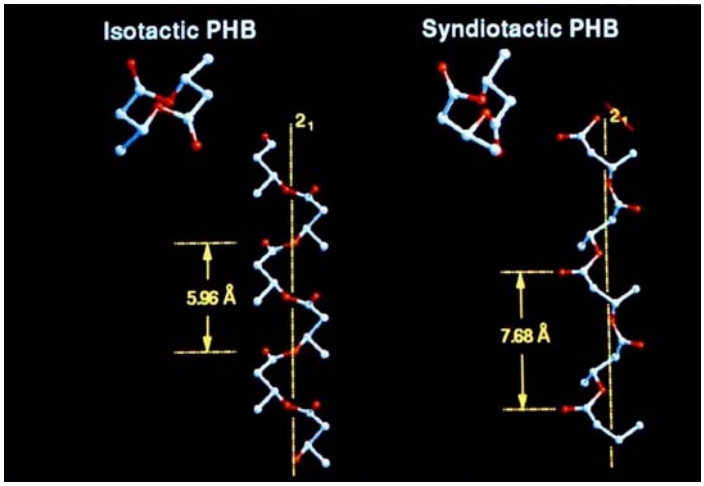

Fig 3.11 Arrangement of molecules and pendant groups in an isotactic and syndiotactic PHB are shown. In isotactic form the pendant group is always away from the main chain and are arranged towards one side of the plain unlike in syndiotactic wherein the pendant groups are alternately arranged.

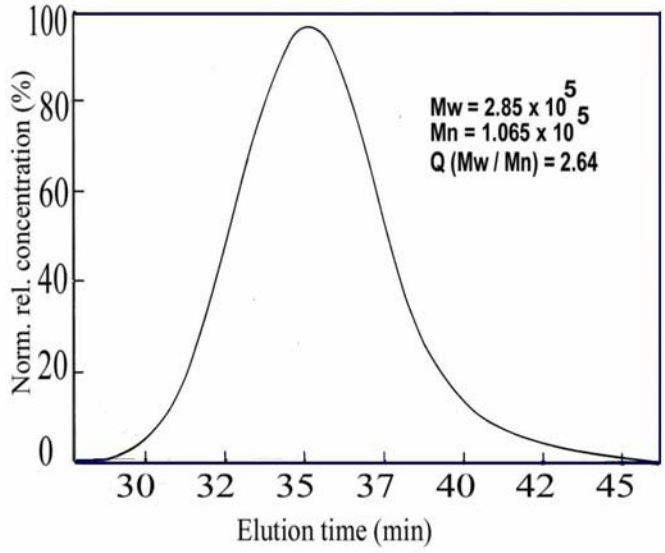

Fig. 3.12 Gel permeation chromatography of the purified PHB sample. Mw and Mn represent weight average and number average molecular weights, respectively. The polydispersity index (*Q*) [Mw/Mn] of 2.64 indicative of the non-uniform PHB polymer chain formation within the cell cytoplasm

chemical shift signal at 7.25 ppm. The signal multiplicity by a proton as a quadruplet or octet in case of protons of CH_2 group is obtained due to proton coupling in isotactic form unlike in syndiotactic where duplet signal is obtained due to proton coupling. From these results, it was concluded that recombinant *E. coli* cells exclusively synthesized isotactic PHB homopolymer (Fig. 3.11).

The thermodynamic properties of a polymer are dependent on its number-average molecular mass and the bulk properties connected with large deformations are largely determined by weight-average molecular mass (Van Krevelen 1972). Molecular weight of PHB is dependent on the physiological background or on the abundance of PHA synthase present within the cell (Rehm and Steinbuchel 1999; Madison and Huisman 1999). In the present study, the purified PHB sample showed weight-average molecular weight (Mw) to be 2.85 x 10^5 and the number average molecular weight (Mn) to be

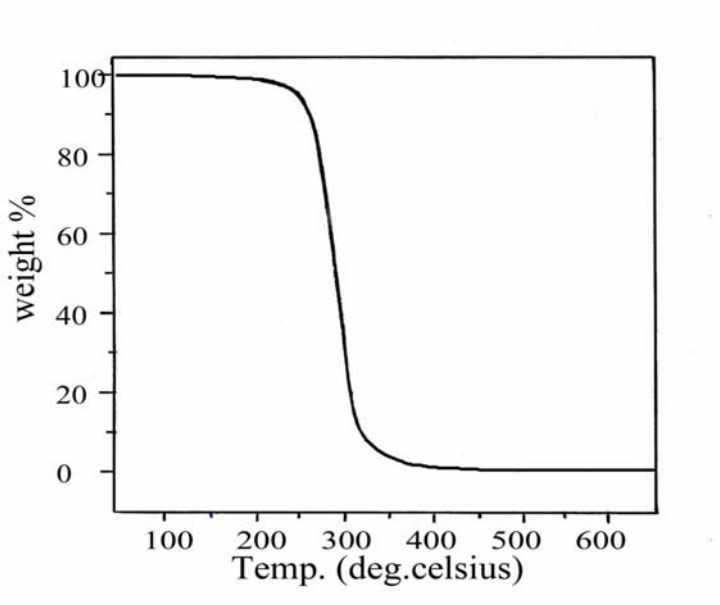

Fig. 3.13 Thermogravimetric analysis of the purified PHB sample. It shows a rapid degradation of the recovered PHB between 250 and 340°C with a peak at 295°C.

1.065 x 10^5. The possible reason for low molecular weight of PHB synthesized by the recombinant *E. coli* may be due to over expression of the PHA synthase gene in heterologous physiological environment (Sim *et al.* 1997; Krank *et al.* 1997).

The polydispersity index (Q) (defined as Mw / Mn) was 2.64 (Fig. 3.12). It has been shown earlier that Q is directly proportional to the hypochlorite treatment time, where in Q increased with the increasing treatment time (Hahn *et al*. 1993, 1995). This is also indicative of the non-uniform chain length of the polymer synthesized and accumulated within the recombinant cells.

The recovered polymer rapidly degraded between 250°C and 340°C with a peak at 295°C (Fig. 3.13). The melting temperature and enthalpy of fusion of the polymer were 173°C and 85.0 J g^{-1} respectively. The high enthalpy of fusion suggests high crystalline nature of the recovered PHB, which was calculated to be of 60-65% assuming the enthalpy of fusion of 100% crystalline sample to be 146 J g^{-1} (Barham *et al.*1984). The

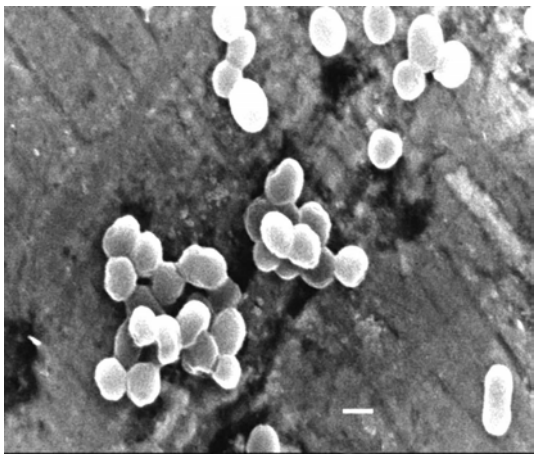

Fig. 3.14 Scanning electron microscopy (SEM) of the gold coated PHB granules. PHB granules of uniform spherical shape with a stable configuration are seen. The size of the granule is ~1.1 μm. Bar represents 1 μm.

difference between the decomposition and the melting temperatures of the recovered PHB was high enough to facilitate processing of the polymer. This was unlike the reported values of 173°C and 200°C, which normally makes processing of the polymer difficult (Lee 1996).

PHB granules coated with and without gold were observed under scanning electron microscope showed a uniform spherical shape and size with a stable configuration. The average diameter of the PHB granule was 1.1 μm (Fig. 3.14).

3.4 CONCLUSIONS

- An approximately 5.0 kb Sau3A I genomic DNA fragment from *Streptomyces aureofaciens* NRRL 2209 responsible for PHB synthesis was isolated.
- This fragment upon cloning and expression in *E. coli* supported 25 - 28 times more PHB accumulation in recombinant *E. coli* than the native organism when glycerol was supplied as the sole carbon source.
- Extraction of the polymer using sodium hypochlorite / chloroform dispersion method and subsequent GC analysis showed the polymer to be of high purity
- The recovered PHB was an isotactic, crystalline homopolymer with granule size of 1.1 μm. DSC and TGA results show a shift in the thermal degradation temperature pattern of the polymer, which is found to be at 295°C.
- Molecular weight of the PHB synthesized and accumulated by *E. coli* was found to be around 3 x 10^5 by GPC.

CHAPTER 4

SEQUENCE ANALYSIS OF A 5.0 KB GENOMIC DNA FRAGMENT FROM *S. AUREOFACIENS* NRRL 2209.

4.1 INTRODUCTION

The precise localization of the limits of a protein coding region of a bacterial DNA sequence often relies initially on the identification of an open reading frame (ORF) enclosed by translational signals similar to those of previously characterized genes (Bibb *et al*. 1984). Computer aided statistical analysis have been developed to assist in the recognition of DNA sequences with a protein coding function (Fickett 1982; Rodier *et al*. 1982; Smith *et al*. 1983) and those based on codon preference are particularly useful (Staden and McLachlan 1982).

Heterologous expression of the genes depends on factors like the codon usage, a characteristic of the overall base composition, the type of promoter and the translational start. Sequence identification and analysis of the DNA fragments gives insight into all the above factors besides sequence homology and phylogenetic distance. Hasegawa and Hashimoto (1993) have shown that the phylogenetic analysis is adversely affected by the base compositional bias, which led to analysis based on the amino acid (AA) sequence of protein. However, Foster and Hickey (1998) have shown that AA sequences can also be affected secondarily by the nucleotide compositional bias wherein, two taxa with large compositional bias were grouped together despite biological relatedness.

4.1.1 Features of Streptomyces genes

Streptomyces sp. DNA typically has a base composition of 68 to 78 mol % G+C (Enquist and Bradley 1971; Hodgson 2000). Due to high G+C content the *Streptomyces* genes show very limited or no sequence similarity to homologous genes from other organisms. Promoter regions also show wide diversity of sequences. Few promoters show sequence similarities to eubacterial promoters having a consensus –35 (TTGACPu) and –10 (TAgPuPuT) boxes (Strohl 1992). However, loci like *vph* (Janssen ad Bibb 1988), *erme* (Bibb *et al*. 1985; Bibb and Janssen 1986), *hrdD* / *bar*, *actI* / *actIII* (Hallam *et al*. 1988; Parro *et al*. 1991) have overlapping promoters which are postulated to be involved in complex regulatory patterns (Beck and Warren 1989; Janssen *et al*. 1989). Promoters like *gal-p2* (Fornwald *et al*. 1987) and *nshR-p* (Li *et al*. 1990, 1991) initiate transcription from the middle of the multi-gene operons in

which other promoters are found upstream of the gene in the operon. A few promoters are seen to lie within the open reading frames of *Streptomyces* genes (Deng *et al*. 1986; Horinouchi *et al*. 1986; Distler *et al*. 1987; Stein *et al*. 1989; Babcock and Kendick 1990; Horinouchi *et al*. 1990; Bedford *et al*. 1991; Raibaud *et al*. 1991).

The distance between the transcription start site and the coding region in *Streptomyces* genes ranges from between 9 to 345 nucleotides (Strohl 1992). It is also observed that this region contains regulatory sites such as antiterminators (Piepersberg *et al*. 1988; Li *et al*. 1990). An analysis of the Shine-Dalgarno (S-D) sequences (Shine and Dalgarno 1975) of Gram positive bacterial mRNAs reveal that they form strong complexes with the 3'end of their 16s rRNAs. However, the ability of *Streptomyces* to express *E. coli* genes suggests that streptomycete ribosomes do not require extensive complimentarity between S-D sequence and the 3' end of 16s rRNA in order to initiate translation (Strohl 1992). In case of a few genes it has been observed that transcription and translation may initiate at the same nucleotide or transcription may start one nucleotide upstream of the translation start site (Bibb *et al*. 1985; Bibb and Janssen 1986; Horinouchi *et al*. 1987; Hoshiko *et al*. 1988; Horinouchi *et al*. 1989, 1991).

4.1.2 Molecular biology of PHA synthesizing genes

Till 1980's research in the area of PHAs was limited to the identification of microorganisms synthesizing PHAs, characterization of different types of PHAs and their co-polymers. With advancements in molecular biology, cloning and characterization of genes involved in PHA biosynthesis gained momentum. Though, the number of microorganisms shown to accumulate PHAs is increasing every year, the genetic data regarding the PHA biosynthesis genes remains rather limited.

Different strategies have been employed to identify PHA biosynthesis genes (Table 4.1). Of these only a few allow successful identification of the PHA biosynthesis genes encoding enzymes with novel and / or unusual features (Rehm and Steinbuchel 2001). The most successful and widely used strategy are the screening of genomic libraries for phenotypic complementation of PHA negative mutants

Table 4.1 Strategies for screening and identification of PHA biosynthesis genes (Source: Rehm and Steinbuchel 2001)

Strategy	Screening technology
A	Enzymatic analysis
B	Homologous gene probes obtained by transposon insertions
C	Heterologous gene probes from well characterized genes
D	Consensus Oligonucleotides derived from multiple alignments
E	Oligo nucleotides derived from N-terminal or internal aa sequences of PHA synthases
F	Opaque and fluorescent colonies in PHA negative host After heterologous expression
G	Growth after detoxification of media due to removal of fatty acids
H	Genome sequence analysis and functional assignments of PHA Biosynthesis genes

(Leibergesell and Steinbuchel 1993; Valentin and Steinbuchel 1993) and hybridization with heterologous probes made from well characterized genes of *R. eutropha* (Leibergesell and Steinbuchel 1992; Ueda *et al*. 1996).

Since the physiology and biochemistry of the PHA biosynthesis is well documented, the molecular genetics provides sound basis for a thorough evaluation of the biochemistry and the catalytic mechanisms of PHA synthesis. In the present chapter, sequencing and sequence analysis of the cloned ~5.0 kb g-DNA fragment from *S. aureofaciens* NRRL 2209 has been dealt with.

4.2 EXPERIMENTAL PROCEDURES

4.2.1 Organisms and growth

pSa240 was grown and maintained as detailed in Chapter 2.

4.2.2 DNA Sequencing

ABI Prism Big Dye Terminator Cycle sequencing kit from Perkin Elmer, USA was used to perform DNA sequencing reactions. The primer extension products were

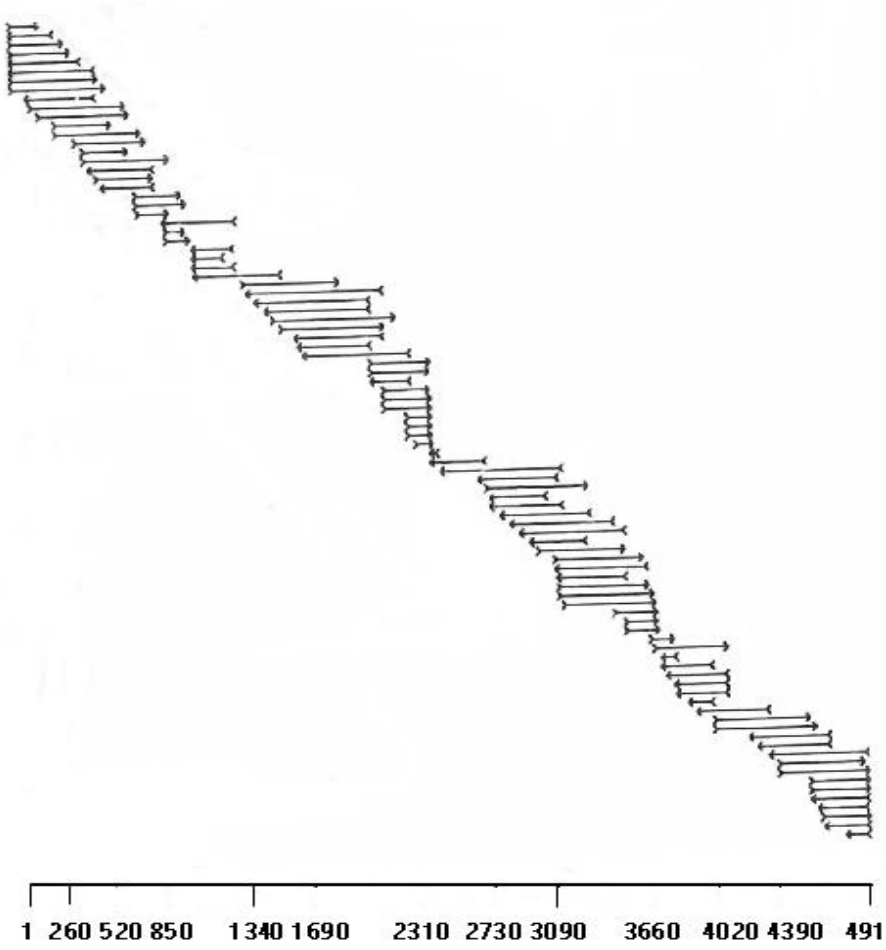

Fig. 4.1 DNA sequencing strategy. DNA sequences generated by transposon mediated insertions. The arrows indicate the direction of DNA sequencing.

separated by gel electrophoresis on an ABI sequencer. A transposon insertion system was used to generate random overlapping sequences. An EZ::TN Kan –2 insertion kit from Epicentre Technologies, USA was used to randomly insert transposon Tn5 into pSa240 plasmid DNA. Colonies resistant to kanamycin and ampicillin were randomly chosen and sequenced using forward (KAN-F) and reverse (KAN-R) primers. Sequences generated were aligned using Sequencher software.

4.2.3 Sequence analysis

Frame plot ver 2.3.2 was used for the sequence analysis due to its high G+C content of the DNA (Bibb *et al*. 1994). Sequence retrieval was from GenBank, NCBI, USA. Sequence alignments were performed using L-FASTA (EMBL), FASTA3 (EMBL), BLAST (NCBI) and CLUSTAL X (Thompson *et al*. 1997).

4.3 RESULTS AND DISCUSSION

Streptomyces genome is known to produce but only short readable sequences due to its high G+C content (Hodgson 2000). The high G+C content affects the polymerase chain reaction due to secondary structure formations (Choi *et al*. 1999). Dideoxy chain termination sequencing of the subclones generated from pSa240 was attempted earlier in our laboratory (Mahishi 2001), however, no more than 100 nucleotides could be read in each pass due to band compactions. Severe band compactions resulting out of intra-molecular base pairing between guanine and cytosine residues unusually lead to unreadable sequencing gel profiles (Motz *et al*. 2000). An *in vitro* transposon insertion kit based on the system developed by Goryshi and Reznikoff (1998) was subsequently used to generate shorter overlapping sequences (Fig 4.1). The contigs generated were joined using Sequencher software.

The complete sequence of the insert in the pSa240 was found to be 4826 bp (Fig. 4.2). This was deposited with GenBank under the accession number AYO32926. The restriction analysis of the sequence done using pDRAW32 (Acalone Software) was found to be in conformity with the restriction endonuclease map generated earlier (see Chapter 3.0).

```
   1 GATCGGCGGC CGGTCGGCGG TGCTGGCCGC GGTGACCCTG GGGGCGCTGG
  51 CCGCTCCGGC GGTGCTGCTG CGCCGGGGGC TGGCGGCCAC CGCGGAGGCG
 101 CTGGCGGCGC TGGCCCTGGT GCTGACGCTG CTGGACGTGT ACGCGGTCCA
 151 CGCGGTGGCC GCGCCGGACA CCGACGGACT CGGCTTCACG GCCCTCGCGT
 201 CGGCGGTGCT CGCGGCGCTG TGGACGGCGT ACGGGCTGGC GCTGGGCAAG
 251 CTGCGCCTGC CGTTGCCGGC CGCCGTGGTG CTGGCCCAGT GGCCGCTGCT
 301 GTTCTGGGCC TGGGCCGTGG GCGCACCGGC GCCGGTGGTC GGGTGGGCGC
 351 TGCTGGCCAC CGCGGTGCTG GACGGGGCGA TCGCCCTGTG GGGCAAGGGC
 401 GCCGGGGTGC GGGTCACGGC GTGCGTCGGT GGAGCGGTGA TGGGCTTCTC
 451 GGCCCTGATG GTGGGCCTGG CGCTGTCCCT GACGGCCCCG GGGCCGCTCG
 501 GGGCGGTGGC TCCGGGCGTG CTGCTGCTGA CGGCCTCGGC GGCGGCCGTG
 551 GCCGGGGCGT GGCGCGCGCC GAAGGGTTTC GCGCGGACGG GTGGTGCGGT
 601 GGCGGGGCTC GCGGCGGTGG CGGCCGTCGG CGGCGTACCG GCGGCGGCGC
 651 TCCCGGCGGG CTGGCGGGTG CTCGCGTACC TGCTGTGCGG TCTCGCGTTG
 701 ACGGCGGTCG TCCGTTCCCG GCTGCCGGGC CACGCCGCGC GCGGGGTACT
 751 GGCGGCGTCG GGGGCGGTGG TGGCCGGCGC GCTGGTGTGG GCGCTGCCGC
 801 CGCTCGCGGC GGTGCTGCTG GGGCCGGTGA CGGTGCTGTC GGACGTGTGG
 851 GCGGGGACGC CGGACGGCTT CCGGTCCGCG CTGGGGTCGA CGCTGCCCTG
 901 GTCGGAGCTG GCCGCGGCCC CGGTGGTGCT CGCGCTGGTG GCCGGGCATG
 951 CTGGGGGCGA GCGTAACCGG AGGTGGCCGT CGGTCGTCCG GCTCCGGCGC
1001 CGTTGGCCGG TCCTTCTGGC TCGACGCCGG CCCCCCGGCA GCACCGGCAG
1051 CGGGAGCCCC GGGCACGGAT GCGCCGGGCG CGGCCGGGGG CGCTGCGCCG
1101 TGGCCCGGCT GGTCCGGCTG GTCCGGCCGG CCCGGTGCGG GGGCCGGTGG
1151 TCGCGGGCGG CCTTCCGCGG CGACGCTGCG CGGGGTCGTC GGCGCGGGCG
1201 CGGTGGCGCT CGGCTGGGGG GCCCTCCTGC TGGCCGGCGC GCTGCTGGAC
1251 GTGCCCCACG CGCTCGCGCT GGCCGGGGAG ACGGCTCTGG TGGGCGTCCT
1301 GCTCGCCCTG GCGGTCCGGG GTGGCGGCGC CGAGCGGGGC GCGACGGCGA
1351 TGCCGGTGAC CGCTCTGGTG GCTTCGGTGG CCGGGGCGGT GAGCGCCGGG
1401 CTGCTGTCGC TGGCGTCCGA GGGGGCCTCG TACGCGGTGT TCGGCGCGCT
1451 GGCGGCGCTG TTCGCCGGGG CCGCTCTGCG GGCGGGCGCC GGGGTGCCGC
1501 GTGCGGTGTT CGCGGTCGCC GCGGTGGTCT GGGGCACCGT GATCACGGGG
1551 TTGGCGGGCC GGTCCCTGGG GCTCGCCCCG CACGAGGCCG CCCCGCTGAT
1601 GCTGCTGGTG CCGGCGCTGA CGGTGCTGCT CGGGGCACGA CTGCGGCGGA
1651 ACCCGGTGGC CTTGCCCGTG GAGCTGACGG GAGCGCTGGG CGCGCTCGTC
1701 GCCGTGGGGC TCGCGGTGTC CGACGCGCCG TTCCTGGCCC TGGTGCTGGC
1751 GCTGTGCGGG GTGCTGGCGG CGGGGACGGC GGTGCGGCCG GAGCGGCGGC
1801 CGGTGGCGGG CTACCTGGCG GCGACGCTGT TCGTGCTGGC CACGTGGGTG
1851 CGGCTGGCGG CCTCGGAGGT GTCGTTCCCG GAGGCGTACA CGCTGCCGGT
1901 GACGGTGCCC GCGCTGCTGG TCGGTGCGGC GCGGCGGCGC CGGGACCCGG
1951 AGGCCTCGTC GTGGACGGCG TACGGGCCGG GGCTCGCGGC GACGCTGCTG
2001 CCCAGCCTGG CGGTCGCCTG GACCGACCCG GACTGGCTCA GGCCGTTGCT
2051 GCTGGGGACG GCGGCGCTGG TGATCACCCT GCTCGGCGCG CGCCACCGGC
2101 TCCAGGCGCT GCTGCTGCTC GGCGGGACGG TGCTGGCACT GGTCGGCCTG
2151 CACGAGCTGG CGCCGTACGT GGTGCAGGTC GCGGGTGCGC TCCCCCGCTG
2201 GCTCCCGCCC GCCCTGGCCG GGCTGTTGTT GCTGGTGGTC GGAGCGACGT
2251 ACGAGCAGCG GCTGCGGGAC GCCCGCCGTC TGAAGGACGC GCTGGGGCGG
2301 ATGCGGTGAG CCGTGCCCGG TCCGGGGGCG CGCAGGTCAC GGCGTCCCCG
2351 GGCCGGGCGC CAGTGGCGTG GGCAACGCAG AGGGCCCGGC CCTCTGTCCG
2401 GGTGGGCGAT ACTGGGTTCG AACCAGTGAC CTCTTCGGTG TGAACGAAGC
2451 GCTCTCCCAC TGAGCTAATC GCCCGGGCGC ACCGCAAACA TTACCCCATG
```

Fig. 4.2 (cont…d)

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2501 TCAGCGGTGC TCCCGGACCG TCCCCGGGCT ACTCGCTGAT CTTCCACGGC
2551 ATGGTGAGCC CGAACTTCCA GACGTAGATC CCGGCCAGCA CCGCCATGAT
2601 CACGAGCCCG AGCGTGGTGA GGATGATGTT GCGCCGCCGG ACCTTGGGAT
2651 CGAGGGCCCG CTGCGCCGCT TCGGTGACCT TGCGCTTGGT CCAGCGCAGC
2701 ACCAGCTGGG CCCAGACGAA CTCGGTCGCC CAGATCGCCA TGCCGCCGAA
2751 GATCACCAGC CAGCCGGGGC CCGGCAGCAC CAGCATGAGC ACACCCGCGA
2801 TCACCACGCC GAGACCGACG ATGAAGACAC CGACCTGCCA GCTCAGGTGG
2851 AGCGCCTTGG ACGCCTTGAT GAAACCCGGC GCCCGCGAGC CCAGCGCGCG
2901 TTCCTCCCGG TCCGATTCCC CCGTGGCGGA TACCGGGGAC GCCTGCTCGG
2951 CGACCTTGCT CCGCTCGTCA CTCTCCGCGT TCATGAAGCT CAACTTACCC
3001 GACCTGTCTC CGTCACTGGA ATGGGCGCAT AACTCAAAGT TACACGCCGC
3051 TGAGCGGGGG ACCCGAAGCG TCACAAATGG GTCAGAGGGG TTTACAACGC
3101 CACCGTAGGT GGCATGTCGA TTTCGCCGAC GTGCGAATCC CCGAGCGCAC
3151 ACTGAGCGAA AGGCCCTGGC GCTTATGAAC ACCACGGTCA GCTGCGAGCT
3201 GCACCTGCGC CTCGTTGTGT CGAGCGAGTC CTCACTGCCT GTACCCGCGG
3251 GCCTGCGGTA TGACACGGCC GATCCCTATG CCGTGCACGC CACCTTCCAC
3301 ACCGGAGCGG AGGAGACGGT CGAATGGGTA TTCGCCCGCG ACCTCCTTGC
3351 CGAGGGGCTG CACCGGCCCA CCGGCACCGG AGACGTCCGC GTCTGGCCAT
3401 CTCGTAGTCA CGGTCAAGGC GTCGTATGCA TCGCCCTGAG CTCCCCAGAG
3451 GGAGAAGCCC TGCTCGAAGC CCCGGCGCGG GCCCTGGAGT CGTTCCTGAA
3501 GAGGACCGAC GCCGCGGTTC CGCCCGGCAC CGAGCATCGT CACTTCGATC
3551 TCGACACGGA GCTCTCCCAC ATCCTGGCCG AGAGCTGAGC CAGGCAGAGA
3601 GCCGCTCTAC GCCGTCCGAC TCGGGGCGAC GGCGTCGTGC TGACAACCGC
3651 ATAGGGCAGA CACCGGCGCC GTCGTCGCGG AATCCACCGC GACGACGGCG
3701 CCGGCGCGTT CCCCGCCGCG CCGCCGGAGG GGTCCGTTCC GCTCTCCGCC
3751 GGGCCCGCAC CGGGCCCGGC ACCGGCCGGC CGAGCCAGTA GAGTCAGCCG
3801 CCATCGGCAG GCGCCCGCCC GCCGGAAGGC CAGGGAGCGA AGCGTGCTGA
3851 TCCCTCACGA CACCCGGATC GCCCTCGACG CGGTGGTCGA TCTGGTGAAC
3901 ACCGCACCGG AGAGCGAGCC GCCGGGGGAC GACCCCGGCG ACAGACACGC
3951 GGGCGGGCCC GAGGACGGTC TCCCCGACAT CGCCGCGCTG TACGCCTTCG
4001 CGGAGCGCCA TCTCATCAGC GGGGTCGGCA CCCTCGGCGA GAAGGACCTC
4051 GGCGCCGTGC GCGACGTCCG GGCCCGCTTC GCCGAGGTCT TCGCGGCGCC
4101 CGACGCCCGC GTCGCCGCCG ACCTGGTCAA CCGGCTCGTC GCGGCGGCCG
4151 GGACCACCCC GCAGCTCACG GACCACGACG GCTACGACTG GCACGTGCAC
4201 TACTTCGCCC CGGACGCCTC GATCGCCGAC CATCTCGCGG CCGACTGCGG
4251 CATGGCGCTG GCCTTCATCA TCGTGGCGGG CGAGCAGGAG CGGCTGCGGC
4301 GCTGCGAGGC CCCGGACTGC GGGCACGCGT TCGTCGACCT GTCGCGCAAC
4351 CGCTCCCGCC GCTACTGCTC CAGCCGTACG TGCGGGAACC GGCTCCACGT
4401 CGCGGCGTAC CGGGCCCGGC GCAAGGAAGC CGCGGGCTGA CGCCCGGCAC
4451 GGTGGCGCGA GGCGTCACAG CACGAAGAGA TCGTGCAGCG CGGCCATCAG
4501 CAGCAGGCCC CCGATCACCG TCAGGAAGAT CATCAGGGGC GGCTGGGAGA
4551 GCGCGAAAAG ACAGCCGCGG GCCTCTTCGG CGGGGGGTGC GGGGGCATCG
4601 CCCCGGGAAG TGTCCACCAT CTCGGGGTGA TCATGACGCA CCGGCGGCGG
4651 TGTTGGCGAT CAACCGGCTT CATTCTCCCG GGAGTTCACC GTCCCGTGGC
4701 CATCGATATT CGCTCCGGCG TACGGGGAGC CGTCAGACAT TCGGACCGCC
4751 GCCCGGAACG CACGCCGGCG GGGCCGGCCG ACGCCTCGGA CGCCGCGCTT
4801 CTCAGATGCC GTGCTTCTTG AGGATC
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Fig. 4.2 4,826 bp sequence of the cloned *S. aureofaciens* g-DNA fragment (GenBank Acc. No. AY032926)

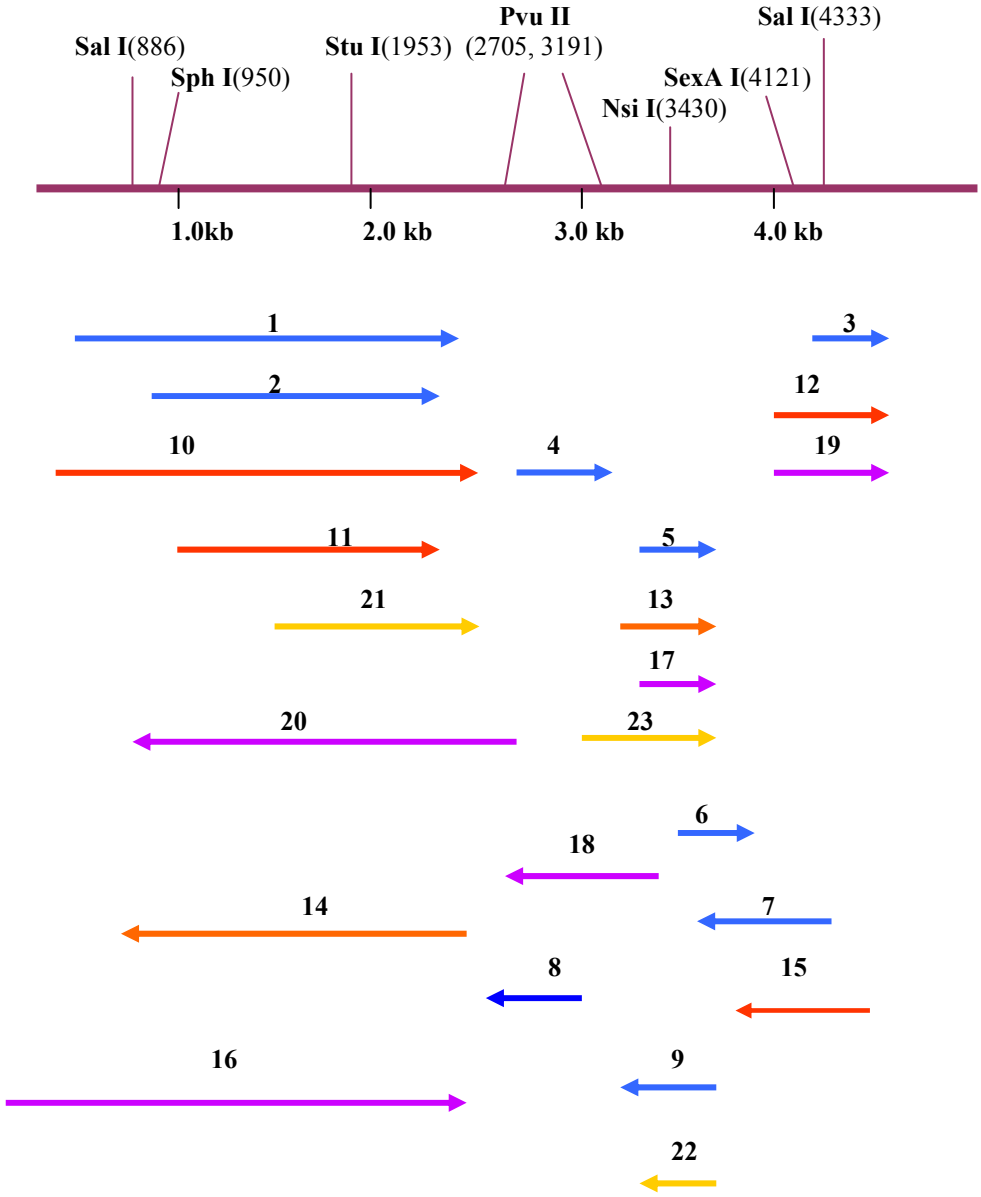

Fig. 4.3 Restrcition endonuclease and ORF map of pSa240 containing a *Streptomyces aureofaciens* ~5.0 kb genomic DNA fragment. → Indicates ORFs starting with ATG as start codon. Similarly →, →, →, represents ORFs starting with CTG, GTG and TTG respectively

4.3.1 Open Reading Frames (ORFs)

The 4.826 kb insert from the pSa240 was found to have a G+C content of 74.6 mol%. Hodgson (2000) had earlier reported the G+C content of *Streptomyces* sp. to be between 69 to 78 mol%. Our results confirm these earlier observations.

ATG is the most often used translation initiation codon in prokaryotes and eukaryotes. However, *Streptomyces* genes are known to use CTG, TTG, GTG and ATT also as the translational initiation codon besides ATG (Bibb *et al*. 1984). In each instance the codon is read as formyl-methionine. The nucleotide sequence of pSa240 was analyzed for putative ORFs using *Frame plot* ver 2.3.2 (Bibb *et al*. 1984) and 23 possible functional ORFs were identified (Fig 4.3). Among these nine were identified as ORFs with ATG as the translation initiation codon. Six ORFs with GTG, five with CTG and three with TGG as the translation start codon were also identified. However, no ORF of any considerable and functional size was identified with ATT as the translation initiation codon.

The nine ORFs with ATG as the start codon spanned from nucleotide numbers:

ORF 1: 440-2443; ORF 2: 948-2309; ORF 3: 4252-4440; ORF 4: 2596-3108; ORF 5: 3175-3588; ORF 6: 3426-3791; ORF 7: 4233-3607; ORF 8: 2984-2529 and ORF 9: 3652-3191.

The ORFs with GTG as the translation start codon were:

ORF 10: 20-2443; ORF 11:1203-2309; ORF 12: 3844-4440; ORF 13: 3109-3588; ORF 14: 2153-416 and ORF 15: 4398-3613,

The ORFs with CTG as the translation start codon were identified as:

ORF 16: 23-2443; ORF 17: 3166-3588; ORF 18: 3191-2531; ORF 19: 3847-4440; ORF 20: 2258-416.

The ORFs with TTG as the translation start codon were:

ORF 21: 1551-2309; ORF 22: 3646-3191; ORF 23: 2629-3588.

4.3.2 Analysis of the ORFs

ORF 1 extends between nucleotides 440 and 2443 with ATG as the translation initiation codon (Fig. 4.3). It encoded a protein of approximately 667 deduced

amino acids. This ORF in the pSa240 clone was in opposite orientation with respect to the *lac*Z promoter of the pGEM-3Z vector and hence, could not be expressed under the control of the *lac*Z promoter. It may , however, be expressed under the control of its own promoter sequence. *Streptomyces* sp. are known to exhibit a wide diversity in promoter sequences (Strohl 1992). Some *Streptomyces* promoters which are temporally regulated e.g. *sapA-pi* (Forsman and Jaurin 1987; Schauer *et al*. 1988), *nsnA-p1* (Li *et al*. 1991) and a*1-p* (Farkasovsky *et al*. 1991) are not active until late in the growth phase of the respective organism. These promoters, however, possess no significant sequence similarity among themselves (Strohl 1992). Promoter sequences showing similarity to –10 (^{298}GCTGTT^{303}) and –35 (^{276}TGGTGC^{281}) motifs were identified in ORF 1 (Fig. 4.4). However, two more sequences showing similarity to *bar-p1* (Janssen and Bibb 1988) and *orf-p3* (Strohl 1992) promoters were also identified spanning nucleotides 384-408 and 20-73 respectively (Fig. 4.4). (The significance of these sequences in PHB synthesis will be discussed in Chapter 5). Nucleotides –9 to –6 upstream of ^{440}ATG is an apparent ribosome binding region (RBS) ^{431}GGAG ^{434} (Fig. 4.4) which shows complimentarity to the 3' end of the 16S rRNA of *S. lividans* (Strohl 1992).

While PHB synthesis in *S. aureofaciens* NRRL 2209 is temporally regulated (since PHB accumulation occurs only during late log phase), the set of genes responsible for its synthesis when expressed heterologously in *E. coli*, express in an unregulated and constitutive manner (since PHB accumulation occurs through the growth cycle of the recombinant organism) (ref. Chapter 3). The gene(s) responsible for PHB synthesis in *S. aureofaciens* might possess promoter sequence(s), which are responsive to *trans*- or *cis*-acting functions. It is a well established fact that the genes which are expressed temporally require the expression of other genes, which provide *trans*- or *cis*-acting functions (Vujaklija *et al*. 1991; Gonzalez *et al*. 1997). Three possibilities exist in the present case. In the first instance, an ORF for the *cis*-acting element might be present in the cloned ~ 5.0 kb g-DNA fragment. In which case it might be expressed constitutively in recombinant *E. coli* due to change in the host environment. This in turn results in expression of the PHB biosynthesis genes and consequent PHB accumulation during the entire growth cycle of the recombinant

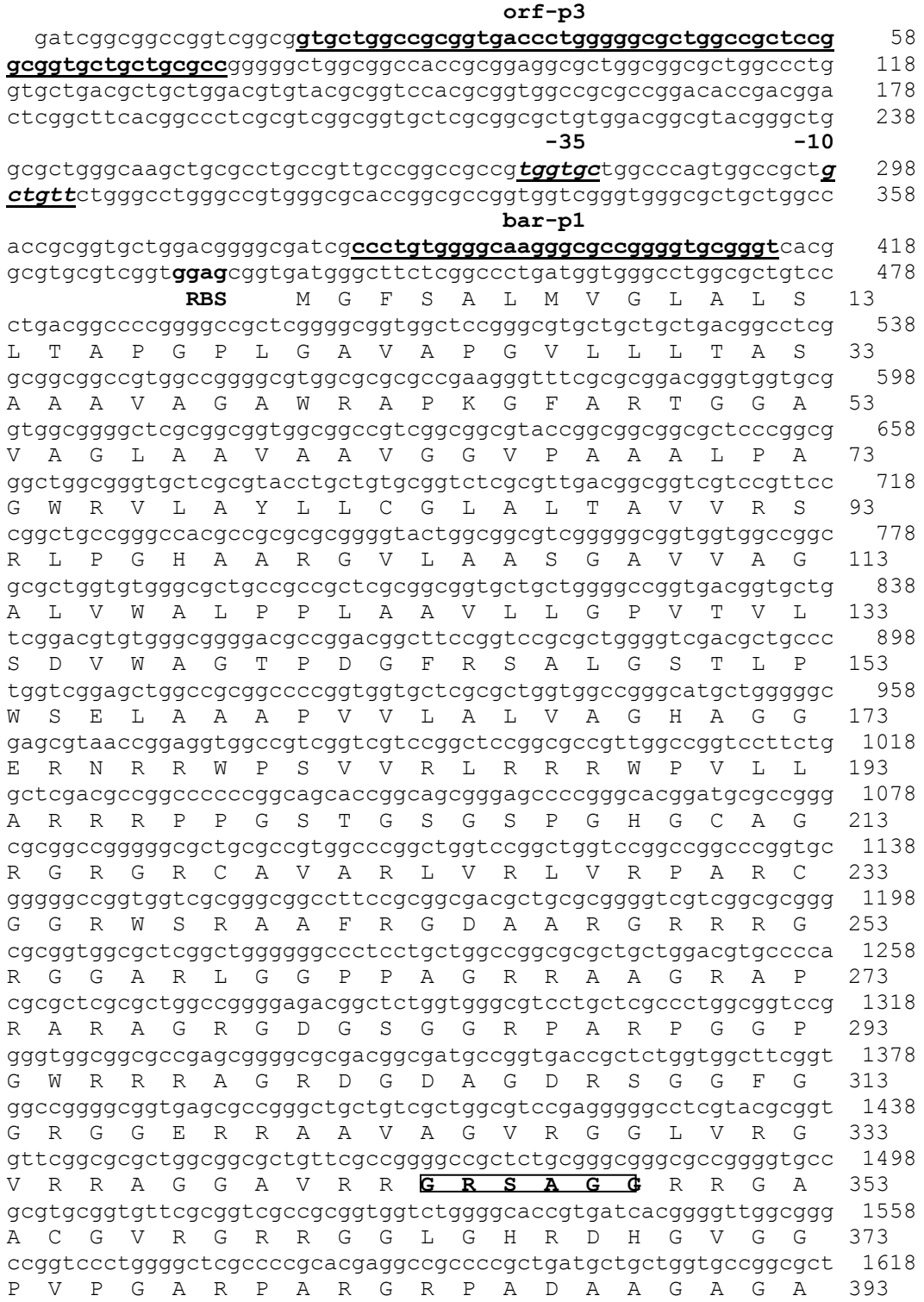

Fig. 4.4 (See next page for legends)

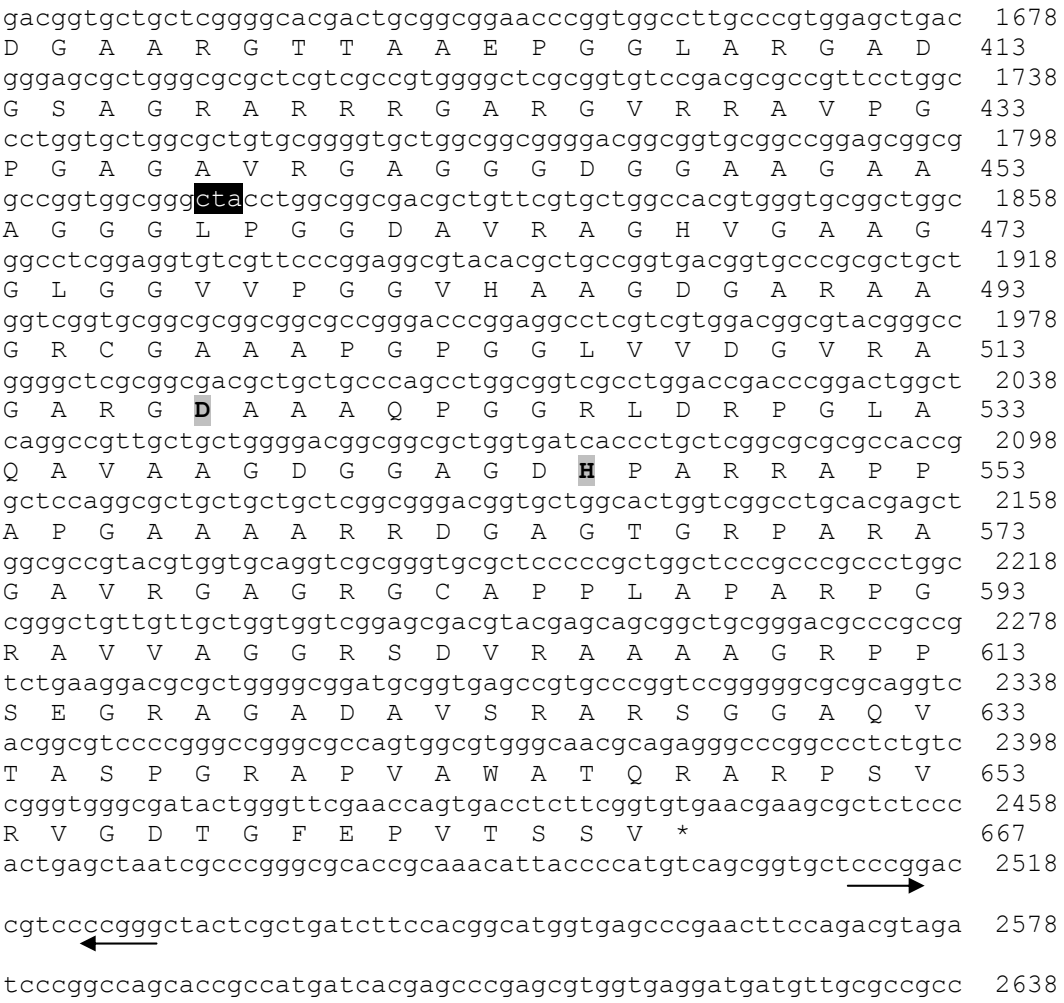

Fig. 4.4 Putative *pha*C_{Sa} with nucleotide and amino acid sequence. CTA coding for rare leucine codon is shown as white letters on black background. Lipase box is shown enclosed in a box, while Aspartic acid and Histidine involved in traid formation are shown in black letters on grey background. Inverted repeats are shown by the arrows.

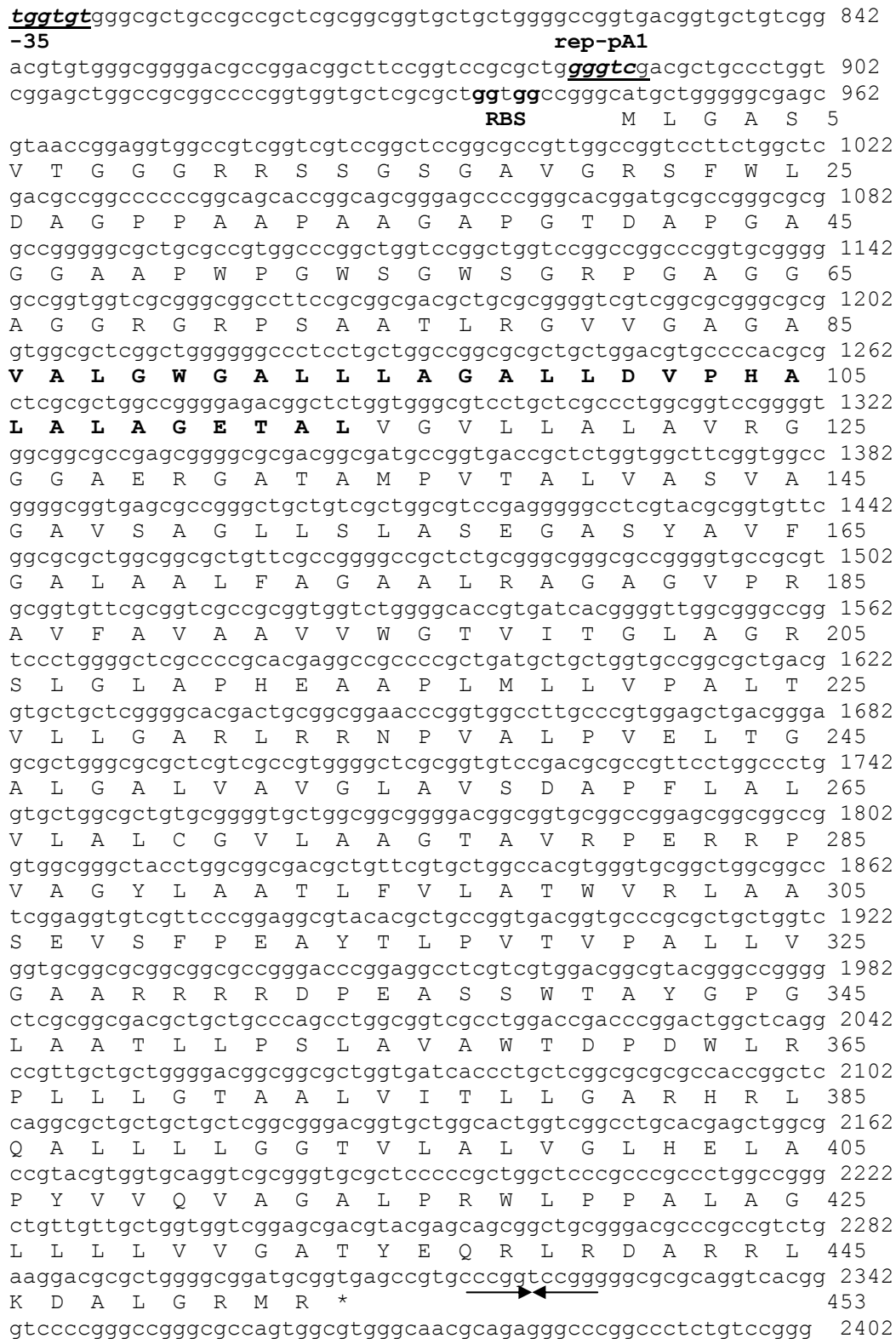

Fig. 4.5 Putative *pha*B_{Sa} with nucleotide and amino acid sequence. NADPH binding site is shown in bold letters. Inverted repeats are shown by arrows.

organism. In the second instance, the regulating element might not be present in pSa240 clone and the host machinery recognizes the PHB biosynthesis genes promoters and aids in their unregulated expression. In the third instance, the PHB biosynthesis genes promoter might be under a tight hormonal regulation in the native organism. But due to the absence of this effect in the host organism the PHB biosynthesis genes are expressed constitutively as seen in A-factor dependent streptomycin production (Vujaklija *et al*. 1991).

PHA synthases from different organisms vary in their given nucleotide size from 1068 bp in *Ectothiorhodospora shaposhnikovii* (GenBank Acc. No. AF307334) to 2011 bp in *Caulobacter crescentus* (Qi and Rehm 2001). Correspondingly, the apparent molecular mass of PHA synthases varies from ~40 kDa to ~73 kDa. The protein encoded by ORF 1 has an apparent molecular mass of 73 kDa. This size compares well with the molecular mass of *C. crescentus* PHA synthase (Qi and Rehm 2001). PHA synthases are members of prokaryotic lipase superfamily (Jia *et al*. 2000) which in turn is a member of α / β hydrolase family (Heinkinheimo *et al*. 1999). A conserved lipase box, G-X-S/C-X-G-G, wherein cystein or serine serves as the active nucleophile is a special feature of the lipases (Steinbuchel and Hein 2001). The deduced amino acid sequence of the ORF 1 showed the presence of the conserved lipase box, ^{344}G-R-S-A-G-G^{349} (Fig. 4.4). While cystein is reported to serve as the active nucleophile in PHA synthases reported so far (Steinbuchel and Hein 2001), the presence of a serine in the present case would make it first amongst the PHA synthases to have it as the active nucleophile. Aspartic acid and Histidine residues present at positions 518 (D^{518}) and 546 (H^{546}) probably form a part of the catalytic triad needed for synthase function (Fig 4.4). Among the reported PHA synthases, only marginal sequence identity of 3% has been reported (Steinbuchel and Hein 2001). Given the observations that ORF 1 shows the presence of: (a) a well defined coding domain, (b) presence of promoter sequences upstream of the coding region, (c) a RBS site in the correct context with regards to the translation start signal, (d) the presence of the conserved catalytic triad and (e) a molecular mass within the accepted limits, this ORF may be the putative PHA synthase gene of *S. aureofaciens* (*pha*C$_{Sa}$).

In ORF 1, besides ^{440}ATG translation start codon, several other in frame translational start codons were identified. While ^{20}GTG (ORF10), ^{23}CTG (ORF 16), ^{275}GTG, ^{365}GTG, ^{407}GTG and ^{437}GTG were found upstream of the ^{440}ATG, ^{458}ATG and ^{461}GTG were identified downstream of it. Since no RBS sites and promoter sequences could be identified upstream of ^{20}GTG (ORF10), ^{23}CTG (ORF 16) the possibility of transcription and subsequent translation was ruled out. Interestingly ^{437}GTG precede ^{440}ATG and ^{461}GTG follows ^{458}ATG. In some instances the *Streptomyces* sp. genes may permit the use of GTG as the translation initiation codon provided it lies in the vicinity of an ATG codon and any ATG or GTG codon that lie downstream of a translation start codon may serve as the secondary start codon (Horinouchi *et al*. 1989). Due to the presence of a strong RBS site near ^{437}GTG or ^{440}ATG, ORF 1 may start at either nt 437 or at nt 440, while ^{458}ATG or ^{461}GTG may serve as the secondary start codons.

The deduced translation product of ORF 2, nucleotides 948 to 2309 (Fig. 4.5), encoded for a protein of approximate molecular mass of 49 kDa. At position 783 a sequence similar to –35 box, ^{783}TGGTGT^{788}, was found (Strohl 1992). However, no corresponding –10 box was identified in its vicinity. A promoter similar to *rep-pA1* –10 promoter sequence (^{884}GGGTC^{888}) was found (Sinclair and Bibb 1989). In *Streptomyces* sp. –10 and / or –35 or both may act as promoter (Strohl 1992). The orientation of ORF 2 in opposite direction to the *lacZ* promoter sequence and the non-presence of a proper promoter sequence upstream of the translation start codon, it is quite likely that the promoter sequence of the putative *pha*C_{Sa} gene may be used much in a way similar to the *pha*B_{Re} gene of *R. eutropha* (Peoples and Sinskey 1989). Upstream of the ^{948}ATG a RBS site with the sequence ^{937}GGTGG^{941} was also present. The nucleotide sequence of ORF 2 (nt 948 – nt 2309) did not show any substantial homology with the reported gene sequences. However, like other NADPH dependent acetoacetyl CoA reductases wherein the NADPH binding motif is present at the N-terminal of the protein, ORF2 also showed the presence of the conserved NADPH binding motif, ^{86}V-A-L-G-W-G-A-L-L-A-G-A-L-L-D-V-P-H-A-L-A-L-A-G-E-T-A-L^{114} (Fig. 4.5) (Oliver *et al*. 1988). By virtue of the conserved NADPH domain, ORF 2 may be the putative *pha*B_{Sa} gene.

Besides ^{948}ATG other translation start sites at ^{1203}GTG (ORF 11), ^{1350}ATG, ^{1506}ATG, ^{1551}TTG (ORF 21) and ^{1596}CTG were also found. However, except for ^{1203}GTG, all other start codons were found down stream of the conserved NADPH binding motif, thus ruling out the possibility of any of these being the translation initiation start of the putative *pha*B_{Sa}.

The 453 AA putative protein encoded by ORF 2 is larger than the gene product of any *pha*B gene reported thus far. In *R. eutropha* and *A. vinelandii* the *pha*B gene codes for 246 and 247 amino acids respectively. However, in the eventuality of ^{1203}GTG (ORF 11) being the true translation initiation codon the gene product would be smaller by 89 AA and have an ~ Mol. Wt. Of 40 kDa.

From the above it is clear that the ORF 2 lies within the ORF 1. The presence of individual RBS sites and promoter sequences for both the ORFs suggests of two transcriptional start points (*tsp*) in the same direction for the above ORFs operating from different frames. This is a rare though not an unusual feature in *Streptomyces* wherein the down stream *tsp* is present within a large ORF that is being transcribed upstream at the same time (Babcock *et al*. 1990). Downstream of the ORF 1 and ORF 2 inverted repeat sequences which could form stem loop structures and serve as the transcription termination signal were identified (Fig. 4.4 and 4.5).

Since the pSa240 clone was isolated using heterologous probes, the putative *pha*C_{Sa} and *pha*B_{Sa} gene sequences were checked for sequence homology to the Nco I / Stu I and XcmI / Nde I fragments respectively of the *pha*C and *pha*B genes from *R. eutropha*. The putative *pha*C_{Sa} gene showed a sequence similarity of 52 % to Nco I / Stu I fragment while the putative *pha*B_{Sa} showed ~51 % similarity to Xcm I / Nde I fragment (Fig. 4.6 and 4.7). Upon analysis, none of the other ORFs of ~5.0 kb pSa240 sequence showed any significant sequence similarity to either *pha*C_{Sa} or *pha*B_{Sa} gene probes.

None of the ORFs of pSa240 insert showed any sequence similarity to the β-ketothiolase gene of *R. eutropha*. It was assumed that the gene for β-ketothiolase is present either distantly or some other ORF might be substituting for *pha*A activity. Also, the possibility of the host *E. coli* supplementing the *pha*A enzyme cannot be ruled out. In the eventuality of the *E. coli* β-ketothiolase not taking part in the PHB

```
      1365          1375          1385          1395          1405          1415
Re3'  TGGAAGACCTGACACGCGGCA - - -AGATCTCGCA GACCGACGAGAGCGCGTTTGAGGTCG
      :  :    :      : :       :  : : : :     : : : :  : :    :    :    :     :    :  :  :  : : :
sa240 TCGCCGCGGTGGTCTGGGGCACCGTGATCACGGGGTTGGCGGGCC GGTCCCTGGGGCTCG
      1080          1090          1100          1110          1120          1130

      1425          1435          1445          1455          1465          1475
Re3'  - GCCGCA - - ATGTCGCGGTGACCGAAGGCGCCGTGGTCTTCGAGAACGAGTACTTCCAG-
         : : : :    :  :  : : :     :  :  : :  :    : :  :   :  :  : :      : : :   : :  : :  :   :
sa240 CCCCGCACGAGGCCGCCCCG – CTGATGCTGCTGGTGCCGGCGCTGACG - GTGCTGCTCGG
      1140          1150          1160          1170          1180          1190

            1485      1495          1505          1515          1525          1535
Re3'  - - - - CTGTTGCAGTACAAGCCGCTGACC - - - GACAAGGTGCACGCGCGCCCGCTGCTGAT
            :    : : :  :    : : :  : :  : :        :   : :  : :     : :  :   : : : : :
sa240 GGCACGACTGCGGCGGAACCCGGTGGCCTTGCCCGTGGAGCTGACGGGAGCGCTGGGCGC
      1200          1210          1220          1230          1240          1250

      1545          1555          1565          1575          1585          1595
Re3'  GGT - GCCGCCGTGCATCAACAAGTACTACATCCTGGACCTGC- AGCCGGAGAGCTCGCTG
      :  :   :  : : : : : :          :    :    :  :  :    :      : :  :    :  : : :  : :  :
sa240 GCTCGTCGCCGTGGGGC- TCGCGGTGTCCGACGCGCCGTTCCTGGCCCTGGTGCTGG C - G
      1260          1270          1280          1290          1300          1310

      1605        1615          1625          1635          1645          1655
Re3'  GTGCGCCATGTGGTGGAGCAGGGACATACGGTGTTTCTGGT GTCGTGGCGCAATCCGGAC
        : :  : :     : : :  : : :  :    : : :      : : : :  :  :  :  :    :   :  : : : :          : :
sa240 CTGTGCGGGGTGCTGGCGGCGGG- - - GACGGCGGTGCGGCCGGAGCGGCGGCCGGTGGCG
      1320              1330          1340          1350          1360

      1665        1675          1685          1695          1705          1715
Re3'  GCCAGCATGGCCG - GCAGCACCT- GGGACGACTACATCGAGCACGCGGCCATCCGCGCCA
      :  :   :  : : : :  :  :  :   : :   :  :  :   :  : : :  :  :      : :  :  :  :   : :  : :
sa240 GGCTACCTGGCGGCGACGCTGTTCGTGCTGGCCACGTGGGTGCGGCTGGCGGCCTCGGAG
      1370          1380          1390          1400          1410          1420

      1725        1735          1745          1755          1765          1775
Re3'  TCGAAGTCGCGCGCGACAT- CA -GCGGCCAGGACAAGATCAACGTGCTCGGCTTCTGCGT
         :   : :   :   :  :  :  : :  : :  : : :  : :   :  :  :      : :  : : :    : : :   :    : :
sa240 GTGTCGTTCCCGGAGGCGTACACGCTGCC – GGTGACGGTGCCCGCGCT - -GCTGGTCGGT
      1430          1440          1450          1460          1470          1480

      1785        1795          1805          1815          1825          1835
Re3'  GGGCGGCACCATTGTCTCGAC CGCGCTGGCGGTGCTGGCCGCGCGCGGCGAGCACCCGGC
      :  :   : :   :    :  :   : : :  :  : :   : : :      : :  :  : :  :   : : : : :    :    :  :
sa240 GCGGCGCGGCGGCGCCGGGACC –CGGAGGC- - - - CTCGTCGTGGACGGCGTACGGGCCGG
      1490          1500          1510          1520          1530          1540

      1845        1855          1865          1875          1885          1895
Re3'  CGCCAGCGTC – ACGCTGCTGACCACGCT - - - GCTGGACTTTGCCGACACGGGCATCCTCG
        : :    : : :  :  : : : : : : : :  : : :    : :      :  :  :  : :      : : : :  : :  :  :     : : :
sa240 GGCTCGCGGCGACGCTGCTGCCCAGCCTGGCGGTCGCCTGGACCGACCCGGACTGGCTC -
      1550              1560          1570          1580          1590          1600

      1905        1915          1925          1935          1945          1955
Re3'  ACGTCTTTGTCGACGAGGGCCATGTGCAGTTGCGCGAGGCCACGCTGGGCGGCG-GCGCC
      :  :  :  : : :  :    :  :  : :  :       :    :  : :    :  :   : : :  : : :   : : : : :  : : : : :
sa240 AGGCCGTTGCTGCTGGGGAC - - - - - GGCGGCGCTGGTGATCACCCTGCTCGGCGCGCGCC
      1610          1620              1630          1640          1650          1655
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Fig. 4.6 (Con…..td)

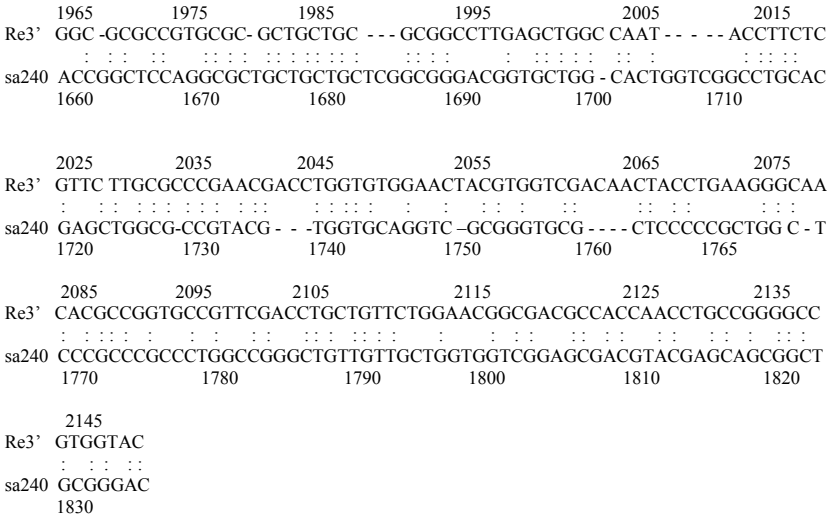

Fig. 4.6 Sequence similarity of putative *pha*C_{Sa} with the 1.0 kb Nco I / Stu I fragment of *Ralstonia eutropha*(Re 3' in the figure). Both share a sequence similarity of 52.3%

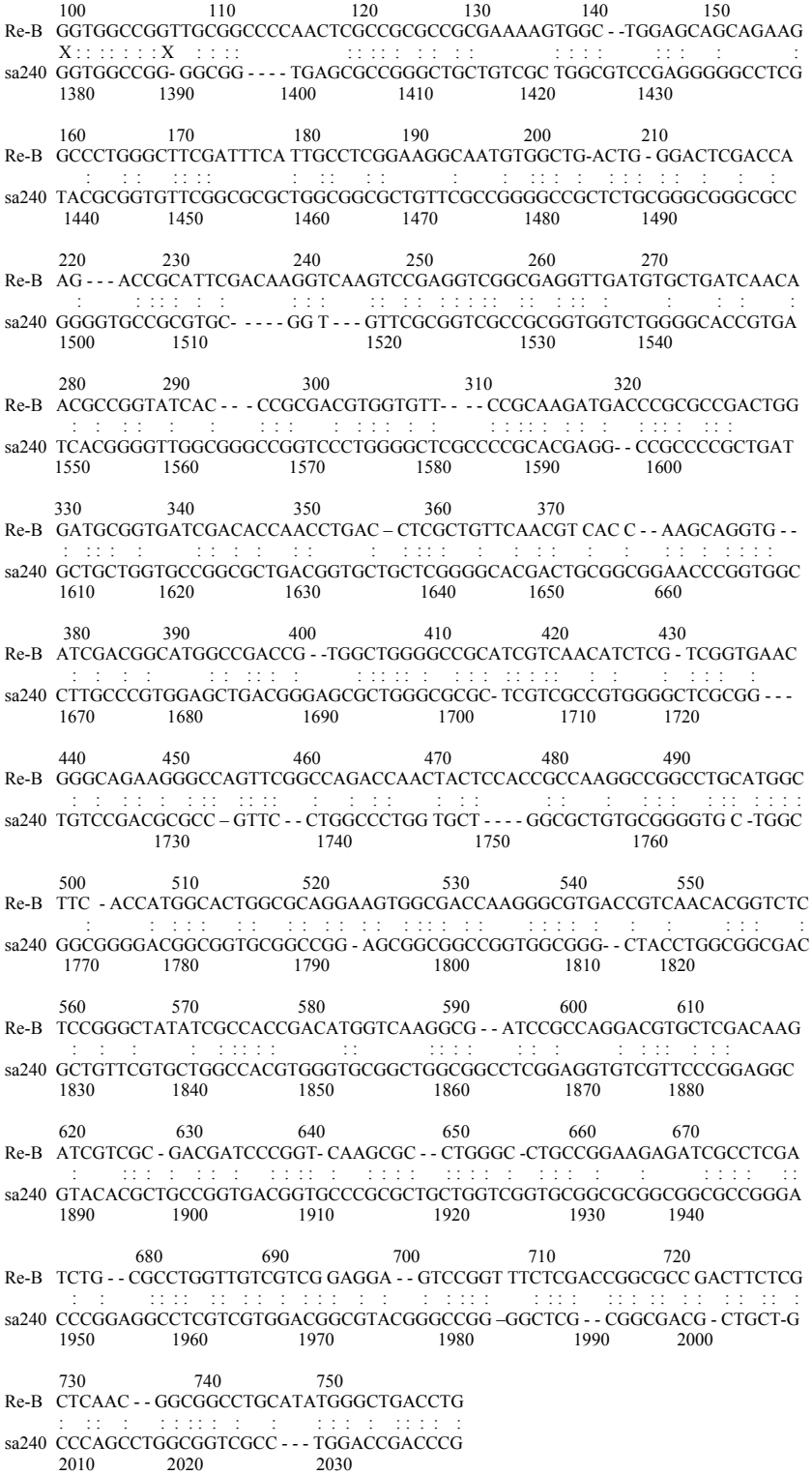

Fig. 4.7 Sequence similarity of putative *pha*B_{Sa} with the 0.74 kb Xcm I /Nde I Fragment of *R. eutropha*(Re-B in the figure). Both share a sequence similarity of 50.9%

*1 *2 *3

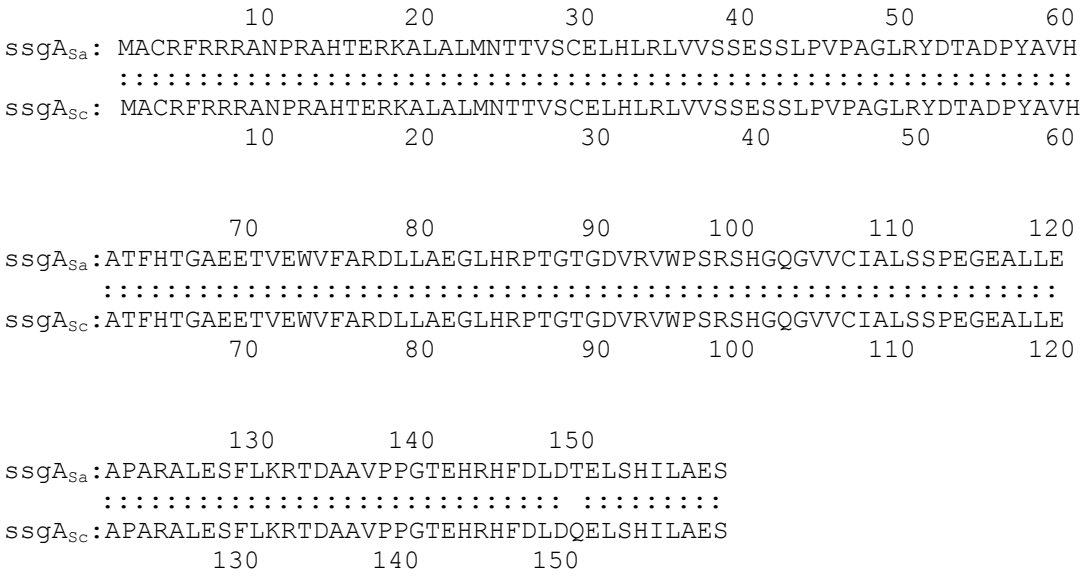

Fig. 4.8 ORF 13 (*ssg*A$_{Sa}$) sequence showing 99.37 % sequence identity to *ssg*A gene of *S. coelicolor*. *1, *2, *3 represent the GTG, CTG and ATG translational start codons respectively.

synthesis, intermediates of the fatty acid β-oxidation or fatty acid synthesis may be channelized to PHB synthesis as in the case of *Pseudomonas aeruginosa* (Langenbach *et al*. 1997).

ORF 5 encoding a protein of 15 kDa starts with ATG as the translation initiation codon at nt 3175 and ends at nt. 3588. Fasta3 analysis of the ORF 5 deduced amino acid sequence showed 75% sequence similarity (Fig 4.8) with SsgA protein of *S. coelicolor* (EMBL Acc. No AL939109 CAB709441). Other inframe translational start codons were found upstream of ORF 5. These were ^{2629}TTG (ORF 23), ^{3109}GTG (ORF 13) and ^{3166}CTG (ORF 17). Upon analysis, a 99.37 % homology to the full SsgA protein sequence was observed with ORF 13 using ^{3109}GTG as the translation start codon. A conserved RBS site, ^{2619}GAGGA^{2624}, is found upstream of ^{2629}TTG but not upstream of ^{3109}GTG translation initiation codon. However, it is suggested that *Streptomyces* ribosomes do not require extensive complimentarity between the S-D sequences and the 3' end of the 16S rRNA in order to initiate translation. A promoter sequence corresponding to –10 (^{2575}TAGATC^{2580}) and –35 (^{2552}TGGTGA^{2557}) motifs

was also found. The SsgA protein is the regulatory protein essential during sporulation and cell division (Wezel *et al*. 2000).

ORF 3 (Fig. 4.3), starting with ATG, reads between nucleotides 4252-4440 and showed 75.2% sequence similarity with the *S. coelicolor* hypothetical protein of unknown function (EMBL Acc. No. AL939109). ORFs 12 (GTG start codon) and 19 (CTG start codon) are located upstream of ORF 3 and read into the same frame. Though a RBS site is found near all the ORFs, a promoter with –10 (^{4011}TCTCAT^{4016}) and –35 (^{3938}CTGTAC^{3943}) was found only upstream of the ^{4252}ATG translation initiation start codon.

ORF 8 encoded between nucleotides 2984-2531 with ^{2984}ATG as start codon shows 73.68% sequence similarity to the putative membrane protein of *S. coelicolor* (EMBL Acc. No. AL939109 CAB70942). A RBS site ^{2994}GAAGA^{2990} and a promoter sequence with –10 (^{3021}TTCCAG^{3016}) and –35 (^{3054}TGTAAC^{3048}) lie upstream of ^{2984}ATG. Though the exact nature of the proteins coded by the ORFs 3 and 8 are not known it is assumed that they might be aiding in regulatory functions.

ORF 6, encoding a 122 amino acids protein product, extends from nucleotides 3426-3791 (Fig 4.3). This protein has shown 33.87% similarity to the P0401G10 protein of *Oryza sativa* (EMBL Acc. No. AP003238 BAB89002), which has got a proline rich region and an eukaryotic RNA binding region in it. Upstream of the translation start codon a RBS site ^{3417}AGGCAG^{3422} and a promoter with –10 (^{3215}TTGTGT^{3220}) and –35 (^{3192}TGCGAG^{3197}) were identified. The proline rich regions that are found in Gram negative *ton* B genes (Moeck and Coulton 1998) are found to bind and transport iron from the host or environment into the cell cytoplasm. This gene product is found to be present in the periplasmic place of the bacterium.

ORF 9 extends from 3652-3191 with ^{3652}ATG as the start codon. It encodes a protein of 16.94 kDa. ORF 22 starting with TTG at 3646 nucleotides was found downstream of the ^{3652}ATG translational start codon. A RBS site (^{3664}GGTG^{3661}) and a promoter like sequence with –10 (^{3840}TCGCTC^{3835}) and –35 (^{3862}TGTCGT^{3857}) were identified upstream of ^{3652}ATG. ORF 9 showed 33.5% sequence similarity to DNA binding protein of *Bifidobacterium* sp. at the amino acid level (EMBL Acc. No. AJ318689 CAC41624). It probably binds to one of the ORFs, thus enabling its

transcription or exerting a regulatory effect.

ORFs 4, 7, 14, 15 and 20 have shown no sequence similarity to the existing sequences in the databanks. Still their involvement in PHB synthesis cannot be ruled out since, PHA synthesizing and regulatory genes are found to be clustered in many bacteria. Phasins, low molecular weight proteins, are proposed to promote PHA synthesis by binding to granules and increasing surface / volume ratio of the granules. Amino acid sequences of phasin proteins are dissimilar even in closely related bacteria (Sudesh *et al* 2000; Steinbuchel and Hein 2001). ORFs 4, 7 and 15 might be coding for phasins.

In many bacteria harboring the PHB synthesizing genes, an ORF located on the opposite strand with regards to the PHB biosynthesis genes has been designated as *pha*Q. Its gene product is speculated to regulate PHA metabolism (Madison and Huisman 1999). ORF 20 encompassed by nt 2258 to nt 416 with translation initiation codon at ^{2258}CTG lies on the negative strand with respect to the putative PHB biosynthesis genes of *S. aureofaciens*. This gene may also use ^{2153}GTG, which lies in frame, as the translation initiation codon. Both the ORFs have their individual RBS sites and possess a promoter sequence with a –10 (^{2420}CTTGGT^{2425}) and –35 (^{2443}TTGCTT^{2448}).

4.3.3 CODON USAGE

Streptomyces genome is highly GC rich with 69 to 78 mol % G+C (Hodgson 2000). Despite extremely high overall G+C content individual genes in Streptomycetes are known to vary in G+C content between 61 to 80 %. The incidence of G or C at the third position of a codon varies between 76 to 98 % (Wright and Bibb 1992). A correlation exists between AT / GC bias at the nucleotide level and the content of AT and GC rich codons (Foster and Hickey 1999). Organisms which are AT-rich have gene sequences encoded by AT-rich codons including the phenylalanine (F), tyrosine (Y), methionine (M), isoleucine (I), asparagine (N) and lysine (K). The same are correspondingly poor in amino acids coded for by GC-rich codons: glycine (G), alanine (A), arginine (R) and proline (P) (Sueoka 1961; Jukes and

Table 4.2: Codon Usage Table for *Streptomyces aureofaciens* [gbbct]: 58 CDS's (20257 codons). Rare leucine codons are shown as white letters on black background

AmAcid	Codon	Number	/1000	Fraction	..	AmAcid	Codon	Number	/1000	Fraction	..
Gly	GGG	369.00	18.22	0.19		Trp	TGG	279.00	13.77	1.00	
Gly	GGA	137.00	6.76	0.07		End	TGA	47.00	2.32	0.81	
Gly	**GGT**	**171.00**	**8.44**	**0.09**		Cys	TGT	9.00	0.44	0.06	
Gly	GGC	1236.00	61.02	0.65		Cys	TGC	135.00	6.66	0.94	
Glu	GAG	1092.00	53.91	0.86		End	TAG	5.00	0.25	0.09	
Glu	GAA	179.00	8.84	0.14		End	TAA	6.00	0.30	0.10	
Asp	GAT	41.00	2.02	0.03		Tyr	TAT	8.00	0.39	0.02	
Asp	GAC	1195.00	58.99	0.97		Tyr	TAC	421.00	20.78	0.98	
Val	GTG	703.00	34.70	0.42		Leu	TTG	49.00	2.42	0.02	
Val	GTA	38.00	1.88	0.02		**Leu**	**TTA**	**1.00**	**0.05**	**0.00**	
Val	GTT	22.00	1.09	0.01		Phe	TTT	8.00	0.39	0.01	
Val	GTC	905.00	44.68	0.54		Phe	TTC	607.00	29.96	0.99	
Ala	GCG	937.00	46.26	0.34		Ser	TCG	279.00	13.77	0.28	
Ala	GCA	87.00	4.29	0.03		Ser	TCA	10.00	0.49	0.01	
Ala	**GCT**	**48.00**	**2.37**	**0.02**		Ser	TCT	4.00	0.20	0.00	
Ala	GCC	1659.00	81.90	0.61		Ser	TCC	392.00	19.35	0.40	
Arg	AGG	55.00	2.72	0.03		Arg	CGG	580.00	28.63	0.36	
Arg	AGA	8.00	0.39	0.00		Arg	CGA	26.00	1.28	0.02	
Ser	AGT	30.00	1.48	0.03		Arg	CGT	124.00	6.12	0.08	
Ser	AGC	276.00	13.62	0.28		Arg	CGC	828.00	40.87	0.51	
Lys	AAG	444.00	21.92	0.97		Gln	CAG	508.00	25.08	0.98	
Lys	AAA	14.00	0.69	0.03		Gln	CAA	12.00	0.59	0.02	
Asn	AAT	5.00	0.25	0.01		His	CAT	23.00	1.14	0.05	
Asn	AAC	364.00	17.97	0.99		His	CAC	464.00	22.91	0.95	
Met	ATG	326.00	16.09	1.00		Leu	CTG	1187.00	58.60	0.59	
Ile	ATA	6.00	0.30	0.01		**Leu**	**CTA**	**2.00**	**0.10**	**0.00**	
Ile	ATT	12.00	0.59	0.02		Leu	CTT	19.00	0.94	0.01	
Ile	ATC	657.00	32.43	0.97		Leu	CTC	740.00	36.53	0.37	
Thr	ACG	354.00	17.48	0.28		Pro	CCG	676.00	33.37	0.56	
Thr	ACA	15.00	0.74	0.01		Pro	CCA	5.00	0.74	0.01	
Thr	ACT	14.00	0.69	0.01		Pro	CCT	20.00	0.99	0.02	
Thr	ACC	867.00	42.80	0.69		Pro	CCC	507.00	25.03	0.42	

Coding GC 72.14%; 1st letter GC 71.83%; 2nd letter GC 50.32%; 3rd letter GC 94.27%

Table 4.3a Deduced amino acid composition of the putative *phaC*$_{sa}$ gene product

Number of amino acids: 667

Theoretical pI: 12.23

Amino acid composition:

Ala (A)	149	22.3%
Arg (R)	102	15.3%
Asn (N)	1	0.1%
Asp (D)	23	3.4%
Cys (C)	7	1.0%
Gln (Q)	4	0.6%
Glu (E)	6	0.9%
Gly (G)	151	22.6%
His (H)	8	1.2%
Ile (I)	0	0.0%
Leu (L)	43	6.4%
Lys (K)	1	0.1%
Met (M)	2	0.3%
Phe (F)	6	0.9%
Pro (P)	**53**	**7.9%**
Ser (S)	26	3.9%
Thr (T)	15	2.2%
Trp (W)	10	1.5%
Tyr (Y)	1	0.1%
Val (V)	59	8.8%

Total number of negatively charged residues (Asp + Glu): 29
Total number of positively charged residues (Arg + Lys): 103
Total number of Arginine and Alanine residues (Arg + Ala): 251
Total number of GC rich residues (G, A, R, P, V): 76.1 %

Table 4.3b: **Deduced amino acid composition of the putative *phaB*$_{sa}$ gene product**

Number of amino acids: 453

Theoretical pI: 11.29

Amino acid composition:

Ala (A)	98	21.6%
Arg (R)	33	7.3%
Asn (N)	1	0.2%
Asp (D)	9	2.0%
Cys (C)	1	0.2%
Gln (Q)	3	0.7%
Glu (E)	11	2.4%
Gly (G)	63	13.9%
His (H)	4	0.9%
Ile (I)	2	0.4%
Leu (L)	79	17.4%
Lys (K)	1	0.2%
Met (M)	4	0.9%
Phe (F)	7	1.5%
Pro (P)	**32**	**7.1%**
Ser (S)	20	4.4%
Thr (T)	22	4.9%
Trp (W)	11	2.4%
Tyr (Y)	6	1.3%
Val (V)	46	10.2%

Total number of negatively charged residues (Asp + Glu): 20
Total number of positively charged residues (Arg + Lys): 34
Total number of Arginine and Alanine residues (Arg + Ala): 131
Total number of GC rich residues (G, A, R, P, V): 60.1 %

Table 4.4a Codon usage table for putative *pha*C_{Sa} gene from *S. aureofaciens*. fields: [triplet] [frequency: per thousand] ([number])

Phe	UUU 0.0(0)	Ser	UCU 9.0(6)	Tyr	UAU 0.0(0)	Cys	UGU 0.0(0)
	UUC 9.0(6)		UCC 7.5(5)		UAC 1.5(1)		UGC 10.5(7)
Leu	UUA 0.0(0)		UCA 0.0(0)	Stop	UAA 0.0(0)	Stop	UGA 1.5(1)
	UUG 1.5(1)		UCG 13.5(9)	Stop	UAG 0.0(0)	Trp	UGG 15.0(10)
	CUU 3.0(2)	Pro	CCU 18.0(12)	His	CAU 1.5(1)	Arg	CGU 19.5(13)
	CUC 16.5(11)		CCC 19.5(13)		CAC 10.5(7)		CGC 58.4(39)
	CUA 1.5(1)		CCA 7.5(5)	Gln	CAA 0.0(0)		CGA 12.0(8)
	CUG 41.9(28)		CCG 34.4(23)		CAG 6.0(4)		CGG 59.9(0)
Ile	AUU 0.0(0)	Thr	ACU 4.5(3)	Asn	AAU 0.0(0)	Ser	AGU 0.0(0)
	AUC 0.0(0)		ACC 3.0(2)		AAC 1.5(1)		AGC 9.0(6)
	AUA 0.0(0)		ACA 0.0(0)	Lys	AAA 0.0(0)	Arg	AGA 0.0(0)
Met	AUG 3.0(2)		ACG 15.0(10)		AAG 1.5(1)		AGG 3.0(2)
Val	GUU 15.0(10)	Ala	*GCU 85.3(57)*	Asp	GAU 9.0 (6)	Gly	GGU 58.4 (39)
	GUC 24.0(16)		GCC 49.4(33)		GAC 25.4 (17)		GGC 92.8(62)
	GUA 10.5(7)		GCA 7.5(5)	Glu	GAA 4.5 (3)		GGA 18.0(12)
	GUG 38.9(26)		GCG 80.8(54)		GAG 4.5 (3)		GGG 56.9(38)

Table 4.4b Codon usage table for putative *pha*B_{Sa} gene from *S. aureofaciens*
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 15.4(7)		UCC 19.8(9)		UAC 13.2(6)		UGC 2.2(1)
Leu	UUA 0.0 (0)		UCA 0.0(0)	Stop	UAA 0.0(0)	Stop	UGA 2.2(1)
	UUG 11.0(5)		UCG 17.6(8)	Stop	UAG 0.0(0)	Trp	UGG 24.2(11)
	CUU 0.0 (0)	Pro	CCU 2.2 (1)	His	CAU 0.0(0)	Arg	CGU 6.6(3)
	CUC 35.2(16)		CCC 19.8(9)		CAC 8.8(4)		CGC 13.2(6)
	CUA 0.0 (0)		CCA 0.0 (0)	Gln	CAA 0.0(0)		CGA 2.2 (1)
	CUG127.8(58)		CCG 48.5(22)		CAG 6.6(3)		CGG 48.5 (22)
Ile	AUU 0.0(0)	Thr	ACU 0.0 (0)	Asn	AAU 0.0(0)	Ser	AGU 0.0(0)
	AUC 4.4(2)		ACC 11.0(5)		AAC 2.2(1)		AGC 6.6(3)
	AUA 0.0(0)		ACA 0.0 (0)	Lys	AAA 0.0(0)	Arg	AGA 0.0(0)
Met	AUG 8.8(4)		ACG 37.4(17)		AAG 2.2(1)		AGG 2.2(1)
Val	GUU 2.2(1)	Ala	GCU 11.0(5)	Asp	GAU 2.2 (1)	Gly	GGU 15.4(7)
	GUC 26.4(12)		GCC 66.1(30)		GAC 17.6(8)		GGC 61.7(28)
	GUA 2.2(1)		GCA 11.0(5)	Glu	GAA 0.0 (0)		GGA 8.8(4)
	GUG 70.5(32)		GCG127.8(58)		GAG 24.2(11)		GGG 52.9(24)

Bhushan 1986; D'Onofrio 1991; Collins and Jukes 1993; Jermiin 1994; Porter 1995; Anderson and Sharp 1996). However, some genes may not conform to the above bias (Hashimoto *et al*. 1994, 1995).

The exact reason for *Streptomyces* sp. exhibiting strong bias for G or C rich codon is not known. It has been attributed to a mutational bias or due to a partial deficiency in the ability to synthesize A or T during evolution (Wright and Bibb 1992). The high G+C content might represent a means of minimizing the potentially lethal and mutational effects of UV irradiation, where adjacent T's constitute the

major mutagenic target (Baltz 1986). The amino acids like alanine and argnine which show GC codon bias contribute to increased stability of the proteins while serine and lysine decreases stability. In *Streptomyces* the relative abundance of tRNAs and their respective codons play a role in gene expressivity (Bibb *et al*. 1984) as is seen in the case of lipase gene (*lip*A) wherein the expression is regulated by the availability of a rare TTA leucine codon (Gonzalez *et al*. 1997).

Table 4.1 shows the codon usage data calculated using the gene sequences available for *S. aureofaciens* (http://www.kazusa.or.jp/codon/). A strong bias towards the use of G or C in the third position of the codon was observed. In the present case, amino acids G, A, R, P and V in the putative *pha*C_{Sa} and the putative *pha*B_{Sa} genes amount to ~76 % (Table 4.3a) and ~60 % (Table 4.3b) of the total amino acids respectively. The extensive use of the GC rich codons for amino acids make these less similar to the other reported sequences.

From the codon usage table (Table 4.2) it was inferred that codons TTA and CTA for leucine are of rare occurrence. Most of the codons used in putative *phaC*$_{Sa}$ gene were found to be of general occurrence. The presence of a single rare codon CTA for leucine was identified in the *phaC*$_{Sa}$ gene (Table 4.4a). From the codon usage table it was also seen that the GCT codon, coding for alanine, which is otherwise rarely used by the *Streptomyces* sp., was the most often used in the putative *pha*C gene sequence (85.3 mol %). From the above it seems reasonable to assume that charged tRNA availability might provide a mechanism against the expression of *pha*C_{Sa} gene, hence leading to limited PHB is accumulation in *S. aureofaciens*. However, since the tRNAs for these rare codons are available in considerable amounts in *E. coli* no limit is exerted on accumulation. The presence of rare codons were not found in the putative *pha*B_{Sa} (Table 4.4b) or in other ORFs except for ORF 4 which contains two rare TTA codons coding for leucine. Hence, as in *lip*A gene (Bibb *et al*. 1984; Gonzalez *et al*. 1997) this rare codon might function as a regulatory protein.

4.4 CONCLUSIONS

- The exact size of the ~ 5.0 kb cloned Sau3A I g-DNA fragment from *S. aureofaciens* NRRL 2209 was found to be 4,826 bp with 74.6 mol % G+C rich.
- From the sequences analysis it was concluded that the above sequence coded for the putative *pha*C_{Sa} and the *pha*B_{Sa} genes but not for the β-ketothiolase gene. The β-ketothiolase activity might be supplemented by the host or an intermediary product of β-fatty acid oxidation may participate in PHB biosynthesis.
- Clustering of the PHB synthesizing genes with the *ssg*A gene and other putative genes for membrane associated proteins suggested its possible role as a reserve food material in septum formation during sporulation and cell division processes in the native organism.
- The interesting feature is the presence of putative *pha*B_{Sa} gene within putative *pha*C_{Sa} gene. Moreover, this kind of arrangement is found in sporulation related genes of *Streptomyces*.
- The presence of a rare codon for leucine suggests temporal expression of the *pha*C_{Sa} gene.

CHAPTER 5

CHARACTERIZATION OF PHA 'C' GENE INVOLVED IN PHB BIOSYNTHESIS OF *S. AUREOFACIENS*.

5.1 INRODUCTION

Intensive research in the physiology, biochemistry and molecular genetics of polyhydroxyalkanoates (PHAs) metabolism has over the years expanded our knowledge of the biosynthesis of these biopolymers in bacteria. Many new enzymes and pathways involved in the metabolic process have been identified. In addition, many genes encoding the enzymes of the pathways have been cloned and characterized (Steinbuchel and Hein 2001).

PHA synthase(s) is the key enzyme in the PHA biosynthesis. It catalyzes the covalent linkage between the hydroxyl group of one and the carboxyl group of another hydroxyalkanoic acid (HA). As detailed earlier three classes of PHA synthases are known to exist. Based on the substrate specificity and size, these have been classified into three different types (detailed in Chapter 1). While Type I and III has a substrate specificity to PHA_{SCL}, type II synthases have PHA_{MCL} as substrates. The substrates for these are the CoA thioesters of HAs. Since, the cloning of *R. eutropha* PHA biosynthesis operon (Schubert *et al*. 1988; Slater *et al*. 1988; Peoples and Sinskey 1989) as many as 50 synthases from 40 different organisms have been cloned and their primary structures deciphered (Steinbuchel and Hein 2001).

The knowledge on PHA synthases has enabled establishment of PHA biosynthesis in many prokaryotic and eukaryotic organisms, thus opening up new perspectives for the production of various PHAs by fermentation biotechnology or agriculture in an economically feasible manner.

Identification of the genes involved in PHA biosynthesis by different strategies (see Chapter 4), revealed varied clustering of these genes in different bacteria. While in *R. eutropha*, the most studied of all, the genes involved in PHB biosynthesis are arranged in an operon as *pha*CAB_{Re}, else where they are clustered in different arrays. In organisms with type III synthases, the synthase gene seems to be separated from the other genes. Other genes e.g. *pha*R, *pha*I, *pha*P, are also located in relative close proximity.

In this Chapter, experiments were carried out to assign the possible role to the various ORFs present on the ~5.0 kb insert of pSa240 in PHB synthesis (discussed in Chapter 4).

5.2 EXPERIMENTAL PROCEDURES

5.2.1 Organisms and Growth

E. coli and pSa240 were grown as described in Chapter 2. Recombinant *E. coli* cells were grown in the medium supplemented with the required amounts of antibiotic.

5.2.2 Nucleic Acid Isolation and Competent cell preparation

The procedures for plasmid DNA isolation and competent cell preparation are detailed in Chapter 2.

5.2.3 Plasmids used in this Chapter

The details of the plasmids used in this Chapter are given in Table 5.1

5.2.4 Complementation by phaC$_{sa}$

The clone pGT07 (ref. Chapter 2) harboring the PHB biosynthesis operon, *pha*CAB$_{Re}$, of the *R. eutropha* was double digested with the restriction endonucleases EcoR V and Kpn I. The larger fragment was gel extracted and treated with DNA polymerase I in the absence of dNTPs to remove the 3' overhang in the Kpn I site (ref. Sambrook *et al*. 1989). The DNA polymerase I was inactivated by heating at 75°C for 10 min and the fragment was self ligated using T4 DNA ligase (NEB, USA) according to the manufacturers instructions. *E. coli* JM109 was transformed with the ligation mix and the deletion clone was denoted as pRC14. The pRC14 clone had the *pha*AB$_{Re}$ genes under the control of the same promoter of the PHB biosynthesis operon and the *pha*C$_{Re}$ gene deleted.

pRC14 harboring *pha*AB$_{Re}$ genes was restriction digested with BamH I and EcoR I. The purified ~3.8 fragment was cloned into the BamH I - EcoR I sites of pET-28a(+) vector (Novagen, USA) and was introduced into *E. coli* JM109. This clone was termed as pRC15.

A 2.7 kb EcoR I - Hind III fragment from the pRC01 (ref. Table 5.1) harboring *pha*CB$_{Sa}$ was cloned into the EcoR I - Hind III sites of the pRC15. The resultant clone was termed as pRC16 and was used for the complementation studies.

Table 5.1 Various plasmids constructed and used in this study

Plasmid	**Description**
pRC01	^{MCS}EcoR I / ^{2705}Pvu II fragment of pSa240 cloned into the EcoR I – Hind III sites in the MCS of pGEM-3Z
pRC02	^{MCS}EcoR I / ^{3430}Nsi I fragment of pSa240 cloned into the EcoR I – Pst I sites in the MCS of pGEM-3Z
pRC03	^{886}Sal I / ^{4333}Sal I fragment of pSa240 cloned in the Sal I site of pGEM-3Z
pRC04	^{886}Sal I / ^{3430}Nsi I fragment of pSa240 cloned into the Sal I – Pst I sites in the MCS of pGEM-3Z
pRC05	^{3430}Nsi I / ^{4333}Sal I fragment of pSa240 cloned into the Pst I – Sal I sites in the MCS of pGEEM-3Z
pRC06	^{3191}Pvu II / ^{4333}Sal I fragment of pSa240 cloned into the Hinc II – Sal I sites in the MCS of pGEM-5Z
pRC07	^{1953}Stu I / ^{3430}Nsi I fragment of pSa240 cloned into the Hinc II – Pst I sites in the MCS of pGEM-3Z
pRC08	^{1953}Stu I / ^{4333}Sal I fragment of pSa240 cloned into the Hinc II – Sal I sites in the MCS of pGEM-5Z
pRC09	^{MCS}EcoR I / ^{4333}Sal I fragment of pSa240 cloned into the EcoR I – Sal I sites in the MCS of pGEM-3Z
pRC10	pSa240 plasmid DNA was cut with Dra III and the large fragment devoid of the Dra III fragment (nt 158-420) was self ligated using T4 DNA ligase
pRC11	pSa240 plasmid DNA was cut with Sty I and the large fragment devoid of the Sty I fragment (nt 2642-2855) was self ligated using T4 DNA ligase
pRC12	pSa240 plasmid DNA was cut with Tth111 I and the large fragment devoid of the Tth111 I fragment (nt 3631-4123) was self ligated using T4 DNA ligase

MCS – multiple cloning site of the vector

pET-28a(+) vector with the *pha*CAB$_{Re}$ PHB biosynthesis operon, termed as pRC17, was used as control.

5.2.5 SDS-PAGE

Preparation of cell free lysate from the bacterial cell pellets and SDS-PAGE were done as enumerated in Chapter 2.

5.2.6 GC Analysis

PHB was isolated and GC analysis done as described in Chapter 2.

5.2.7 Enzymes assays

The cell free lysate was made as detailed in Chapter 2. Cell free lysate for the enzyme assays were made in Tris - buffer (10 mM Tris-Cl pH 7.5, 10 mM β-mercaptoethanol). Activities of PHA synthase, β-ketothiolase and acetoacetyl-CoA reductase were measured as described previously (Nishimura *et al*. 1978; Valentin and Steinbuchel 1993; Belova *et al* 1997).

PHA synthase activity was determined by measuring the increase in absorbance at 412 nm. The reaction mixture contained (1mL): 0.1 M Tris-Cl pH 8.0, 0.7 mM DNTB (5,5'-dithiobis-(2-nitrobenzoic acid), 3.3 μM EDTA pH 8.0 and an aliquot of the cell free lysate. Reaction was started by adding 0.2 μM β-HBCoA (β-hydroxybutyryl CoA). One unit of the PHA synthase activity was defined as the change of 0.001 O.D at 412 nm min^{-1} mg $protein^{-1}$.

β-ketothiolase activity was determined by measuring the decrease in absorbance at 303 nm. The reaction mixture (1mL) contained: 50 μM Tris-Cl, pH 8.0, 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 the β-ketothiolase activity was defined as the change of 0.001 O.D at 340 nm min^{-1} mg $protein^{-1}$.

The activity of NADPH-acetoacetyl-CoA reductase was determined by measuring the decrease in the absorption at 340nm. The reaction mixture (1mL) contained: 50 mM Tris-HCl pH 7.5, 0.5 mM $MgCl_2$, 0.025 mM NADPH and an

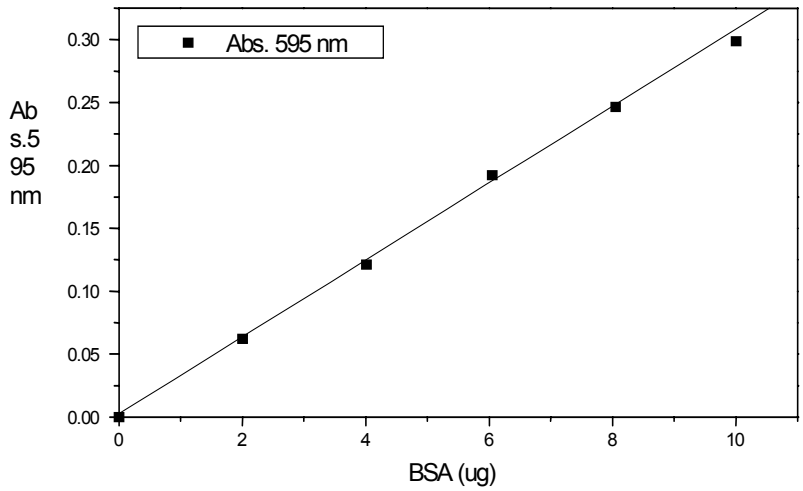

Fig. 5.1 Bradford's method for protein estimation: Protein dye binding response pattern for BSA.

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 activity was defined as the change of 0.001 O.D at 340 nm min^{-1} mg $protein^{-1}$.

5.2.8 Protein estimation

Protein content was determined by Bradford method (Bradford 1976). Protein reagent contained 0.01% Coomassie Brilliant Blue G 250; 4.7% (v/v) ethanol; 8.5% (v/v) phosphoric acid. To 0.2% Coomassie Brilliant Blue-G 250 (made in 50 mL of 95% ethanol) 100 mL of 85% (v/v) phosphoric acid was added and diluted to 1L with deionized water.

Solutions containing 1–10 μg BSA were dispensed into test tubes and volume was adjusted to 0.1 mL with 0.9% NaCl. 1ml of protein reagent was added to the test-tube and the contents 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 the protein was plotted against the corresponding absorbance

resulting in a standard curve (Fig. 5.1) to be used to determine the protein concentration in unknown samples.

5.2.9 Southern hybridization and genome reconstruction studies

The g-DNA of S. aureofaciens *NRRL 2209 was restriction digested with BamH I, Pst I, Nsi I, Sph I, Stu I, and Xba I. The enzyme reactions were carried out as per the manufacturer's instructions. The restriction digested DNA was blotted and Southern hybridization was performed as detailed in Chapter 2. Purified 1.5 kb Dra III – Stu I fragment of pSa240 plasmid DNA was used as the probe.*

For slot blot hybridization *S. aureofaciens* g-DNA (2 μg) was blotted onto Hybond N^+ membrane. Purified 1.5 kb Dra III – Stu I fragment of pSa240 plasmid DNA was also applied to the membrane in quantities that contained the indicated copies per haploid genome equivalent (De Rose *et al.* 1989). The membrane was hybridized with the above Dra III – Stu I fragment. One microgram of *S. aureofaciens* genomic DNA or 205 pg of purified 1.5 kb Dra III – Stu I fragment contain one gene copy equivalent. The gene copy numbers were estimated by densitometric comparison of the hybridization signals.

5.3 RESULTS AND DISCUSSION

In Chapter 4, based on the sequence analysis (ref. Chapter 4) it was concluded that the ~5.0 kb insert in the pSa240 plasmid harbors only the putative *pha*C_{Sa} and the *pha*B_{Sa} genes but not the *pha*A (β-ketothiolase) gene. Also *ssg*A gene was shown to be present downstream of the above mentioned putative genes. To assign functions to the putative genes a series of deletion cloned were constructed as detailed in Table 5.1 and Fig. 5.2 and 5.3.

5.3.1 Analysis of the PHB accumulation by deletion clones

Based on the restriction enzyme sites in the pSa240 insert, twelve deletion clones were constructed (Table 5.1). The *E. coli* cells harboring these were grown on basal medium with glycerol as the sole carbon source for 42 h. GC analysis of the acid-propanolysed cell pellets show that the deletion clones pRC02, pRC09, pRC10,

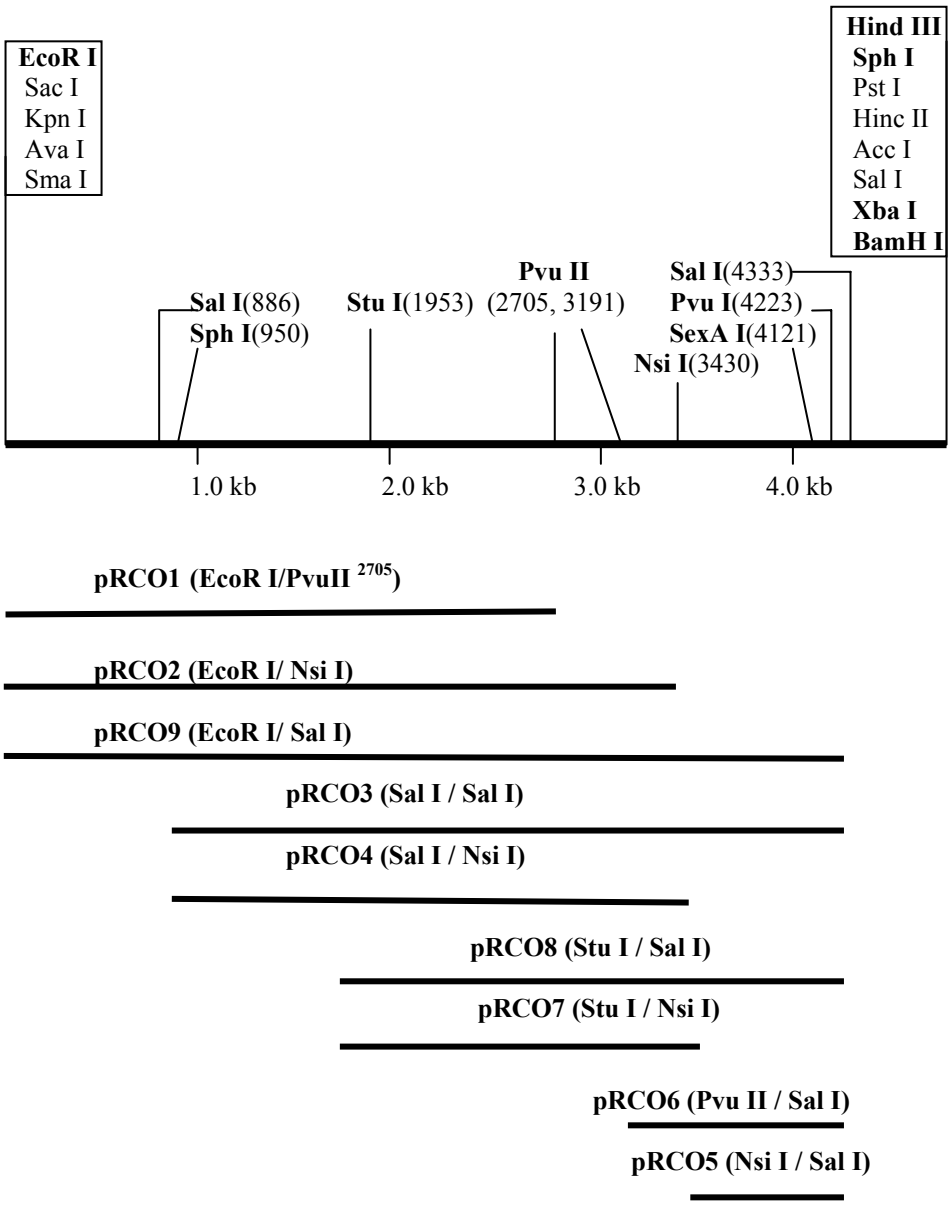

Fig. 5.2 SubClones pRC01 – 09 constructed from pSa240 harboring ~ 5.0 kb genomic DNA fragment from *S. aureofaciens* NRRL 2009.

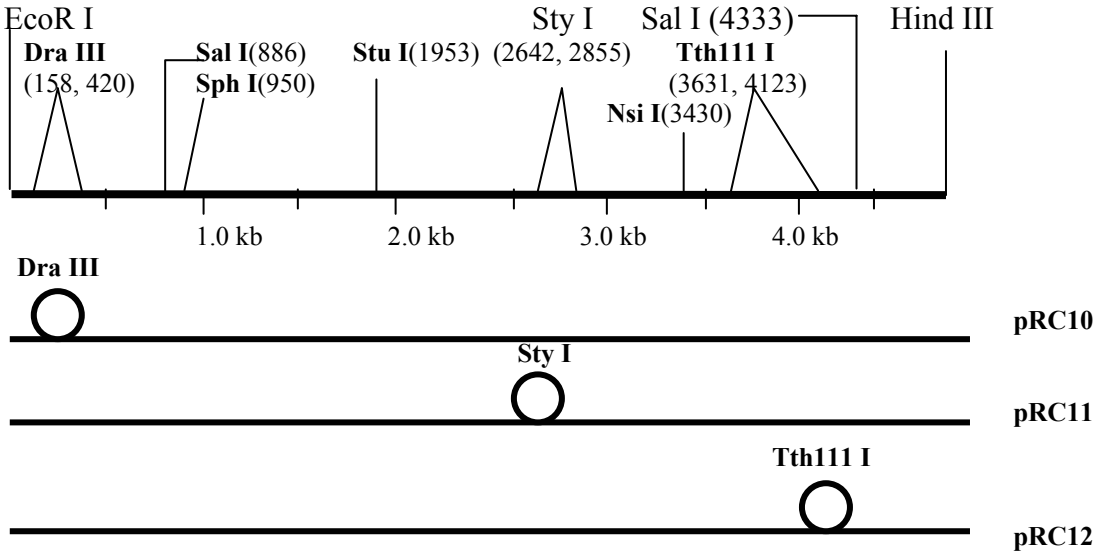

Fig. 5.3 SubClones pRC10 – 12 constructed from pSa240 harboring ~ 5.0 kb genomic DNA fragment from *S. aureofaciens* NRRL 2009.

pRC11 and pRC12 accumulate PHB. All the clones except pRC02 were found to accumulate PHB at par with pSa240. While these clones accumulated 60-65% PHB of the dry cell mass, pRC02 accumulated only 36-42% PHB of the dry cell mass. The clone pRC01 harboring both the putative *pha*C_{Sa} and *pha*B_{Sa} genes accumulated a mere 0.6-1.0% PHB of the dry cell mass.

A critical appraisal of the results obtained with the deletion clones revealed that :

1) pRC09 (EcoR I / ^{4333}Sal I) clone in which the ORFs 3,12,15 and 19 were truncated continued to accumulate PHB at par with full length pSa240 clone, this suggested that these ORFs (3,12,15 and 19) have apparantly no role to play in PHB biosynthesis;
2) in pRC12 clone the deletion of the Tth111 I fragment (nt 3631-4123) resulted in truncation of ORF 7. This deletion had no deleterious influence on PHB accumulation;
3) deletion clone pRC02 (EcoR I / ^{3430}Nsi I) accumulated only 36-42% PHB of the dry cell mass as compared to the 60-65% PHB accumulated by the full-length pSa240 clone.

Since the deletions or truncations of the ORFs enumerated in points 1 and 2 above did not have any effect on PHB accumulation, it was obvious that in pRC02 clone the truncation of any one of the ORFs 5,9,13,17,22 or 23 has substantial influence on PHB accumulation. This argument was further substantiated by the deletion clone pRC11 (nt 2642- nt 2855 Sty I deletion) in which ORFs 4, 8 and 18 were truncated without any influence on accumulation of PHB by the clone.

From the foregoing it may be concluded that any one of the ORFs 5, 9, 13, 17, 22 or 23 may influence PHB accumulation by the recombinant cells harbouring the full length pSa240 clone. As enumerated in Chapter 4, ORF 13 has shown 99.37% sequence similarity to the *ssg*A gene from *S. coelicolor*. ORF 9 also shows 33.5 % sequence similarity with the DNA binding protein of *Bifidobacterium* sp (see Chapter 4). While the former has earlier been implicated in sporulation and cell division in *Streptomyces* sp. (Wezel *et al*. 2000) the latter has benn implicated in control of transcriotion or ecert some form of regulatory influence on gene expression.. The present study is the first report of its kind where the *ssg*A and / or the DNA binding protein genes are suggested to possibly provide *tran* acting factor(s) which influences the expression of the PHA biosynthesis genes. These two genes even in their truncated versions continue to provide the functions. While the ssgA gene runs into the MCS of the cloning vector and utilized a translation and transcriotion stop sequences from there, the DNA binding protein gene which runs in the opposite direction utilizes the translation start signal from the *lacZ* gene and also utilized its promoter.

The near non accumulation of PHB by the pRC01 clone highlighted the above observation.

5.3.2 Enzyme assay analysis of the deletion clones

Enzyme assays for β-ketothiolase, acetoacetyl-CoA reductase and PHA synthase were performed using the crude cell free extracts from all the deletion clones listed above along with pSa240 and *E. coli* JM109 harboring pGEM-3Z plasmid. The results have been shown in the Table 5.2.

Table 5.2 Specific activities of the *E. coli* JM109 with plasmid vector pGEM-3Z, pSa240 and of the deletion clones (in U mg protein $^{-1}$)

	Activity (Units)		
	β-ketothiolase	AcAc-CoA reductase	PHB Synthase
JM109	0.63	6.94	ND
Psa240	1.58	15.85	2.32
PRC01	1.68	17.54	ND
PRC02	1.16	17.61	1.76
PRC03	0.75	14.96	ND
PRC04	0.62	9.89	ND
PRC05	0.57	6.60	ND
PRC06	0.68	6.59	ND
PRC07	0.81	7.19	ND
PRC08	0.74	6.74	ND
PRC09	0.63	18.27	2.60
PRC10	1.15	18.89	1.85
PRC11	0.72	17.53	1.55
PRC12	1.04	15.57	1.88

From the table it is apparent that the β-ketothiolase enzyme activity was detected in the *E. coli* cells harboring the pGEM-3Z vector, the pSa240 plasmid and all the deletion clones. The fact that the enzyme activity was detected in the host *E. coli* JM109 cells, the pRC01 [a pSa240 sub-clone with entire 3' ~2.0 kb fragment (Pvu II – Hind III) deleted] and as well as the pRC05 clones [the pSa240 sub-clone with entire 5' ~3.5 kb fragment deleted] supported the earlier conclusion that the host *E. coli* cells provide the β-ketothiolase activity needed for PHB biosynthesis. In the eventuality of the *E. coli pha*A gene not taking part in the PHB synthesis, intermediates of the fatty acid β-oxidation or fatty acid synthesis may be channelized to PHB synthesis as has been reported to happen in *Pseudomonas aeruginosa* (Langenbach *et al*. 1997).

The host *E. coli* cells harboring the pGEM-3Z plasmid showed acetoacetyl-CoA reductase activity of 6.94 units. The activity was found to be ~1.5 to 2.5 times more in the clones harboring the putative *pha*B_{Sa} gene (pSa240, pRC01-04,

pRC09-12). The other clones which did not harbor the putative *pha*B_{Sa} gene showed specific activity which was at par with the host organism.

As evident from the Table 5.2 PHA synthase activity was observed in the deletion clones pRC02, pRC09, pRC10, pRC11 and pRC12. Though pRC01 accumulated minimal amounts of PHB, PHB synthase activity was not detected in this clone.

From the above results obtained by assaying the enzyme activities of the enzymes involved in PHB biosynthesis it is substantiated that one or more of the ORF(s) downstream of the ^{2703}Pvu II site are essential for the PHB biosynthesis and their gene products might take part in PHB biosynthesis by providing function in *trans*. Since the role of the ORFs 3, 4, 6, 7, 9, 12, 15, 18, 19, 22 was ruled out it has been concluded that PHB accumulation was dependent upon or regulated by the ORF 13 coding for *ssg*A gene.

5. 3.3 Protein profiling

SDS-PAGE profile was done of the crude cell free extracts (Fig. 5.4) from the *E. coli* JM109, pSa240, pRC01, pRC02, pRC05 and pRC06. Three extra bands in pSa240 and pRC02, and two extra bands in pRC01 were visualized. The molecular weight of the first band in pSa240, pRC02 and pRC01 corresponded to the putative *pha*C_{Sa} gene product (~ 73 kDa) while the molecular weight of the second band corresponded to the *pha*B_{Sa} gene product (~ 49 kDa). The third extra band seen in pSa240 corresponded to the $SsgA_{Sau}$ or the gene product of ORF 13 (~ 17 to 18 kDa) (see Chapter 4). In the pRC02 clone a band of ~ 12 kDa was seen. This could be the translation product of the truncated version of either the *ssgA* gene or the DNA binding protein gene. Thus, protein profiling of *E. coli* with pGEM-3Z plasmid vector, pSa240, pRC01, pRC02, pRC05, pRC06 clones point towards the possible role of SsgA and / or DNA binding protein in PHB biosynthesis.

5.3.4 Complementation by phaC$_{Sa}$

The clones pRC15, pRC16 and pRC17 were constructed as detailed in section 5.3.4. pRC16 harbored the *pha*C_{Sa} and *pha*AB_{Re} under their respective promoters.

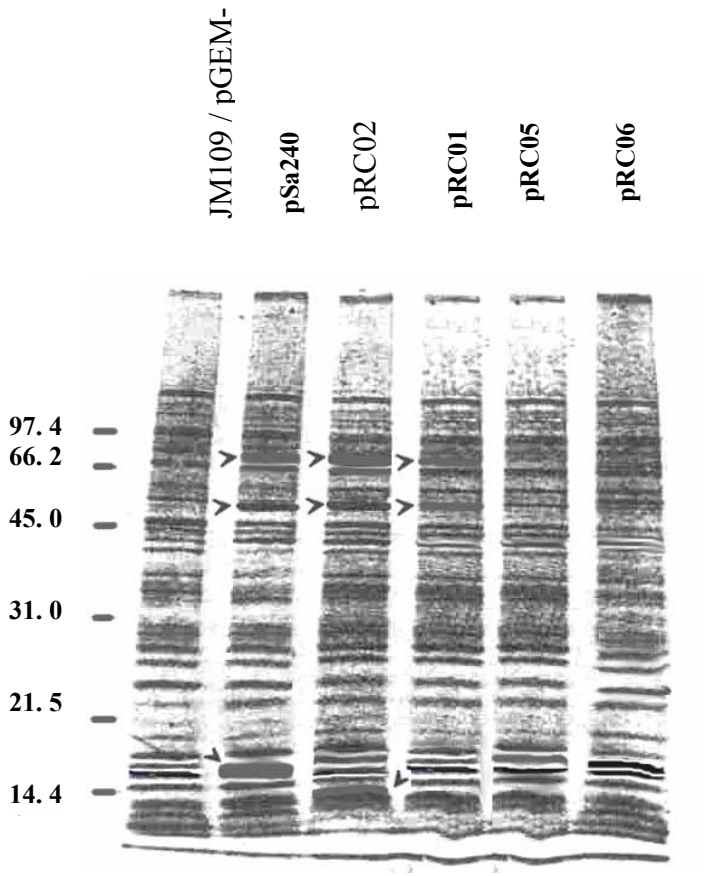

Fig. 5.4 Polyacrylamide gel electrophoresis of the crude cell free lysate from *E. coli* with pGEM-3Z plasmid vector, pSa240, pRC01, pRC02, pRC05, pRC06 clones. Arrows are pointed towards the extra bands. Molecular weight standards are also shown.

They were grown on the basal medium for 42 h and the PHB accumulation was analyzed by gas chromatography. While pRC17 accumulated 70-80% of PHB of dry cell mass, pRC16 accumulated PHB to the extent of 2-4% of dry cell mass. This confirmed that the 2.7 kb EcoR I – Pvu II fragment harbored *pha*C_{Sa} as it supported PHB accumulation in pRC16. This also, showed that despite the presence of a *pha*A_{Re} gene in pRC16 no enhancement in the PHB accumulation occurred suggesting the need of some other ORF(s) for he PHB accumulation thus, substantiating the conclusions drawn in the earlier section regarding the $SsgA_{Sau}$ to take part in the PHB biosynthesis in the recombinant *E. coli* by acting as a *trans* acting element.

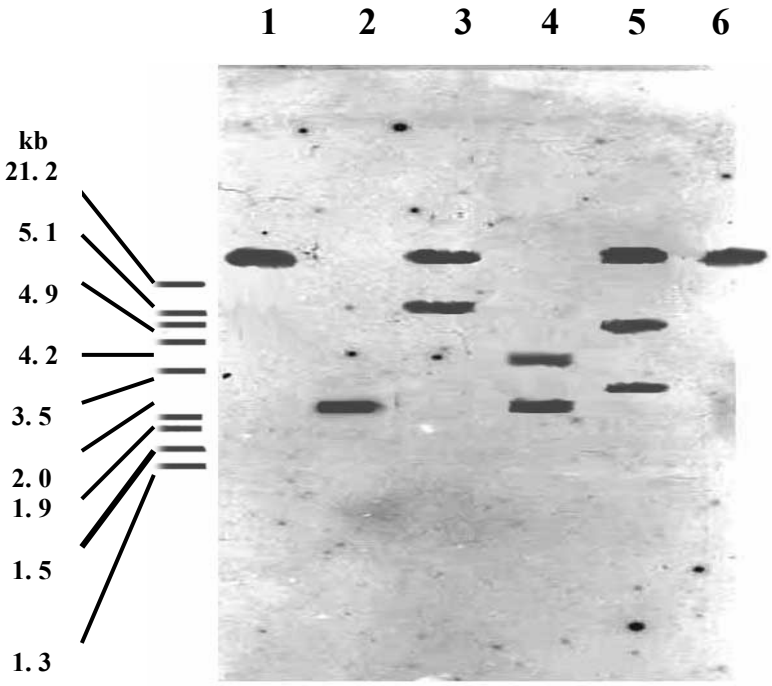

Fig. 5.5 Southern hybridization of the *S. aureofaciens* g-DNA digested with restriction endonucleases. Lanes 1-6 were loaded with 15 µg of DNA and digested with BamH I, Nsi I, Pst I, Sph I, Stu I and Xba I respectively**.**

5.3.5 Southern hybridization and genome reconstruction studies

S. aureofaciens g-DNA was restriction digested with BamH I, Nsi I, Pst I, Sph I, Stu I and Xba I and was blotted onto a nylon membrane. The blot was hybridized with 1.5 kb Dra III – Stu I fragment and the signals as seen in Fig. 5.5 were obtained. From the blot it was clear that no restriction endonuclease sites existed for BamH I, Xba I and Pst I enzymes within the 5.0 kb insert of pSa240 plasmid DNA. A 3.5 kb band was seen in Nsi I digested g-DNA lane (lane 2) which suggested that another Nsi I site was present on the 5' end upstream of the of the 5.0 kb insert. It also suggested of a single copy of *pha*C_{Sa} being present in *S. aureofaciens*. Lane 4 and 5 with g-DNA digested with Sph I and Stu I showed two clear bands. This suggested that Sph I and Stu I enzymes have a site each on 3' and 5' end.

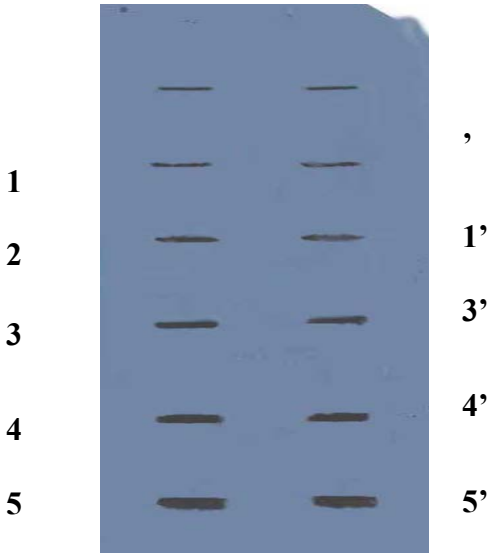

Fig. 5.5 Reconstruction analysis of *S. aureofaciens* NRRL 2209 g-DNA. Lanes S and S' contain 2 μg of *S. aureofaciens* g-DNA ($8x10^{-3}$ pg per haploid genome). Number indicates the amount (in copies per haploid equivalent) of purified 1.5 kb Dra III – Stu I digested *pha*C_{Sa} gene fragment from *S. aureofaciens.*

The hybridization of the slot blot using the same 1.5 kb Dra III – Stu I fragment as probe revealed *S. aureofaciens* NRRL 2209 to harbor a single copy of the of the *pha*C_{Sa} gene confirming of the single band obtained with Nsi I restriction enzyme. As the probe also had the 5' region of *pha*B_{Sa} the results were extrapolated to conclude that a single copy of *pha*B_{Sa} was present on the g-DNA of *S. aureofaciens*.

5.4 CONCLUSIONS

- Twelve deletion clones were constructed from the pSa240 plasmid DNA and were checked for PHB accumulation and activities of the enzymes involved in PHB biosynthesis.
- Deletion clones pRC01, pRC02, pRC09-12 showed PHB accumulation.
- Enzyme activities for acetoacetyl-CoA and PHA synthase enzymes were found in pRC02, pRC09-12. In pRC01, however, acetoacetyl-CoA enzyme activity was found but not the synthase activity.
- Protein profile of the crude cell free extracts from *E. coli* with pGEM-3Z vector, pSa240, pRC01, pRC02, pRC05 and pRC06 showed two extra bands each which were common for pSa240, pRC01 and pRC02, and a third extra band in pSa240 and pRC02.
- The two extra bands common for pSa240, pRC01 and pRC02 were identified as $PhaC_{Sa}$ and $PhaB_{Sa}$. The third band found in pSa240 corresponded to full length $SsgA_{Sau}$ or the DNA binding protein. The third extra band in pRC02 was the truncated version of the same.
- It was concluded that $SsgA_{Sau}$ played an important role in the PHB biosynthesis and that the ~ 5.0 kb g-DNA insert of *S. aureofaciens* harbored only *pha*CB_{Sa} genes but not the β-ketothiolase.
- From the Southern blot it was concluded that a single copy of the *pha*C_{Re} was present with Nsi I site being present in the 5 ‘ upstream end of the 5.0 kb fragment and Sph I and Stu I enzymes having sites at both 3’ and 5 ‘ ends of the 5.0 kb insert.
- From the slot blot studies it became evident that g-DNA of *S. aureofaciens* possessed a single copy of both *pha*C_{Sa} and *pha*B_{Sa}.

CHAPTER 6

SCREENING FOR THE DEPOLYMERASE GENE IN *STREPTOMYCES AUREOFACIENS* NRRL 2209

6.1 INTRODUCTION

The primary reason, which stimulated interest in PHAs, is their synthesis from renewable sources and biodegradation to water and carbon dioxide (Jendrossek and Handrick 2002). PHA depolymerases have the property of being water-soluble and the ability to bind specifically to polyester surfaces (Briese and Jendrossek 1998). Biodegradation may be enzymatically-catalyzed hydrolysis or simple chemical hydrolysis of the polymer. Enzymatic degradation may be further *via* either intracellular (Foster *et al*. 1994, 1996; Handrick *et al*. 2001) or extracellular PHA degradation (Jendrossek and Handrick 2002).

6.1.1 Terminology

Intracellular PHA degradation is a process of active degradation wherein endogenously stored reserve is mobilized by the accumulating bacterium itself. Enzymes catalyzing the intracellular degradation of PHA are intracellular PHA depolymerases denoted as i-PHA depolymerases. Conversely, extracellular degradation and utilization of an exogenous polymer may occur by a microorganism (not necessarily an accumulator) that secretes extracellular PHA depolymerases (denoted e-PHA depolymerases). The source of the extracellular polymer is PHA released by accumulating cells after death and cell lysis. Intracellular PHAs or the native PHAs (nPHAs) exists in an amorphous state within the cell cytoplasm, while the extracellular PHAs, also known as denatured PHAs (dPHAs), are partially crystalline. Hence, PHA depolymerases, which hydrolyze nPHAs, are designated as nPHA depolymerases and those hydrolyzing dPHAs are designated as dPHA depolymerases.

6.1.2 Extracellular depolymerases

The ability to degrade extracellular PHA is widely distributed among bacteria and fungi inhabiting soil, compost, aerobic and anaerobic sewage, fresh and marine water, estuarine sediment and air (Saito *et al*. 1989; Matvulj and Molitoris 1992; Jendrossek *et al*. 1993; Briese *et al*. 1994; Jendrossek *et al*. 1995 b; Schirmer *et al*. 1995; Klingbeil *et al*. 1996; Kusuya *et al*. 1997; Shinomiya *et al*. 1997; Han *et al*.

1998; Kobayashi *et al*. 1999; Gonda *et al*. 2000; Handrick *et al*. 2001). All purified dPHA depolymerases are specific either for $dPHA_{SCL}$ (EC 3.1.1.75) or $dPHA_{MCL}$ (EC 3.1.1.76) respectively. The dPHA depolymerases depolymerize the terminal ends of dPHAs and produce monomers and dimers (Jendrossek and Handrick 2002). In contrast to a large variety of well-characterized $dPHA_{SCL}$ depolymerases (Saito *et al*. 1989; Jendrossek *et al*. 1995; Kasuya *et al*. 1997; Kita *et al*. 1997; Shinomiya *et al*. 1997; Kobayashi *et al*. 1999; Ohura *et al*. 1999; Takeda *et al*. 2000) only few $dPHA_{MCL}$ depolymerases have been studied. *P. fluorescens* GK13 dPHO depolymerase is the only $dPHA_{MCL}$ depolymerase that has been purified and studied at the molecular level (Schirmer *et al*. 1993; Schirmer and Jendrossek 1994). Depolymerases isolated from *Streptomyces exfoliatus* K10 strain (Klingbeil *et al*. 1996) and *Streptomyces hygroscopicus* var. *ascomyceticus* (Wu *et al*. 2000) are found to be $dPHA_{SCL}$ specific. *Paucimonas lemoigne* (earlier *Pseudomonas lemoigne*) is the most interesting of the PHA degrading bacteria (Jendrossek and Handrick 2002). It is unique among PHA degrading bacteria as it possesses atleast seven e-PHA depolymerase genes (phaZ1 to phaZ7). Six of these code for e-dPHA depolymerases specific for dPHA, while the seventh e-PHA depolymerase is specific for amorphous PHB.

Structural analysis of the $dPHA_{SCL}$ depolymerases has revealed that all enzymes have a N-terminal catalytic domain, a C-terminal substrate binding domain and a linker region connecting the two domains (Sudesh *et al*. 2000). The N-terminal domain comprises of strictly conserved amino acids serine, aspartate and histidine, wherein Serine is a part of the lipase-box pentapeptide **G-X-S-X-G** (Jaeger *et al*. 1994, 1999). Since the removal of the C-terminal domain resulted in loss of the hydrolyzing activity of the enzyme towards the insoluble PHA, it has been suggested that this domain acts as the substrate-binding domain. The linking domain contains a fibronectin type III (Fn3) fingerprint in all $dPHA_{SCL}$ depolymerases (Jendrossek and Handrick 2002). Structural studies of $dPHA_{MCL}$ depolymerase have not been undertaken so far.

6.1.3 Intracellular depolymerases

Since PHAs are storage compounds for excess carbon, microorganisms accumulating it are equipped with intracellular depolymerizing system to recover the stored carbon. The depolymerases that act on nPHAs, decrease the molecular weight of the polymer. However, in contrast to the e-depolymerases the i- depolymerases are not well characterized.

Earlier studies on enzymatic depolymerization of PHA inclusions involved the utilization of soluble enzymes from *R. rubrum*. It was used to degrade nPHB inclusions isolated from *B. megaterium* KM (Merick and Doudoroff 1964). The soluble fraction of *R. rubrum* contained a thermolabile depolymerase whose activity increased upon adding small amounts of trypsin. In *Z. ramigera,* also the depolymerase activity was found in the soluble fraction. However, the PHB inclusions needed to be pretreated with protease (Siato *et al*. 1992). This led to the conclusion that native PHA granules in the microbial cell might be protected from the attack of i-PHA depolymerases by surface proteins.

In comparison to the extracellular depolymerase which attacks crystalline PHA, the mechanism for intracellular depolymerase is presumed to be different because of the amorphous nature of the nPHAs. The characteristics of lipases such as lipase box were presumed to be preserved (Sudesh *et al*. 2000). However, Saegusa *et al*. (2001) have shown that *R. eutropha* H16 intracellular depolymerase lacks the lipase box and specifically degrades nPHAs. However, detailed mechanistic and structural studies need to be done.

Since only two reports exists with regards to the presence of a PHA depolymerase in *Streptomyces* sp., it was worth a while to check *S. aureofaciens* NRRL 2209 for the presence of depolymerases (both intracellular and extracellular) since it accumulates and utilizes PHB during its growth cycle (See Chapter 3).

6.2 EXPERIMENTAL PROCEDURES

6.2.1 Organisms and growth

Recombinant *E. coli* pSa240 and *Streptomyces aureofaciens* NRRL 2009 were grown and maintained as described in Chapter 2. Recombinant *E. coli* harboring the

intracellular depolymerase gene of *R. eutropha* H16, designated as pAE171 (Saegusa *et al*. 2001) was maintained on LB plates supplemented with 100 μg mL^{-1} ampicillin.

6.2.2. Nucleic Acid extraction

g-DNA of *S. aureofaciens* NRRL 2209, and plasmid DNA from pSa240 and pAE171 was extracted according to the procedures detailed in Chapter 2.

6.2.3 PHA depolymerase synthesis medium

PHA depolymerase synthesis medium contained 0.3% PHB (w/v) in 100 mM Tris-HCl, pH 8.0 and 1 mM $CaCl_2$. The PHA used in the medium was either crystalline PHB (dPHB) or commercially available amorphous PHB (nPHB). PHB was dispersed in the media by sonication at 20 kHz for 3 cycles of 1 min each. This served as the sole carbon source for the microorganism. However, the above medium was supplemented with 0.5% glucose or yeast extract or both to facilitate initial growth of the organism (Klinbeil *et al*. 1996). Agar (1.5%) was added to the medium as the gelling agent. The cultures were incubated at 37°C.

6.2.4 Oligonucleotide probe and its labelling

Based on the consensus sequence of the e-PHA depolymerases a 30-mer oligonucleotide having the following nucleotide sequence was synthesized. This was designated as ‘oligoZ’.

5’(d) GAUAGCGGGCUCUCGUCCGGUGGGUACAUG 3’ “oligo Z”

The “oligoZ” was 5’ end labelled as detailed in Chapter 2 and used as the hybridization probe.

6.2.5 Genome reconstruction studies

For slot blot hybridization *S. aureofaciens* g-DNA (4 μg) was blotted onto Hybond N^+ membrane. Purified 1.0 kb Pst I fragment of *Ralstonia eutropha* H16 intracellular depolymerase gene (pAE171) was also applied to the membrane in

quantities that contained the indicated copies per haploid genome equivalent (De Rose *et al.* 1989). The membrane was hybridized with the above Pst I fragment. One microgram of *S. aureofaciens* genomic DNA or 137 pg of purified 1.0 kb Pst I fragment contain one gene copy equivalent. The gene copy numbers were estimated by densitometric comparison of the hybridization signals.

Hybridization was done as described in Chapter 2.

6.3 RESULTS AND DISCUSSION

Streptomyces are known to accumulate PHB during their late log phase (Hodgson 2000). The amount of PHB accumulated is far less than other reserve materials (Kannan and Rehacek 1970; Ranade and Vining 1993; Hodgson 2000). In the present study it was found that with the onset of the stationary phase the percentage of the stored PHB decreases to undetectable levels (Chapter 3). It was thus assumed that PHB in *Streptomyces* is mobilized with the approach of the stationary phase for possible use in secondary metabolite production or in sporulation. This would be possible with the presence of intracellular PHA depolymerase(s). Also, considering the soil habitat of *Streptomyces* sp. it is likely to produce extracellular depolymerase.

Attempts were made to identify and isolate extracellular PHA depolymerase of *S. aureofaciens* NRRL 2209 because of the ease with which it could be determined (Jendrossek and Handrick 2001). *S. aureofaciens* cells were grown on PHB agar plates and liquid medium as mentioned in section 6.2.3. Initial absorbance was recorded for liquid cultures and cells were cultivated at 28°C with 200 rpm shaking in baffled flasks. The cells on agar plates were grown at 28°C in an incubator for 3- 6 days for the zone of clearance.

After 6 days of incubation growth of *S. aureofaciens* was scanty on the PHB agar plates as well in the liquid medium. No zone of clearance was seen on the PHB plates. Even the liquid cultures did not show any decrease in turbidity. Thus, it was concluded that *S. aureofaciens* NRRL 2209 probably lacks extracellular depolymerase(s) (e-PHB_{SCL}) which could depolymerize extracellular dPHB_{SCL} or nPHB_{SCL}.

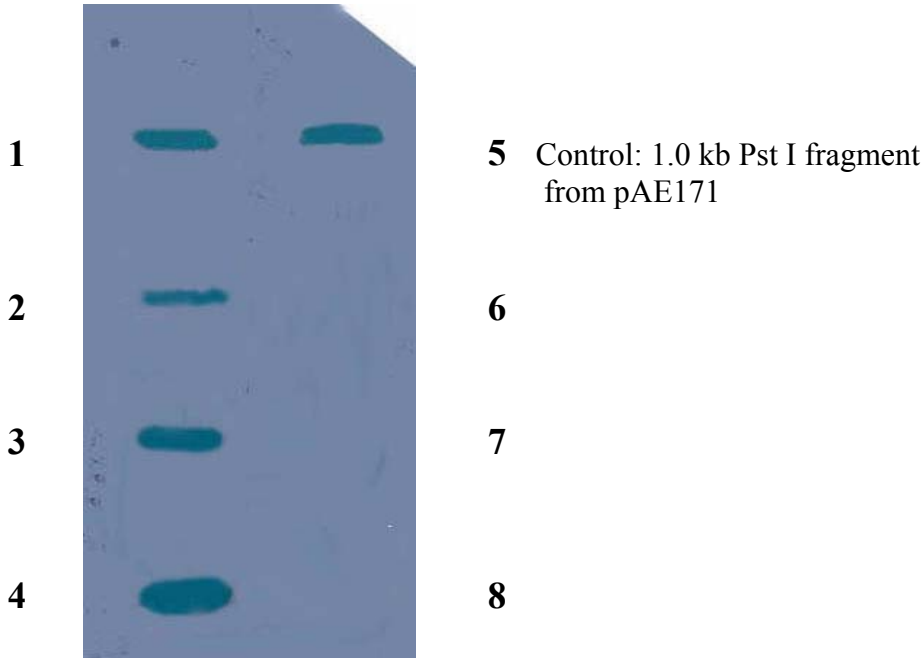

Fig 6.1 Slot blot hybridization analysis. Lanes 1 and 5 are controls. Lanes 2, 3 and 4 contain 2, 4 and 6 µg of *S. aureofaciens* g-DNA respectively. Lanes 6, 7 and 8 contain 2, 4 and 6 µg of pSa240 plasmid DNA respectively. The blot was hybridized with 1 kb Pst I fragment from pAE171.

g-DNA of *S. aureofaciens* and also the plasmid DNA of pSa240 were hybridized with the **oligoZ** (consensus e-PHA depolymerase sequence). No positive signals were observed. This ruled out the possibility of *S. aureofaciens* to contain an e-PHA depolymerase.

Since, the organism accumulated and metabolized intracellular native PHB (i-nPHB), it was assumed to possess an i-PHA depolymerase. As the percentage PHB accumulation decreases after late log phase attempts to identify the intracellular depolymerase were made.

The 1.0 kb Pst I fragment from pAE171 harboring the *R. eutropha* H16 i-PHB_{SCL} (Saegusa *et al*. 2001) was used as a heterologous probe to detect the

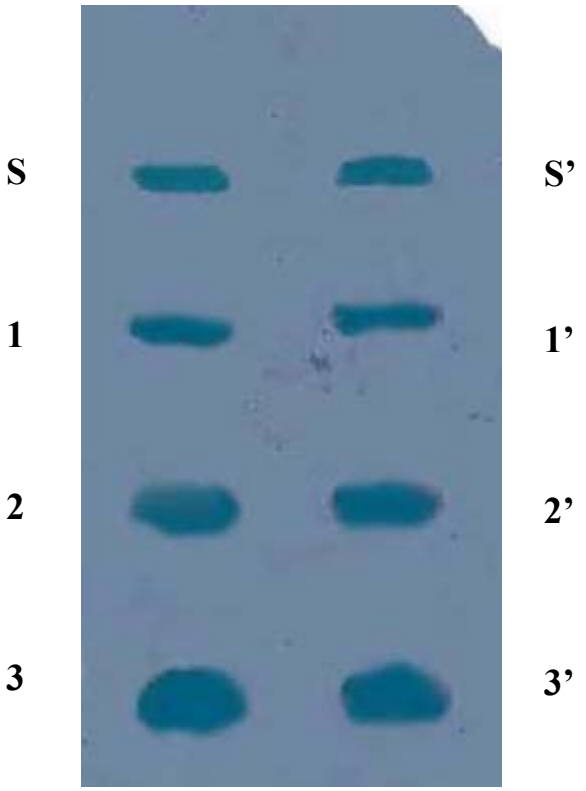

Fig 6.2 Reconstruction analysis of *S. aureofaciens* NRRL 2209 g-DNA. Lanes S and S' contain 4 μg of *S. aureofaciens* g-DNA ($8x10^{-3}$ pg per haploid genome). Number indicates the amount (in copies per haploid equivalent) of purified Pst I digested intracellular depolymerase gene fragment from *Ralstonia eutropha.*

presence of the i-PHA depolymerase gene in *S. aureofaciens* NRRL 2209 as well as on the plasmid pSa240 harboring ~5.0 kb insert from *S. aureofaciens*. The latter was analyzed since the *phaZ* gene is reported to occur either in tandem with the PHB biosynthesis genes or in its close proximity (Madison and Huisman 1999; Sudesh *et al*. 2000). A positive signal was obtained from the *S. aureofaciens* g-DNA. The hybridization signal persisted even after high stringency washes of the blots. This indicated the possible presence of the *phaZ* gene in the *S. aureofaciens* NRRL 2209 genome (Fig. 6.1). However, no signal was observed with the pSa240 indicating that

the *phaZ* gene was not present on the ~ 5 kb cloned *S. aureofaciens* g-DNA fragment. It also suggested that the gene is possibly located rather distantly from the PHB biosynthesis genes.

Next the genome was checked for the copy numbers of the i-PHB depolymerase gene. Hybridizations revealed presence of only one copy of the gene per haploid genome (Fig. 6.2).

6.4 Conclusions

- *S. aureofaciens* NRRL 2209 possibly lacks extracellular depolymerase(s) gene(s) which could depolymerize d- or n-PHAs.
- It possesses a single copy of intracellular depolymerase gene possibly homologous to *R. eutropha* intracellular depolymerase gene.

CONCLUSIONS

- An approximately 5.0 kb Sau3A I genomic DNA fragment from *Streptomyces aureofaciens* NRRL 2209 responsible for PHB synthesis was isolated.
- This fragment upon cloning and expression in *E. coli* supported 25- 28 times more PHB accumulation in recombinant *E. coli* than the native organism when glycerol was supplied as the sole carbon source.
- Extraction of the polymer using sodium hypochlorite / chloroform dispersion method and subsequent GC analysis showed the polymer to be of high purity
- The recovered PHB was an isotactic, crystalline homopolymer with granule size of 1.1 μm. DSC and TGA results show a shift in the thermal degradation temperature pattern of the polymer which is found to be at 295°C.
- Molecular weight of the PHB synthesized and accumulated by *E. coli* was found to be around 3 x 10^5 by GPC.
- The exact size of the ~ 5.0 kb cloned Sau3A I g-DNA fragment from *S. aureofaciens* NRRL 2209 was found to be 4,826 bp with 74.6 mol% G+C rich.
- From the sequences analysis it was concluded that the above sequence coded for the putative *pha*C_{Sa} and the *pha*B_{Sa} genes but not for the β-ketothiolase gene. The β-ketothiolase activity might be supplemented by the host or by an intermediary product of β-fatty acid oxidation may participate in PHB synthesis.
- Clustering of the PHB synthesizing genes with the *ssg*A gene and other putative genes for membrane associated proteins suggested its possible role as a reserve food material in septum formation during sporulation and cell division processes in the native organism.
- The interesting feature is the presence of putative *pha*B_{Sa} gene within putative *pha*C_{Sa} gene. Moreover, this kind of arrangement is found in sporulation related genes of *Streptomyces*.
- The presence of a rare codon for leucine suggested temporal expression of the *phb*C_{Sa} gene.
- Twelve deletion clones were constructed from the pSa240 plasmid DNA and were

checked for PHB accumulation and specific activities of the enzymes involved in PHB biosynthesis.

- Deletion clones pRC01, pRC02, pRC09-12 showed PHB accumulation.
- Enzyme activities for acetoacetyl-CoA and PHA synthase enzymes were found in pRC02, pRC09-12. In pRC01 however, acetoacetyl-CoA enzyme activity was found but not the synthase activity.
- Protein profile of the crude cell free extracts from *E. coli* with pGEM-3Z vector, pSa240, pRC01, pRC02, pRC05 and pRC06 showed two extra bands each which were common for pSa240, pRC01 and pRC02, and a third extra band in pSa240 and pRC02.
- The two extra bands common for pSa240, pRC01 and pRC02 were identified as $PhaC_{Sa}$ and $PhaB_{Sa}$. The third band found in pSa240 corresponded to the full length $SsgA_{Sau}$ (ORF 13) or DNA binding protein (ORF 9). The third extra band in pRC02 was the truncated version of the same.
- It was concluded that $SsgA_{Sau}$ played an important role in the PHB biosynthesis and that the ~ 5.0 kb g-DNA insert of *S. aureofaciens* harbored only *pha*CB_{Sa} genes but not the β-ketothiolase.
- From the Southern blot it was concluded that a single copy of the *pha*C_{Re} was present with an other Nsi I site being present onto the 5 ‘ end of the 5.0 kb fragment and Sph I and Stu I enzymes having sites on both 3’ and 5 ‘ sides of the 5.0 kb insert.
- From the slot blot studies it became evident that g-DNA of *S. aureofaciens* possessed a single copy of both *pha*C_{Sa} and *pha*B_{Sa}.
- *S. aureofaciens* NRRL 2209 possibly lacks extracellular depolymerase(s) gene(s) which could depolymerize d- or n-PHAs.
- It possesses a single copy of intracellular depolymerase gene possibly homologous to *R. eutropha* intracellular depolymerase gene.

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1. Synthesis of PHB from a recombinant *E.coli* harboring an approximately 5.0 kb Genomic DNA fragment from *Streptomyces aureofaciens* NRRL 2009. **TVN Ramachander**, D. Rohini, A Belhekar, SK Rawal. *Int. J. Biol. Macromol.* 2002; **31**: 63-69.

2. Construction of a *Streptomyces sp.* - *Escherichia coli* conjugative shuttle vectors and its application for recombinant biosynthesis of poly(3-hydroxyalkanoic acid). G Tripathi, LH Mahishi, **TVN Ramchander**, SH Phadnis, OGB Nambiyar and SK Rawal. *Biotech Lett.* 2002; **24**: 213-218.

3. Molecular cloning, characterization and tissue specific expression of an elongation factor 1A gene in sugarcane. D Vijaykumar*, **TVN Ramachander*,** LH mahishi, R Kaul, P Pyati, B Paul and SK Rawal. *Plant Science* 2002; **162(2)**: 315-321.

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4. A 4.3 kb g-DNA fragment from *Streptomyces aureofaciens* supports PHB synthesis in recombinant *E. coli.* **T.V. N.Ramachander,** D. Rohini Devi, Manish Arha and S.K Rawal. *Poceedings of the 5th National Symposium on Biochemical Engineering and Biotechnology*. 2003.

5. Novel *Escherichia coli* having accession number PTA 1579 and its use to produce polyhydroxybutyrate (**US patent published – 2002/0146785**).

6. A process for the production of polyhydroxyoctanoate by *Streptomyces lividans* (**US patent published – 2002/0090687).**

RESEARCH SUBMITTED AND IN PREPARATION

1. Organization and Disribution of a 299-bp tandem repeat from *Saccharum officinarum* L. D. Vijay Kumar*, **T. V. N. Ramachander***, L. H. Mahishi, P. S. Kulkarni and S. K. Rawal. (Manuscript submitted)
   **equal authorship*

2. Sequence analysis and Characterization of a phaC gene from *Streptomyces aureofaciens* 2209. **T. V. N. Ramachander** and S. K. Rawal (Manuscript in preparation).

PAPERS PRESENTED AT CONFERENCES AND WORKSHOPS ATTENDED

1. Poster Presented at the *5th National Symposium on Biochemical Engineering and Biotechnology* held at IIT Delhi, India. (March, 2003). Ist prize.

2. Accepted for Oral Presentation at the *International Symposium on Biological Polyesters* held at Munster, Germany (22-26 September, 2002).

3. Accepted for poster presentation at the *7th World Conference on Biodegradable Polymer and Plastics* held at Pisa, Italy (June, 2002)

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

5. Poster presented at national symposium on "Development of microbial pesticides and insect pest management" held at Hotel Pride, Pune, India (November 12-13, 1998)

6. Attended workshop on "Parasite Bioinformatics" held at Bioinformatics Centre, University of Pune Campus, Pune, India from 28-31 January 2002.