CHAPTER 5
PHA GENE LOCUS FROM
*BACILLUS THURINGIENSIS* R1:
ISOLATION, CLONING, SEQUENCING AND
EXPRESSION IN *E. COLI*
5.1 INTRODUCTION

The class of PHA synthesized by a bacterium is dictated by the organism’s metabolic background and its PHA-biosynthesis genetic composition and function. The PHA biosynthesis genetic system (pha gene locus) is divided into four classes on the basis of the organization of the gene locus and the structure – function properties of the PHA synthase (polymerase) enzymes. The class I pha gene locus, as exemplified by that of Waustersia eutropha, consists of phaC (coding for PHA synthase), phaA (β-ketothiolase), and phaB (acetoacetyl-CoA reductase) genes. The class II pha operon, found in Pseudomonads, comprises of two PHA synthase genes (phaC1 and phaC2) flanking a PHA depolymerase gene (phaZ). The class III pha operon is distinguished by the presence of phaC and phaE genes coding for the two hetero-subunits of its PHA synthase. Adjacent to the two synthase genes are the phaA and phaB genes that are transcribed in the opposite direction. The class IV pha locus found in Bacillus species constitute the phaR and phaC genes coding for the two hetero-subunits of the PHA synthase and a phaB between the two genes (McCool and Cannon 1999; McCool and Cannon 2001; Satoh et al. 2002).

5.1.1. Molecular biology of PHA synthesizing genes

Till 1980’s research was limited to the identification of microorganisms synthesizing PHAs, characterization of the PHAs and their copolymers. With advancements in the molecular biology tools, characterization of the genes involved in PHA biosynthesis gained momentum. Though, the number of microorganisms shown to accumulate PHAs is increasing every year, the molecular data regarding the PHA biosynthesis genes remains rather limited.

Different strategies have been employed to identify PHA biosynthesis genes (Table 5.1). Of these only a few allow successful identification of the PHA biosynthesis genes encoding enzymes with novel and / or unusual features (Rehm and Steinbüchel 2001).

Timm et al. (1994) found that the class I phaC gene of W. eutropha was not useful as a general hybridization probe to identify PHA synthase genes. Based on conserved regions of the PHA synthases of W. eutropha and Pseudomonas
oleovorans, these authors designed 30-mer oligonucleotides for use as a universal probe for \textit{phaC}, these researchers detected and subsequently cloned the class II \textit{pha} gene loci of \textit{Pseudomonas citronellolis}, \textit{Pseudomonas} sp. DSM 1650, \textit{Pseudomonas mendocina}, and both the class I and class II \textit{pha} loci of \textit{Pseudomonas} sp. GP4BH1. The probe was not tested on classes III and IV \textit{pha} genes and most likely would not detect these genes by virtue of the basis of their design.

Table 5.1 Strategies for screening and identification of PHA biosynthesis genes  
(Source: Rehm and Steinbüchel 2001)

<table>
<thead>
<tr>
<th>Strategy</th>
<th>Screening technology</th>
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<tbody>
<tr>
<td>A</td>
<td>Enzymatic analysis</td>
</tr>
<tr>
<td>B</td>
<td>Homologous gene probes obtained by transposon insertions</td>
</tr>
<tr>
<td>C</td>
<td>Heterologous gene probes from well characterized genes</td>
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<tr>
<td>D</td>
<td>Consensus oligonucleotides derived from multiple alignments</td>
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<tr>
<td>E</td>
<td>Oligo nucleotides derived from N-terminal or internal amino acid sequences of PHA synthases</td>
</tr>
<tr>
<td>F</td>
<td>Opaque and fluorescent colonies in PHA negative host after heterologous expression</td>
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<tr>
<td>G</td>
<td>Growth after detoxification of media due to removal of fatty acids</td>
</tr>
<tr>
<td>H</td>
<td>Genome sequence analysis and functional assignments of PHA biosynthesis genes</td>
</tr>
</tbody>
</table>

Polymerase chain reaction (PCR) provides a means for rapid detection of specific genes in organisms. Lopez \textit{et al.} (1997) reported the use of PCR to identify \textit{phaC} positive organisms in river water environmental samples using nondegenerate primers derived from \textit{W. eutropha}. However, they obtained an essentially nonspecific PCR product mixture which necessitated further confirmation of the \textit{phaC} gene by Southern blot hybridization. Solaiman \textit{et al.} (2000) subsequently developed the first PCR procedure that specifically detects class II \textit{pha} genes, wherein the primers were designed on basis of the highly conserved nucleic acid sequences of the class II \textit{pha} loci of \textit{Pseudomonas}. Zhang \textit{et al.} (2001) reported PCR cloning of the class II \textit{pha} genes on basis of degenerate primers derived from the consensus sequences of ORF1,
phaZ and phaD genes of Pseudomonas. Sheu et al. (2000) designed a broad specificity PCR procedure using degenerate semi-nested primer pairs and isolated 38 PHA-positive strains by colony PCR and semi-nested PCR. PCR detection of the pha genes in Bacillus was reported by Shamala et al. (2003) using non-degenerate primer pairs designed solely on the basis of the class IV phaC of Bacillus megaterium by employing a primer-design program. However, because of the simplistic approach by which these primers were designed, a global detection of class IV pha genes could not be expected (Solaiman and Ashby 2005). This scenario necessitated homologous probes to screen the PHA gene locus from the Bacillus thuringiensis R1 genomic library.

The scope of the present chapter was to obtain a homologous probe to locate the phaC gene and subsequently use it to isolate the pha gene locus. In the present study different primers were used to amplify the phaC gene from Bacillus thuringiensis R1. The putative phaC gene was sequenced and upon sequence conformity it was used as a probe to screen the subgenomic library of Bacillus thuringiensis R1 for the pha operon.

5.2 EXPERIMENTAL PROCEDURES

5.2.1 Organisms and growth

Bacillus thuringiensis R1 and E. coli were grown and maintained as detailed in Chapter 2.

5.2.2 DNA extraction and manipulation

Genomic DNA, plasmid DNA extraction, Southern hybridization were done as described in Chapter 2.

5.2.3 Polymerase Chain Reaction (PCR)

Two sets of primers were used to amplify phaC gene from B. thuringiensis R1. The first set of primers (all 26-mers) were based on the multiple sequence alignment results of 19 phaC genes (Sheu et al. 2000). The primer set I sequences are as below:

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Forward primer I phaC F1 (26 mer):
5'(d)ATCAACAA(GGG/A)T(TT/A)CTAC(AA/G)TC(CC/T)T(CC/G)GACCT3'

Forward primer II phaC F2 (26 mer):
5' (d)GT(CCC/GG)TTC(GGG/AA)T (GGG/CC) (AAA/GG) T(CC/G)(TT/A)
(CCC/GG)CTGGCGCAACCC 3'

Reverse primer phaC R4 (26 mer):
5'(d)AGGTAGTTGT(TT/C)GAC(CCC/GG)(AAA/CC)(AAA/CC)(GGG/A)
TAG(TTT/G)TCCA3'

Two reactions were set up using the two different forward primers.

The second set of primers used earlier to screen the sub-genomic library of Bacillus sp. INT005 (Satoh et al. 2002) for phaC gene was a kind gift from Dr. Yasuharu Satoh (Division of Molecular Chemistry, Graduate school of Engineering, Hokkaido university, Sapporo 060-8628, Japan).

The primer set II sequences are as below:

Forward primer P5 (30 mer):
5' (d)AAGGATCCACTACATTCGCAACAGAATGGG 3'

Reverse primer P6 (30 mer):
5' (d)AACTGCAGTTACTTAGAGCGCTCGTCAAGC 3'

The PCR reaction mixture consisted of:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMQ</td>
<td>5.2 µL</td>
</tr>
<tr>
<td>Template DNA</td>
<td>3.0 µL (10 ng)</td>
</tr>
<tr>
<td>DNTPs</td>
<td>2.0 µL (266 µM)</td>
</tr>
<tr>
<td>10X Taq Pol. Buffer</td>
<td>1.5 µL</td>
</tr>
<tr>
<td>15 mM MgCl₂</td>
<td>1.0 µL</td>
</tr>
<tr>
<td>Forward primer</td>
<td>1.0 µL (10 picomoles)</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>1.0 µL (10 picomoles)</td>
</tr>
<tr>
<td>Taq Polymerase</td>
<td>0.3 µL (1U)</td>
</tr>
<tr>
<td>Total volume</td>
<td>15.0 µL</td>
</tr>
</tbody>
</table>

The PCR reaction was set up as below.

\[
\begin{array}{cccccc}
95°C & 94°C & 72°C & 72°C & 4°C \\
/ 5' & 1' & / 54°C & 2' & 6' & \alpha
\end{array}
\]

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5.2.4 Cloning the PCR product

The PCR amplicons were gel purified (Section 2.3.3) and cloned into pGEMT-Easy Vector (Promega, USA).

5.2.5 Restriction mapping

Plasmid DNA from recombinant *E. coli* was digested with different restriction enzymes. Single and double digestions were done as recommended by the manufacturer to generate the restriction enzyme map of the insert DNA.

5.2.6 DNA Sequencing and sequence analysis

Beckman Coulter CEQ™ 8000 Genetic Analysis System was used to perform DNA sequencing reactions using CEQ™ DTCS Quick Start Kit Dye Terminator Cycle sequencing kit provided by the manufacturer. Sequencing was bi-directional and accomplished by primer walking. Database searches and sequence analysis were performed using BLAST (National Center for Biotechnology Information, National Institute of Health, Bethesda, MD, USA).

5.2.7 Southern hybridization

The genomic DNA from *Bacillus thuringiensis* R1 was restriction digested with *Sac I*, *Cla I*, *BamHI*, *Xba I*, *Hind III*, *EcoRI*, and their combinations. Southern blotting, random primer labeling and Southern hybridization were done as described in sections 2.6, 2.7 and 2.8 respectively.

5.2.8 Sub-genomic library construction

Sub-genomic library construction and transformation were done as described in Section 2.4 and 2.5 of Chapter 2. *Bacillus thuringiensis* R1 genomic DNA (100 µg) was digested to completion with the restriction enzymes *Sac I/Cla I* (double digestion). The restriction fragments were size separated by agarose gel electrophoresis. The fragments in the size range of 4-6 kb were purified by gel extraction and cloned into the *Sac I/Cla I* digested pBluescript KS+ plasmid vector.
5.2.9 Screening for PHA synthesizing genes

Recombinants from the sub-genomic library were screened by amplifying the partial phaC gene sequence with primer set II (P5 forward and P6 reverse). Colony hybridization was done as described in section 2.9 of Chapter 2. The recombinant clones were streaked on basal media agar plates supplemented with glycerol (1% v/v) and Nile red (0.5 μg/mL media) (Spiekermann et al. 1999). In addition to the above tests the PHB synthesis was checked by extraction of the polymer from recombinant E. coli as described in Section 3.2.5.

5.3 RESULTS AND DISCUSSION

5.3.1 PCR amplification of the putative PHA synthase gene

Using the PCR set I primers phaC F1 and phaC R4 no amplification was observed, whereas an amplicon of ~600 bp was observed when the combination of phaC F2 and phaC R4 primers were used (Fig. 5.1). The amplicon was cloned into pGEMT vector (Promega, USA), designated as pRDC1 and the insert sequenced.

The sequence of the pRDC1 insert and its homology results are presented in Figures 5.2, 5.3a and 5.3b respectively. A search with the BLAST program with the deduced amino acid sequence of the 657 bp insert from pRDC1 revealed 100% amino acid sequence similarity, and 99% nucleotide sequence similarity with the DNA-directed RNA polymerase, beta’ subunit of Bacillus sps.

Fig. 5.1 PCR amplification of Bacillus thuringiensis R1 genomic DNA. Lanes:

1 & 2 - phaC F2 & phaC R4 amplified PCR fragment; 3 & 4 - phaC F1 & phaC R4 amplification; 5 - λ Eco RI/Hind III marker.
Fig. 5.2 Nucleotide sequence of pRDCl insert

1 CGATTTCATT GCTGGCGCAA CCCCACCTGT ATGGAACGT CGCATCGTTA
51 ACCTGCTGCC TCGGCTCACCC ATAGACTGAG CTGCGATAAT ACCTACCGCT
101 TTCACCAAC ACCGCCAGTT GCCAAATAT GACCGATTAC TCCGAACTAT
151 AAAACCTGATG TACCTCGCGA TTTTCAGGAT GTTTTACAGT
201 TTTTCTGGAA AAGAGCTCGA CAGACGATC ATATAACGCT TCAAACTC
251 CATTACCCCT ATTTAAATCGA CCGATTTAATA ACCTACCGCT TTTTCTTGCA
301 AAACGTCCAA CAAGACGATC ATATAATGAC TCAATAACTT
351 CATTACCCTC TTTAAATCGC CCAATTAATA AACCGCGATC TGTTCCACAA
401 TTTTCTTGCA AAACGTCCAA CAAGACGATC ATATAATGAC TCAATAACTT
451 TCATTACCGA GAGCTGCGCTAAC CCGCGATC TGTTCCACAA
501 TCATTACCGA GAGCTGCGCTAAC CCGCGATC TGTTCCACAA
551 AACCGCGATC TGTTCCACAA
601 CAGCCCGCAA ATACACGGAT GCTGAGTAAC GTCCAGTGG GTACCACGGG
651 CAGCCCGCAA

Fig. 5.3a Nucleic acid sequence similarity of pRDCl with partial sequence of DNA-directed RNA polymerase, beta’ subunit from Bacillus sps. (represented as Bac).

- **pRDCl**
  - 17 GCAACCCACCTGTATGGAACGT-CGCATCGTTAACTGTGTACCTGGCTCACCGATAGAT
  - 76 TCGCTCGGATAATACCTACCGCTTTCCCTACTCTCTGCTGTTCTCCAGTTGCTAAGTTAGTA
  - 136 CGACCGTAGAATCTTCTTACAATACTACCGGTTCGTTACAGTGTTAACGCTGAAGCAATG
  - 196 TTTACAGTTTCAACACCGAGTTTTCACAAATA7GAGCGATATCTTACGTAATTTAGATA
  - 256 TTTTCAGACCACTATAATACCTACCTGCTGTTCCAGGATGTTTTACAGTTTTTCTCCGAAACGT
  - 316 CCAACAAGACGATCATATAATGACTCAGTTACACACCCCTCTTTAATCGCAGGAAT
  - 376 AATAACCCCGAGCTCAGTTCCCAATAATACCTACCTTTACGTTGCGAACGTCA
  - 436 ACAAGACGAGCTGTTAAGATAAAGCTAAGATCCTTTACGTTTCGCGGCAAGACTCT
  - 496 TTAAGCGGCAACATGTGCCGAGATAGTTTACCACGACTCTTTACCGAAGAACTTT

- **Bac**
  - 114795 GCACCCCGACCTGTAAGGAGCT-GAGCTGATTCTGATGCTTCTGAGTCTCCAGGATAGAT
  - 114735 TGAACCCGCAATGCTGACGTGCTTCCCTACTCTCTGCTGTTCTCCAGTTGCTAAGTTAGTA
  - 114675 CGACCGTAGAATCTTCTTACAATACTACCGGTTCGTTACAGTGTTAACGCTGAAGCAATG
  - 114615 TTTACAGTTTCAACACCGAGTTTTCACAAATA7GAGCGATATCTTACGTAATTTAGATA
  - 114555 TTTTCAGACCACTATAATACCTACCTGCTGTTCCAGGATGTTTTACAGTTTTTCTCCGAAACGT
  - 114495 CCAACAAGACGATCATATAATGACTCAGTTACACACCCCTCTTTAATCGCAGGAAT
  - 114435 AATAACCCCGAGCTCAGTTCCCAATAATACCTACCTTTACGTTGCGAACGTCA
  - 114375 ACAAGACGAGCTGTTAAGATAAAGCTAAGATCCTTTACGTTTCGCGGCAAGACTCT

The sequences show high similarity, indicating a possible functional relationship between the pRDCl insert and the DNA-directed RNA polymerase, beta’ subunit from Bacillus sps.
Fig. 5.3b Amino acid sequence similarity of pRDCl with partial sequence of DNA-directed RNA polymerase, beta' subunit from Bacillus sps. (represented as Bac).

Sheu et al. (2000) designed primers phaC F1, phaC F2 & phaC R4 based on the multiple sequence alignment of 13 phaC genes from 13 Gram-negative bacteria. They used these primers to screen 38 PHA producing environmental isolates (30 Gram-positive, 5 Gram-negative and 3 Gram variable strains) and reported that the individual PCR products are suitable for use as specific probes for genomic library screening. Interestingly, one of the bacterial test samples used by these authors was Bacillus megaterium, however, the amplicon obtained was not sequenced to confirm its classification (Solaaiman and Ashby 2005). The present study proves that phaC F1, phaC F2 and phaC R4 primers cannot be used to amplify the class IV phaC gene, since the nucleic acid sequence is not homologous to those of class I and class II genes (Solaaiman and Ashby 2005). Hence, the primers designed by Sheu et al. (2000) cannot be used as universal degenerate primers to amplify the phaC gene from Bacillus sp. Shamala et al. (2003) designed non-degenerate primers based on sequence analysis of class IV synthases from Bacillus sps., however, their standard species (B. circulans and B. brevis) did not produce the expected amplicons (Solaaiman and Ashby 2005).

With the primer set II an ~1 Kb DNA fragment was amplified (Fig. 5.4). The amplicon was cloned into pGEMT vector and the clone designated as pRDC2. The
Clone pRDC2 was digested with different restriction enzymes (Fig. 5.5a & Fig. 5.5b) and a restriction map generated for the insert DNA (Fig. 5.6). The nucleotide sequence of pRDC2 is presented in Fig. 5.7. The homology search of the 1083 nucleotide sequence showed 99% homology to the nucleotide and amino acid sequences with poly(R)-hydroxyalkanoic acid synthase (phaC) gene of different Bacillus sps. (Fig. 5.8a and 5.8b). However, the sequence showed only 82% similarity to the nucleotide sequence of Bacillus megaterium. The putative lipase box (G-X-C-X-G-G) of the PHA synthase is represented in the figure. Because of high sequence similarity with other phaC gene sequences from Bacillus sps., the insert from pRDC2 (putative phaC gene) could be used as probe to locate the pha operon and screen the Bacillus thuringiensis R1 genomic library.

**Fig 5.4** PCR amplification of with set II Bacillus thuringiensis R1 genomic DNA. Lane1: λ/Hind III marker, Lanes 2 & 3: P5P6 amplified PCR fragment.
Fig. 5.5a Agarose gel electrophoresis of restriction digested pRDC2 plasmid


Fig. 5.5b Agarose gel electrophoresis of restriction digested pRDC2 plasmid

Fig. 5.6 Restriction map of pRDC2 insert DNA (not to scale)

\[ \begin{array}{cccccc}
\text{Hind III} & \text{Hinc II} & \text{Acc I} & \text{Nde I} & \text{Pvu II} \\
(163) & (464) & & & \\
\hline
1(ACT) & \text{ORF in frame 1} & & & 1081 (TAA) \\
\end{array} \]

Fig. 5.7 Nucleotide sequence of pRDC2 insert DNA.

**P5 forward**

1 ACTACATTTCGCAACAGAATGGAAAAGCAATTAGAGCTATATACCCAGAAGA
51 GTATCGAAAGCATACCAGCCGAGTGAAAAGGGCAGTGAAAATTTTTATTAC
101 GTGAAACCAGAGCCCAAGTGGATTAACGCCGAAGAGGTATTATTGGGACG
151 AAGAATAAGCAGAAGCCTTTATATCGCTACATCTCCAAAAACAAGAAAAACACA
201 AAGAGTTCCAATTCATTATATATGCTCTTATATTAATATACACTCATATATA
251 TGGACTTAACCTCCTGGAATAATTAGTTTAGTGAAATATCAGTATGGAACCCTG
301 TTTGATGTGTATATGCTTGATTGGGCATATTTGGTTTAAAGAGGATATGCA
351 TTTGAAATTTTGATGATTTCTCTGTTTGTATATATGGGCAAAACGAGTGA
401 AAGTAATACCGAGACTCGCAAAATCGGAGATCTTTTTACTTTATTGGTATTGC
451 ATGGGTTGGAAGCTTTTCTTTATTTATCGGCAACTTCTCCTCCACATGCCC
501 AATTCGCAACTTATCTTTATGAGACAGCTCTTTTTGTATTCTCTGGAACAG
551 GATTATATGGTTCTTTACTAGTAAAGATTATTTTAACCTAGATAAAAAGCA
601 GTTGATACATTTGCGAATATCTCCGCCGAAGATGATTGTATTCGGAACCAA
651 AATGATTAAAAAACCATAAAACTTTTTTGGAGATTACGTCTTTATTAGT
701 ATCGTTAGAGAATGAGCTCTGTGTGATATCTGGAAGCTGAGCTTGTTAAG
751 TGGTTGGCGATGCGCATTCCGCTTTCCAGGTGAAATCAGACAGCTGGAT
801 TGCTGATTTTTTATCAAAATTAAACTGTTAAGGGTGAAACTCGTTATTC

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Fig. 5.8a Nucleic acid sequence similarity of pRDC2 insert DNA with the *phaC* gene from *Bacillus* *sps.* (represented as Bac).

pRDC2 1  ACTACATTGCACAAGAAGGAAGCTATACCCAGAAGAGTATCGAAAA
Bac 113451 ACTACATTGCACAAGAAGGAAGCTATACCCAGAAGAGTATCGAAAA
pRDC2 61  GCATACCGCCGAGTGAAAAGGGCGAGTGAAATTTTATTACGTGAACCAGAGCCGCAAGTC
Bac 113511 GCATACCGCCGAGTGAAAAGGGCGAGTGAAATTTTATTACGTGAACCAGAGCCGCAAGTC
pRDC2 121  GGATTAACGCCGAAAGAGGTTATTTGGACGAAGAATAAGACGAAGCTTTATCGCTACATT
Bac 113571 GGATTAACGCCGAAAGAGGTTATTTGGACGAAGAATAAGACGAAGCTTTATCGCTACATT
pRDC2 181  CCAAAACAAGAAAAAACACAAAGAGTTCCAATTCTATTAATATATGCTCTTATTAATAAA
Bac 113631 CCAAAACAAGAAAAAACACAAAGAGTTCCAATTCTATTAATATATGCTCTTATTAATAAA
pRDC2 241  CCATATATTATGGACTTAACTCCTGGAAATAGTTTAGTGGAATATCTAGTGGACCGTGGT
Bac 113691 CCATATATTATGGACTTAACTCCTGGAAATAGTTTAGTGGAATATCTAGTGGACCGTGGT
pRDC2 301  TTTGATGTGTATATGCTTGATTGGGGCACATTTGGTTTAGAAGATAGTCATTTGAAATTT
Bac 113751 TTTGATGTGTATATGCTTGATTGGGGCACATTTGGTTTAGAAGATAGTCATTTGAAATTT
pRDC2 361  GATGATTTCGTGTTTGATTATATTGCAAAAGCAGTGAAAAAAGTAATGCG7y\CTGCAAAA
Bac 113811 GATGATTTCGTGTTTGATTATATTGCAAAAGCAGTGAAAAAAGTAATGCGAACTGCAAAA
pRDC2 421  TCGGACGAGATTTCTTTACTTGGTTATTGCATGGGTGGAACGTTAACTTCTATTTATGCG
Bac 113871 TCGGACGAGATTTCTTTACTTGGTTATTGCATGGGTGGAACGTTAACTTCTATTTATGCG
pRDC2 481  TCTGAAACAGGATTATATGGTCCTTTACTAGATGAAAAATATTTTAACTTAGATAAAGCA
Bac 113931 TCTGAAACAGGATTATATGGTCCTTTACTAGATGAAAAATATTTTAACTTAGATAAAGCA
pRDC2 541  GCACTTCATCCGCACATGCCAATTCGCAACTTAATCTTTATGACAAGTCCTTTTGATTTC
Bac 113991 GCACTTCATCCGCACATGCCAATTCGCAACTTAATCTTTATGACAAGTCCTTTTGATTTC
pRDC2 601  TCTGAAACAGGATTATATGGTCCTTTACTAGATGAAAAATATTTTAACTTAGATAAAGCA
Bac 113991 TCTGAAACAGGATTATATGGTCCTTTACTAGATGAAAAATATTTTAACTTAGATAAAGCA
Fig. 5.8b Amino acid sequence similarity of pRDC2 with poly(R)-hydroxyalkanoic acid synthase from *Bacillus sps.* (represented as Bac).

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PRDC2   601  GTTGTGATACATTTGGCAATATTCCGCCAGAAATGATTGATTTCGGAAACAAAATGTTAAAA
Bac 114051 GTTGTGATACATTTGGCAATATTCCGCCAGAAATGATTGATTTCGGAAACAAAATGTTAAAA

PRDC2   661  CCATTACAAACTCTGTGGGACCATACGTTGCTTTAGTAGATCGTTCAGAGAATGAGCGT
Bac 114111 CCATTACAAACTCTGTGGGACCATACGTTGCTTTAGTAGATCGTTCAGAGAATGAGCGT

PRDC2   721  TTTGGAAGCTGGAGGTTAGTTCAAAAGTGGGTTGGCGATGGCATTCCGTTCCCAGGT
Bac 114171 TTTGGAAGCTGGAGGTTAGTTCAAAAGTGGGTTGGCGATGGCATTCCGTTCCCAGGT

PRDC2   781  GAATCATACGAGCTTGATTCCGCCAGAAATGATTGATTTCGGAAACAAAATGTTAAAA
Bac 114231 GAATCATACGAGCTTGATTCCGCCAGAAATGATTGATTTCGGAAACAAAATGTTAAAA

PRDC2   841  CTCGTTATTCGCGGACAAAAGGTAGACCTTGCAAATATTAAGGCGAATGTCTTAATT
Bac 114291 CTCGTTATTCGCGGACAAAAGGTAGACCTTGCAAATATTAAGGCGAATGTCTTAATT

PRDC2   901  TCCGGGAAACGTGATCATATCGCTCTGCCATGCCAAGTAGAAGCATTGCTAGATCATATT
Bac 114351 TCCGGGAAACGTGATCATATCGCTCTGCCATGCCAAGTAGAAGCATTGCTAGATCATATT

PRDC2   961  TCTAGCACAGATAAACAATATGTATGTTTACCAACAGGGCATATGTCTATCGTTTACGGC
Bac 114411 TCTAGCACAGATAAACAATATGTATGTTTACCAACAGGGCATATGTCTATCGTTTACGGC

PRDC2  1021  GGAACAGCTGTAAAACAAACGTATCCGACGATTGGAGATTGGCTTGACGAGCGTTCAAAG
Bac 114471 GGAACAGCTGTAAAACAAACGTATCCGACGATTGGAGATTGGCTTGACGAGCGTTCAAAG

PRDC2  1081  TAA 114533

Putative lipase box
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PRDC2  62  PKQETQRPILLIYALINKYIMDLTPGNSLVEYLVDRGFDVYMLDWTGFGLEDSHLKF
Bac 122  DDFVFDYIAKAVKKVMRTAKSDEISLLGYCMGGTTLTSTIYAALHPHMPIRNLHMTSPFDF

PRDC2  361  DDFVFDYIAKAVKKVMRTAKSDEISLLGYCMGGTTLTSTIYAALHPHMPIRNLHMTSPFDF
Bac 122  DDFVFDYIAKAVKKVMRTAKSDEISLLGYCMGGTTLTSTIYAALHPHMPIRNLHMTSPFDF

PRDC2  541  SETGLYGPLLDEKYFNLDKAVDTFGNIPPEMIDFGNKMLKPITNFVGPYPVYVALDERSNER
Bac 182  SETGLYGPLLDEKYFNLDKAVDTFGNIPPEMIDFGNKMLKPITNFVGPYPVYVALDERSNER

PRDC2  721  FVESWRVQKWGWGPFPGFESRYQWIRDFTQNYQNKLVKGVELVIRQKGVDPGNIKANVLNI
Bac 242  FVESWRVQKWGWGPFPGFESRYQWIRDFTQNYQNKLVKGVELVIRQKGVDPGNIKANVLNI

PRDC2  901  SGKRDHALPQCQVEALLLDHISSTDQYVCLFTGHSIVYGYGGTAVKQYPTIGWDLERSK
Bac 302  SGKRDHALPQCQVEALLLDHISSTDQYVCLFTGHSIVYGYGGTAVKQYPTIGWDLERSK

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5.3.2 Construction of Bacillus thuringiensis R1 sub-genomic library

Bacillus thuringiensis R1 genomic DNA was digested with restriction enzymes Sac I, Cla I, BamH I, Xba I, Hind III, EcoR I, and their combinations (Fig. 5.9) and hybridized with $^{32}$P labelled ~1 kb insert from pRDC2. The Southern hybridization showed positive signals in all the lanes (Fig. 5.10). The probe hybridized to restriction fragments of different sizes (Table 5.2). The pRDC2 plasmid with the putative phaC gene served as the positive control. To construct a sub-genomic library of Bacillus thuringiensis R1, genomic DNA was digested with Cla I and Sac I (Fig. 5.11). Fragments ranging from 4-6 kbp were shotgun cloned into Cla I and Sac I digested pBluescript KS+.

5.3.3 Screening the sub-genomic library of Bacillus thuringiensis R1 for PHB operon

The recombinant clones were PCR screened for the phaC gene operon using set II P5 and P6 primers. With one of the clones an approximately 1 kb fragment (Fig. 5.12) was amplified, which upon sequencing was homologous to the pRDC2 insert. The recombinants were further screened by colony hybridization using putative phaC gene probe from pRDC2. The probe hybridized to one recombinant colony of the Bacillus thuringiensis R1 sub-genomic library (Fig. 5.13). The same colony also showed weak fluorescence when plated on Basal medium with Nile red and exposed to ultraviolet light. The positive clone was checked by growing the recombinant E. coli cells in Basal media and PHB extraction by cell lysis. However, the recombinant clone accumulated only minimal amount of PHB (3.0% of dry cell mass) as compared to Bacillus thuringiensis R1 (34.0% of dry cell mass) in basal media with glycerol as the carbon source. The PHA+ clone was designated as pSVA10. The clone harbors an ~5.0 Kb genomic DNA fragment from Bacillus thuringiensis R1.

5.3.4 Restriction mapping of pSVA10

Plasmid DNA from pSVA10 was digested with Apa I, Kpn I, Hinc II, Nde I, Xho I, Acc I, Xba I, Hind III, Sac I, Cla I, EcoR I, Pst I, Sma I, BstB I, BstY I, Xcm I, Nsi I, Sty I, EcoR V, Sca I, Mlu I, Bgl II. The representative photographs are shown in Figures 5.14a, b, c, d. The results of the digestions are summarized in Table 5.3.
Fig. 5.9 Agarose gel electrophoresis of restriction digested genomic DNA of *Bacillus thuringiensis* R1 (Bt)


Fig. 5.10 Southern hybridization of the gel shown in Fig 5.9 with the insert from pRDC2
Table 5.2 Summary of Southern blot analysis of the *Bacillus thuringiensis* R1 digests using putative *phaC* gene as the probe.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Restriction enzymes used</th>
<th>Hybridization signals (approx. size)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.</td>
<td><em>Cla I</em></td>
<td>~9.0 kb</td>
</tr>
<tr>
<td>3.</td>
<td><em>Sac I</em></td>
<td>~7.0 kb</td>
</tr>
<tr>
<td>4.</td>
<td><em>Cla I &amp; Sac I</em></td>
<td>~5.0 kb</td>
</tr>
<tr>
<td>6.</td>
<td><em>BamH I</em></td>
<td>~10.0 kb</td>
</tr>
<tr>
<td>7.</td>
<td><em>Xba I</em></td>
<td>&gt;10.0 kb</td>
</tr>
<tr>
<td>8.</td>
<td><em>BamH I &amp; Xba I</em></td>
<td>~6.0 kb</td>
</tr>
<tr>
<td>10.</td>
<td><em>EcoR I</em></td>
<td>&gt;10.0 kb</td>
</tr>
<tr>
<td>11.</td>
<td><em>Hind III</em></td>
<td>~1.4 kb</td>
</tr>
<tr>
<td>12.</td>
<td><em>EcoR I &amp; Hind III</em></td>
<td>~1.4 kb</td>
</tr>
</tbody>
</table>

Fig. 5.11 Agarose gel electrophoresis of restriction digested genomic DNA of *Bacillus thuringiensis* R1

Lanes: 1, 2, 4 and 5 - Bt/Cla I/Sac I; 3- λ/Hind III marker
Fig. 5.12 PCR amplification using with P5 P6 set II primers

Lanes: 1: Bt; 2: pBSK+; 3: pRDC2; 4: pSVA10 as template DNA;
5: λ/Hind III marker

Fig. 5.13 Colony hybridization representative autoradiogram for screening of subgenomic library of Bacillus thuringiensis R1. The 1 kb fragment from pRDC2 was used as the probe for hybridization.
**Fig. 5.14a** Restriction digestions of pSVA10


**Fig. 5.14b** Restriction digestions of pSVA10

Fig. 5.14c Restriction digestions of pSVAlO


Fig. 5.14d Restriction digestions of pSVAlO

Table 5.3 Summary of restriction digestions of pSVA10

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Restriction enzymes</th>
<th>Approx. fragment sizes in basepairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Apa</em>I</td>
<td>8000</td>
</tr>
<tr>
<td>2.</td>
<td><em>Kpn</em>I</td>
<td>8000</td>
</tr>
<tr>
<td>3.</td>
<td><em>Hinc</em>II</td>
<td>3500 2200 1500 800</td>
</tr>
<tr>
<td>4.</td>
<td><em>Nde</em>I</td>
<td>4500 3500</td>
</tr>
<tr>
<td>5.</td>
<td><em>Xho</em>I</td>
<td>8000</td>
</tr>
<tr>
<td>6.</td>
<td><em>Acc</em>I</td>
<td>5000 1800 1200</td>
</tr>
<tr>
<td>7.</td>
<td><em>Xba</em>I</td>
<td>8000</td>
</tr>
<tr>
<td>8.</td>
<td><em>Hind</em>III</td>
<td>6600 1400</td>
</tr>
<tr>
<td>9.</td>
<td><em>Sac</em>I</td>
<td>8000</td>
</tr>
<tr>
<td>10.</td>
<td><em>Cla</em>I</td>
<td>8000</td>
</tr>
<tr>
<td>11.</td>
<td><em>Bst</em>YI</td>
<td>4500 2700 900</td>
</tr>
<tr>
<td>12.</td>
<td><em>Nsi</em>I</td>
<td>8000</td>
</tr>
<tr>
<td>13.</td>
<td><em>Sty</em>I</td>
<td>8000</td>
</tr>
<tr>
<td>14.</td>
<td><em>EcoRV</em></td>
<td>7500 500</td>
</tr>
<tr>
<td>15.</td>
<td><em>Sca</em>I</td>
<td>5400 2600</td>
</tr>
<tr>
<td>16.</td>
<td><em>Mlu</em>I</td>
<td>8000</td>
</tr>
<tr>
<td>17.</td>
<td><em>Bgl</em>II</td>
<td>8000</td>
</tr>
<tr>
<td>18.</td>
<td><em>Cla</em> I + <em>Sac</em> I</td>
<td>5000 3000</td>
</tr>
<tr>
<td>19.</td>
<td><em>Cla</em> I + <em>Mlu</em> I</td>
<td>5200 2800</td>
</tr>
<tr>
<td>20.</td>
<td><em>Cla</em> I + <em>Bgl</em> II</td>
<td>4500 3500</td>
</tr>
<tr>
<td>21.</td>
<td><em>Cla</em> I + <em>Sty</em> I</td>
<td>4000 4000</td>
</tr>
<tr>
<td>22.</td>
<td><em>Xho</em> I + <em>Hind</em> III</td>
<td>5800 1400 800</td>
</tr>
<tr>
<td>23.</td>
<td><em>Hind</em> III + <em>Nde</em> I</td>
<td>4000 2600 900 500</td>
</tr>
<tr>
<td>24.</td>
<td><em>Xho</em> I + <em>Nde</em> I</td>
<td>3500 3200 1300</td>
</tr>
<tr>
<td>26.</td>
<td><em>Xho</em> I + <em>Xba</em> I</td>
<td>4300 3700</td>
</tr>
<tr>
<td>27.</td>
<td><em>Sac</em> I + <em>Sca</em> I</td>
<td>5400 1600 1000</td>
</tr>
<tr>
<td>28.</td>
<td><em>Sac</em> I + <em>Sty</em> I</td>
<td>7100 900</td>
</tr>
<tr>
<td>29.</td>
<td><em>Sac</em> I + <em>Xba</em> I</td>
<td>6550 1300 150</td>
</tr>
<tr>
<td>30.</td>
<td><em>Sac</em> I + <em>Bgl</em> II</td>
<td>6400 1600</td>
</tr>
<tr>
<td>31.</td>
<td><em>Cla</em> I + <em>EcoRV</em></td>
<td>3900 3600 500</td>
</tr>
<tr>
<td>32.</td>
<td><em>Sac</em> I + <em>EcoRV</em></td>
<td>6600 900 500</td>
</tr>
<tr>
<td>33.</td>
<td><em>Sac</em> I + <em>Nsi</em> I</td>
<td>7400 600</td>
</tr>
</tbody>
</table>
Fig. 5.15 Restriction map of pSVA10 (not to scale)

Among the restriction enzymes used Apa I, Kpn I, Xho I, EcoR I, Pst I, Sma I and BstB I do not cut the insert. The enzymes Sca I, Mlu I, Bgl II, Sty I, Nsi I cut the insert only once while EcoR V and Hind III have cut the insert twice. The predicted restriction map is presented in Figure 5.15.

5.3.5 Sequence of ~5.0 kb insert from pSVA10

The complete sequence of the insert in the pSVA10 was found to be 4787 bp (Fig. 5.16). This was deposited with NCBI GenBank and is available under the accession number DQ000291.

Fig. 5.16 4,787 bp sequence of the cloned Bacillus thuringiensis R1 g-DNA fragment (GenBank Acc. No. DQ000291)

1 GAGCTCCAC ATTTTTCTTA CTGAGATGGAA GTAAATAGAT TTTTTGAGTT
51 TTCCCTGAAAC TTTTTTGCGA GTTCCAACTTG CTTAATTCTT CAACTCCTCA
101 AAAAACCTTC TAACTGTTTT TGAGCCTCTT CACGTTGTAA TTGTGTTGTT
151 TCAATAATTT GTTTTTGTGT TCTCTCAAAT TGCCCCTGTG TTTGAGTTAAG
201 AATAGACAAA GATGTTTTTCG TAGGAGAAG ACAGTAGCTGC TGGCATATGAG
251 CAGAAAGTTTC CTTCCATTTT CTTGCGACT CATTAATTTT ATCATTTAAG
2201 TAATTGACGT GAACCTTAAGC AGTGTTTATTA ATACGACAGA CGCGGTACTT
2251 GCATACATA CCAGAGCGAGA AGAAGGTGAA ATTATTAGCA TTTCTTCTAT
2301 TATTGGTCAA GCGGGTGGAA TTGGACAAAC AAACTACTCA GCAGCAGAAT
2351 CAGGTATGCT AGGATTTACA AAATCATTAG CATTAGAGCT TGCAAAAACA
2401 AATGTAACTG TAAACGCTAT TTGCCCAGGA TTTATTGATA CTGAAATGGT
2451 AGCAGAAGTA CCAGAAGAGA TAGCTCAAAAA AATCGTTGCA AAAAAAGGGA
2501 AAAAAAGTTC TGGTCAAGCA GATGAAAATT CAAAAGGGTT AGTATACCTA
2551 TGCCTGAGCC GTGCTTATAT TACAGGTCAG CAATTAAACA TTAACGGCGG
2601 ATTATATATG TAATGAAGTA AGAAAAAGT GCATATCCCA AAAAAATGGAC
2651 TACATTGGCA ACAGAATGGG AAAAGCAAAT AGAGCTATAC CCAGAAGAGT
2701 ATCGAAAAAG ATACGCCGCA GTGAAAAGGG CGAGTGAAAT TTTATTACGT
2751 GAACGACGAC GCAGCTTACTG CTGAAAATTT TACCCAGTTT GACCCCTTAT
2801 TGGTGCTGAT ATGCTTGATT GGGCACATT TGGTTTAGAA GATACCTATT
2851 TGAATGGTTA TTTCACTTTA TTTATCGGAC ACTTCATCGGA CACCGCAGAA
2901 TTCGAATTTT AATTTTATAG ACAAGCTTTT TTTATCTTTG TAAACAGGTA
2951 TTTATATGCT CTTTACTAGA TTTATTTTAT TTTATTTTAT GTAAAACAGGTA
3001 TGATAATATTG TTTAAACTTT TTTAAATTTT TTTAAATTTT TTTAAATTTT
3051 TTATGCTTAT TTTTTTATTT TTTTTTATTT TTTTTTATTT TTTTTTATTT
3101 GTATAATATTG TTTAAACTTT TTTAAATTTT TTTAAATTTT TTTAAATTTT
3151 GTAAATTTTT TTTTTTATTT TTTTTTATTT TTTTTTATTT TTTTTTATTT
3201 GTAAATTTTT TTTTTTATTT TTTTTTATTT TTTTTTATTT TTTTTTATTT
3251 GTAAATTTTT TTTTTTATTT TTTTTTATTT TTTTTTATTT TTTTTTATTT
3301 GTAAATTTTT TTTTTTATTT TTTTTTATTT TTTTTTATTT TTTTTTATTT
3351 GTAAATTTTT TTTTTTATTT TTTTTTATTT TTTTTTATTT TTTTTTATTT
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3951 GTAAATTTTT TTTTTTATTT TTTTTTATTT TTTTTTATTT TTTTTTATTT
4001 GTAAATTTTT TTTTTTATTT TTTTTTATTT TTTTTTATTT TTTTTTATTT
4051 GTAAATTTTT TTTTTTATTT TTTTTTATTT TTTTTTATTT TTTTTTATTT
5.4 CONCLUSIONS

- The set I primers (phaC F1, phaC F2 & phaC R4) could not amplify the phaC gene. However, phaC F2 & phaC R4 amplified a different gene i.e., DNA-directed RNA polymerase, beta' subunit of the Bacillus sps. genome.

- An ~1 kb gDNA fragment amplified with set II primers (P5 and P6) coded for the Bacillus thuringiensis putative phaC gene which could be used as probe to locate the PHA gene locus.

- The putative phaC gene is designated as pRDC2 has the putative lipase box (G-X-C-X-G-G) of the PHA synthase.

- An ~5 kb Sac I – Cla I insert form Bacillus thuringiensis R1 confers the ability of PHA accumulation to recombinant E. coli designated as pSVA10. PHB accumulation, however, was in negligible amounts.

- A restriction map was generated for pSVA10.

- The complete sequence of the insert in the pSVA10 was found to be 4787 bp and the sequence was deposited with NCBI GenBank and is available under the accession number DQ000291.