Short Communication

Genetic Determination of Polyhydroxyalkanoate Metabolism in *Rhodobacter capsulatus* SB1003

(bacterial genomes / biotechnology / gene organization / polyhydroxyalkanoates)

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Abstract. A cluster of four genes was identified in the *Rhodobacter capsulatus* genome that is involved in PHA metabolism. These genes encode the PHA granule-associated protein (*pha2*), the regulator for granule formation (*pha1*), the PHA synthase (*phaC*) and the PHA depolymerase (*orfX*). Two other genes, namely those encoding β-ketothiolase (*phaA*) and acetoacetyl-CoA reductase (*phaB*), are not linked to this cluster.

Depending on the available substrates, certain bacteria are able to synthesize various polyhydroxyalkanoates (PHAs). The PHA biosynthetic pathway in *R. capsulatus* consists of three reactions catalyzed by at least three enzymes, namely the β-ketothiolase, NADPH-dependent acetoacetyl-CoA reductase and PHA synthase. These enzymes are encoded by the *phaA*, *phaB* and *phaC* genes, respectively. The first step of the PHA biosynthesis, catalyzed by β-ketothiolase, is condensation of two acetyl-CoA molecules to acetoacetyl-CoA. This compound is reduced by the action of NADPH-dependent acetoacetyl-CoA reductase to 3-hydroxyacyl-CoA. The final polymerization is catalyzed by PHA synthase.

Under optimal conditions PHA can form up to 80% of the dry cell mass (Kusaka et al., 1997). The polymer is normally dispersed within bacterial cells and additional enzymes are needed to granulate it. The bacteria use PHAs mainly as a storage material, but also as a sink for reducing equivalents (Steinbuchel, 1991).

PHAs have properties similar to polyethylene and polypropylene (Holmes, 1985). Compared to traditional oil-derived plastics, PHAs have better physical properties and are absolutely non-toxic. They are thermoplastic and biodegradable, with no xenobiotics remaining in the environment (Kusaka et al., 1999). They can be used in many chemical, medical and pharmaceutical applications (Holmes, 1985).

Rhodobacter capsulatus can produce PHAs when grown photosynthetically on acetone with limiting concentration of nitrogen (Madigan, 1990). It was found that *R. capsulatus* SB 1003 accumulates PHA up to 52% of the cell dry weight, while strain B10 accumulates 90% under the same conditions (Kranz et al., 1997). Comparisons of the appropriate genes from the two strains may help explain this difference.

Because of the potential economic benefits, genes regulating PHA synthesis have been studied in numerous bacterial strains. We characterized genes in *R. capsulatus* SB 1003 and compared them with genes of other bacteria.

Material and Methods

DNA cloning

In the course of the *Rhodobacter capsulatus* genome project, a cosmid library was constructed covering the entire bacterial chromosome (Fonstein and Haselkorn, 1993). The PHA gene cluster was found on the 1G5 cosmid. This DNA was isolated and partially digested with the *Sau*3A restriction endonuclease. After agarose gel electrophoresis fragments of 2 to 3.5 kb were isolated using the QIAquick Gel Extraction kit (QIAGEN, Valencia, CA). The DNA fragments were ligated to *Bam*HI-linearized vector pGEM 3Zf+ (Promega, Madison, WI). *E. coli* DH5α (Life Technologies, Paisley, GB) was transformed by these constructs. DNA was isolated using R.E.A.L.® Prep 96 System (QIAGEN).

DNA sequencing

Individual subclones were sequenced by the Sanger method from both ends using universal and reverse primers. Sequencing reactions were prepared using a thermosequenase kit for asymmetric PCR with fluorescently labelled dye terminators (ABI Prism®

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Abbreviations: ORFs – open reading frames; PHA – polyhydroxyalkanoate.

BigDye™ Terminator Cycle Sequencing Kit, Applied Biosystems, Foster City, CA) and analysed in the sequencer ABI Prism®377XL.

Analysis and annotation of the nucleotide sequence

The DNA STAR programme package was used to assemble individual nucleotide sequences into contigs. To obtain the final contiguous sequence primer walking and targeted cloning was applied to fill remaining gaps. Open reading frames (ORFs) were found using start and stop codon identification, Shine-Dalgarno sequences, and the CodonUse programme (Pačes and Pačes, 2002). Structural motifs in the sequenced DNA were characterized by Clone Manager 5 software (Scientific & Educational Software, Durham, CA). Amino acid sequences translated from the predicted ORFs were compared with sequences in the SwissProt or Trembl databases using BlastP, psiBlast and Fasta3 programmes. Nucleotide sequences were compared with the Blast2 programme. For annotations of ORFs the programme package assembled in the WIT suite (http://wit.mcs.anl.gov/WIT2/) was used.

The accession number of the nucleotide sequence presented in this communication is AJ315564.

Results and Discussion

When analysing nucleotide sequences of the *Rhodobacter capsulatus* SB1003 genome, the cluster of four genes was found that are highly homologous to genes involved in PHA metabolism of *Rhodobacter sphaeroides* and *Paracoccus denitrificans* (Hustede and Steinbüchel, 1993; Maehara et al., 1999). Figure 1 shows this gene cluster. Functions were ascribed to individual genes by homology search (Table 1).

The PHA cluster consists of genes encoding the PHA synthase (*phaC*), a regulator of PHA granule formation (*pha1*), a granule-associated protein (*pha2*) and a gene with homology to PHA depolymerases from *Methylobacterium extorquens* and *Ralstonia eutropha* (*orfX*) (Korotkova and Lindstrom, 2001; Saegusa et al., 2001).

In addition, we found four *phaA* genes encoding ketoacyl-CoA thiolase in other chromosomal locations (Fig. 2). This is in agreement with the results of Kranz et al. (1997) indicating that *R. capsulatus* has several ketothiolases differing probably in substrate specificity.

Pha1 and Pha2 gene products were identified as proteins important for PHA granule formation and granule size (Maehara et al., 1999). Pha1 is a transcriptional regulator and Pha2 is a member of the phasins family. Proteins with similar functions were found in *Paracoccus denitrificans, Ralstonia eutropha, Rhodococcus rubber*, *Methylobacterium rhodesenium*, *Acinetobacter* sp., and *Aeromonas caviae* (Pieper-Fürst et al., 1994; Föllner et al., 1995; Schembri et al., 1995; Föllner et al., 1997).

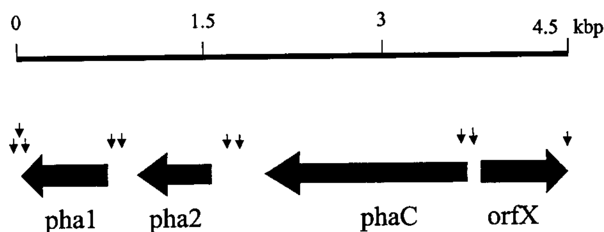

Fig. 1. Gene cluster of *R. capsulatus* involved in PHA metabolism. The arrows show the ORFs orientation. The small arrows indicate positions of the regions encoding stem-loop structures.

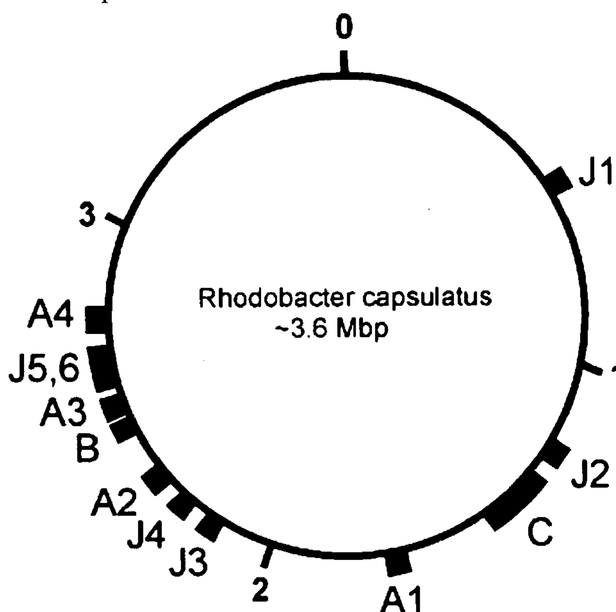

Fig. 2. Positions of the PHA metabolism genes in the *R. capsulatus* genome. A1-A4: genes for β-ketothiolases; B: gene encoding acetoacetyl-CoA reductase; C: PHA cluster described in this communication; J1-J6: genes for enoyl-CoA hydratases. The numbers indicate Mbp. This map is derived from the nucleotide sequences obtained in the genome project and from the cosmid map prepared by Fonstein and Haselkorn (1993).

Upstream of the *phaC* gene there is, in opposite orientation, the *orfX* gene. Homologous genes are present in PHA gene clusters in several PHA-producing bacteria (Hustede and Steinbüchel, 1993; Maehara et al., 1999). Recently, a homologous gene encoding PHA depolymerase was identified in *Methylobacterium extorquens* and *Ralstonia eutropha*.

Table 2 shows homologies of individual proteins involved in PHA metabolism among various bacterial strains.

We identified several possible loops in the predicted secondary structure of RNA corresponding to the intergenic DNA regions (Fig. 1). These loops could be signals for transcription termination or could have other regulatory roles. There are several *phaJ* genes in the *R. capsulatus* genome. These genes encode enoyl-CoA hydratases that are involved in an alternative pathway of PHA synthesis. Positions of all genes in question in the *R. capsulatus* genome are in Fig. 2.

Table 1. Functions of ORFs in the PHA metabolism gene cluster in R. capsulatus.

ORF	Encoded protein	Number of aa	ID	Orientation	GC (%)
Pha1	Regulator for granule-associated protein	180	Q9WX82	-	64.1
Pha2	Granule-associated protein	148	Q9WX81	-	61.7
PhaC	PHA synthase	583	O05334	-	61.9
OrfX	PHA depolymerase	422	Q9WX79	+	63.1

aa – amino acids, ID – SWISS-PROT accesion number

Table 2. The best homologies of proteins involved in PHA metabolism to database entries

Protein	Bacterium	Function	Identity / similarity[a]
PhaA1	*Pseudomonas aeruginosa*	thiolase	59 / 72
PhaA2	*Ralstonia eutropha*	thiolase	53 / 67
PhaA3	*Paracoccus denitrificans*	thiolase	79 / 87
PhaA4	*Pseudomonas aeruginosa*	thiolase	58 / 66
PhaB	*Paracoccus denitrificans*	acetoacetyl-CoA reductase	79 / 86
Pha1	*Paracoccus denitrificans*	granule-associated protein regulator	69 / 77
Pha2	*Paracoccus denitrificans*	granule-associated protein	68 / 85
PhaC	*Rhodobacter sphaeroides*	PHA synthase	62 / 80
OrfX	*Paracoccus denitrificans*	protein of unknown function from PHA cluster	72 / 84
	Rhodobacter sphaeroides	protein of unknown function from PHA cluster	70 / 81
	Methylobacterium extorquens	intracellular PHB depolymerase	41 / 58
	Ralstonia eutropha	intracellular PHB depolymerase	40 / 56

[a]percentage of identical amino acids / percentage of similar amino acids

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