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Poly(3-Hydroxybutyrate) Synthesis Genes in *Azotobacter* sp. Strain FA8

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Genes responsible for the synthesis of poly(3-hydroxybutyrate) (PHB) in *Azotobacter* sp. FA8 were cloned and analyzed. A PHB polymerase gene (*phbC*) was found downstream from genes coding for β-ketothiolase (*phbA*) and acetoacetyl-coenzyme A reductase (*phbB*). A PHB synthase mutant was obtained by gene inactivation and used for genetic studies. The *phbC* gene from this strain was introduced into *Ralstonia eutropha* PHB-4 (*phbC*-negative mutant), and the recombinant accumulated PHB when either glucose or octanoate was used as a source of carbon, indicating that this PHB synthase cannot incorporate medium-chain-length hydroxyalkanoates into PHB.

Polyhydroxyalkanoates (PHAs) are a group of polyesters produced by a large number of bacteria, which accumulate them in intracellular granules as a response to environmental stress and nutrient imbalance (2, 7). These thermoplastic polymers have drawn great interest since their discovery due to their degradability and the potential to produce them from renewable carbon sources. *Azotobacter* sp. FA8 is an aerobic, nitrogen-fixing bacterium that accumulates poly(3-hydroxybutyrate) (PHB) when cultivated on several carbon sources, including sucrose (13). Although the capacity of *Azotobacter* strains to accumulate PHAs is well known (2, 9), except for a recently described β-ketothiolase from *Azotobacter vinelandii* (18), the genes responsible for their synthesis have not yet been identified. In this paper we report the identification, cloning, and molecular analysis of the *phb* gene cluster of *Azotobacter* sp. FA8.

Cloning and molecular analysis of the PHB synthase gene from *Azotobacter* sp. FA8. Genomic DNA of *Azotobacter* sp. FA8 was partially digested with *Xho*I and ligated to the mobilizable cosmid pVK102 (5) digested with the same enzyme and dephosphorylated. The resulting ligation mixture was packaged using an in vitro packaging system (Stratagene, La Jolla, Calif.) and used to transfect *Escherichia coli* S17-1, a strain that contains the *tra* genes of plasmid RP4 integrated into the chromosome (19). Transductants were selected on Luria-Bertani plates containing 10 μg of tetracycline/ml. The library was screened for the presence of the PHB synthase (*phbC*) gene by complementation analysis. *Ralstonia eutropha* PHB-4 (17), a *phbC* mutant, was used as recipient. The complementation was carried out on a mineral salts medium (16) containing 1.5% (wt/vol) agar, 0.05% (wt/vol) NH_4Cl, 0.5% (wt/vol) fructose, and 5 μg of tetracycline/ml. One of the *E. coli* clones, C1, gave rise to opaque, PHB-producing transconjugants. The polymer produced by the recombinant was extracted from lyophilized cells with hot chloroform and ethanol precipitated, and the methyl ester derivatives were analyzed by gas chromatography (1) using a Gow Mac Series 580 gas chromatograph (Bridgewater, N.J.) equipped with a flame ionization detector and a packing column of Carbowax 20 M-TPA–Chromosorb W-AW (SUPELCO, Bellefonte, Pa.). The polymer obtained was found to be a homopolymer of 3-hydroxybutyrate.

The recombinant cosmid from clone C1, pC1 (Table 1), was purified using the Concert High Purity system (BRL, Rockville, Md.) and subjected to restriction analysis with several enzymes, giving an estimated insert size of around 20 kb. Digestion with *Bam*HI originated two bands of 6 and 3 kb. The cosmid cut with this enzyme was diluted, religated, and used to transform *E. coli* S17-1, giving rise to cosmid pC1B, containing a 9-kb deletion, which was not able to complement the *R. eutropha phbC* mutation. The two *Bam*HI restriction fragments were then subcloned in pBluescript SK (Stratagene) to obtain the recombinant plasmids pAC3 and pAC6, containing the 3- and 6-kb *Bam*HI fragments, respectively (Table 1), and the ends of the inserts from both plasmids were sequenced using M13 forward and reverse universal sequencing primers. An ABI 373 A automatic sequencer was used for sequencing. The sequences obtained were compared with sequences available in the databanks using the Blast program (Bioinformatics Center, Institute for Genomic Research, Kyoto, Japan [http://www.blast.genome.ad.jp]), and a region highly homologous to the amino-terminal end of PHA synthase genes occurred 41 bp downstream from one end of the 6-kb insert. Sequencing of the complete corresponding 1,700-bp open reading frame (ORF) was obtained from this clone by primer walking. The deduced amino acid sequence from this ORF (ORF1) showed a striking homology with the PhbC gene product of *Pseudomonas* sp. 61-3 (GenBank accession no. AB014757) (67% identity; 82% similarity). ORF1 also showed great homology with other PHB synthases, such as PhbC from *R. eutropha* (GenBank accession no. J05003) (55% identity; 73% similarity) and PhaC from *Burkholderia* sp. (GenBank accession no.

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TABLE 1. Recombinant plasmids used in this work

Plasmids	Relevant characteristics	Source or reference
pC1	pVK102 containing a 20-kb chromosomal fragment from *Azotobacter* sp. FA8 inserted at the *Xho*I site	This work
pC1B	a deletion derivative of pC1, obtained by self-ligation after restriction with *Bam*HI	This work
pAC3	pBluescript containing a 3-kb *Bam*HI fragment from pC1	This work
pAC6	pBluescript containing a 6-kb *Bam*HI fragment from pC1	This work
pATpoliK	pAT18 (21) containing a 578-bp amplification fragment from the *Azotobacter* sp. FA8 *phbC* gene, interrupted by a kanamycin cassette	This work
pRKpolC1	pRK404 (3) containing a 2.3-kb fragment from pAC6 containing the *phbC* coding region downstream of *lac* promoter	This work
pHP1014::2000	Tc^r Cm^r $phaC1_{Pa}$ $phaD_{Pa}$ $phaC2_{Pa}$ ORF1 ORF2 ORF3 ORF4′	21

AF153086) (52% identity; 71% similarity), and was consequently designated *phbC*.

No genes related to PHB synthesis were found downstream from the *phbC* gene. Sequencing of the upstream region from cosmid pC1 revealed the presence of the carboxy-terminal region of an ORF (ORF2) with great (>80%) homology with previously described β-ketoacyl-coenzyme A (CoA) thiolase genes.

Cloning of the β-ketoacyl-CoA thiolase and acetoacetyl-CoA reductase genes from *Azotobacter* sp. strain FA8. In order to clone the rest of ORF2, presumably the *Azotobacter* sp. FA8 β-ketoacyl-CoA thiolase (*phbA*) gene, primers were designed from the conserved sequences corresponding to the amino-terminal region of several β-ketoacyl-CoA thiolase genes available in the databanks and used together with C1 (5′ GAC ATT GAT CCT GAA AAG CG 3′), a primer corresponding to the region immediately upstream from the *phbC* gene from *Azotobacter* sp. FA8 (Fig. 1), but no amplification fragment was obtained. *pha* genes are normally organized in clusters (7). *phb* genes were not found downstream of *phbC*, and we hypothesized that the acetoacetyl-CoA reductase gene (*phbB*) might be upstream from ORF2. Based on this hypothesis, several primers were designed from conserved regions of *phbB* genes. The following primers were used to obtain a 1,500-bp amplification fragment: R1 [5′ GCN GA(C/T) TT(G/T) (A/T)(G/C)N (G/T /C)TN AA(C/T) GGN GG 3′], a degenerate primer corresponding to the conserved carboxy-terminal region of available *phbB* genes, and C1, described above. The 1,500-bp amplification fragment was cloned into vector pGemT-Easy (Promega, Madison, Wis.) and sequenced as indicated previously. The analysis of its sequence revealed the presence of a 1,176-bp ORF (ORF2) highly homologous to previously described *phbA* genes. The deduced amino acid sequence of ORF2, designated *phbA*, comprised 392 residues and showed similarity with other β-ketoacyl-CoA thiolases, such as the biosynthetic ketothiolase (PhbA) of *A. vinelandii* (GenBank accession no. AF267243) (94% identity; 96% similarity), the acetyl-CoA acetyltransferase of *Pseudomonas aeruginosa* PAO1 (AtoB) (GenBank accession no. C83396) (83% identity; 91% similarity), and the β-ketoacyl-CoA thiolases (PhbA) of *Pseudomonas* sp. strain 61-3 (GenBank accession no. T44362) (80% identity; 89% similarity) and *R. eutropha* (GenBank accession no. J05003) (67% identity; 79% similarity).

A similar approach was used for the cloning of the acetoacetyl-CoA reductase gene (*phbB*) from *Azotobacter* sp. FA8. A degenerate primer, R2 (5′ TNA CNG GNG GNA TGG GNG G 3′), was designed from a conserved region situated approximately 30 bp downstream from the amino-terminal end of available acetoacetyl-CoA reductase genes and used together with primer R3 (5′GCA TGT TCA GAC CAC CGT TG 3′), corresponding to the already sequenced carboxy-terminal end of the gene (Fig. 1), to obtain a 714-bp amplification fragment, which was cloned and sequenced as indicated above. The analysis of its sequence revealed the presence of an ORF (ORF3) highly homologous to previously cloned *phbB* genes. The inverse PCR technique was used to obtain the first 30 bases from the nonconserved amino-terminal end of ORF3. *Bgl*I-digested genomic DNA from *Azotobacter* sp. FA8 was treated with T4 DNA ligase (New England Biolabs, Beverly, Mass.) at a final concentration of approximately 10 ng/μl. Two microliters of the ligation mixture was used for PCR amplification with primers R1 and R4 (5′CGG TCA CGC CCT TGC TGG 3′), corresponding to the already sequenced part of ORF3 (Fig. 1). The 1,724-bp amplification fragment that was obtained (which was cloned and sequenced as previously described) contained an incomplete ORF corresponding to the amino-terminal end of ORF3. The deduced amino acid sequence of ORF3 showed great homology with previously de-

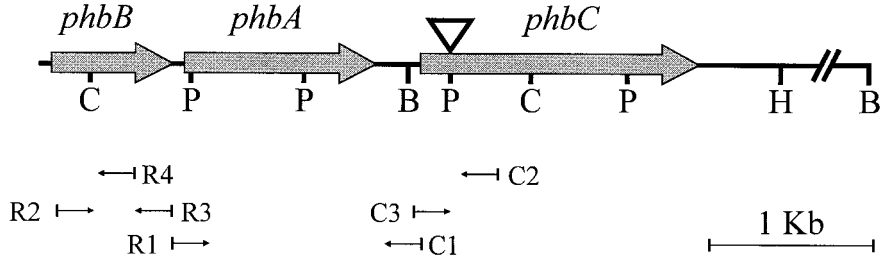

FIG. 1. Organization of the *Azotobacter* sp. FA8 genomic region containing the genes *phbA*, *phbB*, and *phbC*. Relevant restriction sites are indicated with capital letters (B, *Bam*HI; C, *Cla*I; H, *Hin*dIII; P, *Pst*I). Thin black arrows indicate the positions and directions of primers used in this work. The restriction site used for mutagenesis by gene disruption is indicated by an inverted triangle.

TABLE 2. Analysis of polymer production from different carbon sources in *Azotobacter* sp. FA8 and several recombinant strains[a]

Strain	Plasmid	Carbon source(s) (% concn)	PHA produced	PHB content (% [wt/wt] of CDW)[b]
Azotobacter sp. FA8	None	Glucose (3)	PHB	38
		Glucose (3) + octanoate (0.125)	PHB	26
		Glucose (0.5)	PHB	4.5
		Glucose (0.5) + octanoate (0.125)	PHB	3.5
Azotobacter sp. UBA 60-3	None	Glucose (3)	ND[c]	ND
Azotobacter sp. UBA 60-3	pC1	Glucose (3)	PHB	25
		Glucose (3) + octanoate (0.125)	PHB	14
Azotobacter sp. UBA 60-3	pHP1014::2000	Glucose (3)	ND	ND
		Glucose (3) + octanoate (0.125)	ND	ND
Azotobacter sp. UBA 60-3	pRK404	Glucose (3)	ND	ND
Azotobacter sp. UBA 60-3	pRKpolC1	Glucose (3)	PHB	28.5
R. eutropha PHB-4	None	Glucose (1.5)	ND	ND
R. eutropha PHB-4	pRKpolC1	Glucose (1.5)	PHB	4
		Octanoate (0.1)	PHB	1.9
		Octanoate (0.25)	PHB	3.9
		Octanoate (0.5)	PHB	5

[a] *Azotobacter* strains were grown for 24 h in Burk's medium supplemented with carbon sources as indicated. *Ralstonia eutropha* recombinants were grown for 48 h in mineral salts medium supplemented with carbon sources as indicated.
[b] CDW = cell dry weight.
[c] ND, not detected (≤1% [wt/wt] of CDW).

scribed *phbB* genes, such as the *phbB* genes from *Pseudomonas* sp. strain 61-3 (GenBank accession no. T44362) (76% identity; 87% similarity), *Burkholderia* sp. (GenBank accession no. AF153086) (67% identity; 82% similarity), and *R. eutropha* (GenBank accession no. J05003) (64% identity; 81% similarity), and was consequently designated *phbB*. Several consensus sequences of σ^{70}-dependent promoters were found in the region upstream from *phbB*.

Construction of a PHB synthase mutant by gene inactivation. A 578-bp fragment from the *Azotobacter* sp. strain FA8 *phbC* gene containing a *Pst*I internal restriction site was obtained by PCR amplification of genomic DNA using primers C2 (5′CGC AAT CCC GTT GAT AAG 3′) and C3 (5′CGC TTT TCA GGA TCA ATG TC 3′) (Fig. 1). The amplification fragment was cloned in the pGEM-T Easy cloning vector (Promega), digested with *Pst*I, and ligated with a kanamycin cassette obtained from plasmid pUC4K (Pharmacia, San Francisco, Calif.) cut with *Pst*I. A 1,750-bp *Eco*RI fragment carrying the whole insert (*phbC* gene fragment with kanamycin cassette) was cloned into the mobilizable Em^r plasmid pAT18 (22), which cannot replicate in *Azotobacter*, and used to transform *E. coli* S17-1. Km^r, Em^r transformants were selected on Luria-Bertani plates containing 50 μg of kanamycin/ml and 100 μg of erythromycin/ml, and their recombinant plasmids were purified using standard techniques and checked by restriction analysis. One of these transformants was used as a conjugation donor in order to introduce the recombinant plasmid, designated pATpoliK (Table 1), into *Azotobacter* sp. FA8. Transconjugants were selected on Burk's medium (9) containing 1.5% (wt/vol) agar, 0.5% (wt/vol) sucrose, and 5 μg of kanamycin/ml. Their PHB phenotype was verified by gas chromatography of the methyl ester derivatives as previously described (Table 2). One PHB-negative mutant, *Azotobacter* sp. UBA 60-3, was chosen for further studies. In order to characterize the mutant by complementation, we cloned a 2.3-kb *Hin*dIII fragment from pAC6, containing the complete *phbC* gene, into the mobilizable Tet^r plasmid pRK404 (3), downstream from the *lac* promoter, giving rise to plasmid pRKpolC1 (Table 1). This construction was introduced in *E. coli* S17-1 by transformation and transferred to mutant *Azotobacter* sp. UBA 60-3 by conjugation. All the Tet^r transconjugants were able to accumulate PHB, indicating that the mutation in *Azotobacter* sp. UBA 60-3 was complemented by the *phbC* gene (Table 2). The capacity of the transconjugants to accumulate PHB was analyzed by gas chromatography as indicated above.

Production of polyhydroxyalkanoates from different carbon sources in *Azotobacter* sp. strain FA8. Bacterial PHA synthases can be classified in three classes, depending on the type of hydroxyalkanoates they can use as substrates and their subunit composition (14). PHA synthases that prefer three to five carbon substrates and are composed of one type of subunit are called short-chain-length (SCL) PHA synthases and belong to class I. PHA synthases that use substrates with 6 to 14 carbons are also composed of one type of subunit, are called medium-chain-length (MCL) PHA synthases, and belong to class II. The best known example of class I PHA_{SCL} synthases is the *R. eutropha* PHB synthase. Most pseudomonads have PHA synthases belonging to the second group, PHA_{MCL} synthases. Class III PHA synthases prefer three to five carbon substrates and are composed of two different subunits: PhaC (PHA synthase homologue) and PhaE (20). An exception of the class III enzymes is the *Thiocapsa pfennigii* PHA synthase, which also accepts six to eight carbon substrates (6). Class II PHA_{MCL} synthase genes expressed in *E. coli* facilitated the accumulation of PHA_{MCL} from fatty acids when fatty acid β-oxidation was truncated (10, 11, 12).

To determine if *Azotobacter* sp. FA8 could produce MCL polyhydroxyalkanoates, polymer formation in cultures grown in octanoate-supplemented medium was evaluated. Growth of this strain was not observed in Burk's medium containing this fatty acid or hexanoic acid as sole carbon sources. When *Azotobacter* sp. strain FA8 was grown in Burk's medium containing glucose and sodium octanoate, PHB was the only polymer detected (Ta-

ble 2). This result could be due to (i) the inability of the cells to metabolize MCL fatty acids to intermediates that could be used by the PHA polymerase, or (ii) the inability of the PHA polymerase to incorporate MCL monomers into the polyhydroxyalkanoate. As an approach to studying these possibilities, plasmid pHP1014::2000 (21), containing genes encoding PHA_{MCL} synthases PhaC1 and PhaC2 from *P. aeruginosa*, was introduced in *Azotobacter* sp. UBA 60-3 by conjugation. The recombinants were grown in Burk's medium with the addition of octanoate, and their polymer contents were determined (Table 2). No polymer was detected in mutant *Azotobacter* sp. UBA 60-3 containing pHP1014::2000. We cannot rule out the possibility that the *Pseudomonas* genes could not be expressed in this strain. *R. eutropha* is able to use octanoate as a carbon source, and, when grown in a medium supplemented with this fatty acid, recombinants containing a PHA_{MCL} synthase are able to synthesize PHAs containing 3-hydroxyhexanoic acid and 3-hydroxyoctanoic acid (21). Plasmid pRKpolC1, containing the *Azotobacter* sp. strain FA8 *phbC* gene downstream from the *lac* promoter (Table 1) was introduced into *R. eutropha* PHB-4 to determine if the *Azotobacter* sp. FA8 synthase could incorporate MCL monomers into the polymer. The only polymer produced by the recombinant was PHB (Table 2).

These collective findings indicate that the product of the *phbC* gene from *Azotobacter* sp. FA8, situated downstream of the genes for a β-ketoacyl-CoA thiolase and acetoacetyl-CoA reductase (Fig 1), is a class I PHA synthase. This conclusion is supported by the fact that (i) this enzyme cannot incorporate MCL hydroxyalkanoates into PHA, and (ii) the product of the *phbC* gene reestablishes the capacity of *R. eutropha* PHB-4 to accumulate PHB. The *Azotobacter* sp. FA8 *phb* genes showed great homology to the *phb* genes of a PHB-producing *Pseudomonas* strain, *Pseudomonas* sp. 61-3 (8). Similarities in the genes between members of these genera have been previously described (4) and could be due to a common phylogenetic origin or horizontal gene transfer.

The importance of PHAs to the fitness and survival of bacteria during periods of starvation has been proposed (2, 7). Recent work has established a correlation between polymer utilization and nucleotide accumulation and provides insight into the mechanism by which PHAs enhance the survival capabilities of bacteria (15). The isolation of the genes described in the present study will facilitate both applied and basic research on polyhydroxyalkanoates in nitrogen-fixing soil bacteria.

Nucleotide sequence accession numbers. The nucleotide sequences reported in this paper have been deposited in the EMBL database under the accession numbers AJ319748 (corresponding to the *Azotobacter* sp. FA8 *phbC* and *phbA* genes) and AJ311166 (corresponding to the *Azotobacter* sp. FA8 *phbB* gene).

While the manuscript of this report was in the process of being evaluated, the sequences corresponding to the *phb* gene cluster of *A. vinelandii* were released. These sequences, annotated under the GenBank accession number AF267243, include the *phbC* and *phbB* genes, which exhibit high amino acid homology (91% identity and 95% similarity for *phbC*; 94% identity and 97% similarity for *phbB*) with the corresponding *Azotobacter* sp. FA8 genes described in this study.

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