J Microbiol Immunol Infect
2006;39:18-27

Original Article

Identification of two gene loci involved in poly-beta-hydroxybutyrate production in *Rhodobacter sphaeroides* FJ1

Mei-Kwei Yang, Yu-Chih Lin, Che-Hung Shen

Department of Life Science, Fu-Jen University, Taipei, Taiwan

Received: August 9, 2005 Revised: September 10, 2005 Accepted: October 5, 2005

Background and Purpose: Polyhydroxyalkanoates (PHAs), biopolyesters of hydroxyl fatty acids, are synthesized and deposited as cytoplasmic inclusions in many bacteria. We isolated a poly-beta-hydroxybutyrate (PHB)-producing bacterium designated *Rhodobacter sphaeroides* FJ1. To characterize PHB biosynthesis in this organism, we isolated the genes encoding proteins involved in PHB metabolism.
Methods: The genes responsible for the synthesis, accumulation, and degradation of PHB in *R. sphaeroides* FJ1 were cloned and characterized.
Results: Genes involved in the biosynthesis and metabolism of PHB were found to be located in 2 different loci in the genome of *R. sphaeroides* FJ1. One locus contained genes encoding PHB depolymerase (*phbZ*), PHB synthase (*phbC*), phasin (*phbP*) and the regulator protein (*phbR*). The other locus contained the beta-ketothiolase gene (*phbA*) and the acetoactyl-CoA reductase gene (*phbB*). The *phbZ* gene was orientated in an opposite direction to that of *phbC*, *phbP* and *phbR* genes that were located in the same cluster. *R. sphaeroides* FJ1 was able to grow in wastewater released from the human waste treatment plant of Fu-Jen University. Optimal growth and PHB production were achieved when *R. sphaeroides* FJ1 was grown in tryptic soy broth containing 50% wastewater. PHB production by *R. sphaeroides* FJ1 varied in media with different carbon to nitrogen ratios, but the level of PHB synthase was constant, suggesting that PHB production depends mainly on substrate supply.
Conclusions: Six genes encoding proteins related to PHB metabolism are clustered in 2 separate loci, *phbZCPR* and *phbAB*, in a PHB-producing bacterium *R. sphaeroides* FJ1 isolated from wastewater. PHB synthase, the key enzyme for PHB synthesis, is constitutively expressed, and its expression level is not affected by different growth conditions.

Key words: Bacterial genes, molecular sequence data, poly-beta-hydroxybutyrate polymerase, poly-beta-hydroxybutyrate, *Rhodobacter sphaeroides* FJ1

Introduction

Polyhydroxyalkanoates (PHAs) are high-energy polyesters of hydroxyalkanoic acids and are synthesized and deposited as intracellular granules in many different microorganisms under unbalanced growth conditions [1-3]. The production of PHA increases bacterial survival in nutrient-depleted environments because PHAs can be degraded to provide energy and carbon source. More than 150 different hydroxyalkanoic acid derivatives have been identified as constituents of PHA.

Corresponding author: Dr. Mei-Kwei Yang, Department of Life Science, Fu-Jen University, 510 Chun-Chen Road, Taipei 242, Taiwan.
E-mail: mkyang@mails.fju.edu.tw

Natural PHAs have the thermoplastic properties of chemically synthesized polymers such as polyethylene or polypropylene. PHA polymers are biodegradable and have great potential for industrial and medical applications [4,5]. Among various PHA polymers, poly-beta(β)-hydroxybutyrate (PHB), homopolymer of β-hydroxybutyrate, is the most well characterized polymer. PHA heteropolymers of β-hydroxybutyrate and β-hydroxyhexanoate or β-hydroxybutyrate and β-hydroxyvalerate have also been found [6].

PHB is synthesized from acetyl-coenzyme A (acetyl-CoA) in 3 steps. A PHA-specific β-ketothiolase, encoded by *phbA*, catalyzes the condensation of 2 molecules of acetyl-CoA to form acetoacetyl-CoA. Acetoacetyl-CoA is then reduced by acetoacetyl-CoA reductase, encoded

by *phbB*, to produce β-hydroxybutyryl-CoA which is then polymerized by PHB synthase to become high molecular weight PHB, encoded by *phbC* [7,8]. Formation of PHA granules is enhanced by a granule-associated protein referred to as phasin, which binds to PHA granules or interacts with PHA synthase [9,10]. Phasin is encoded by *phaP* [11,12] which is negatively regulated by a regulator protein, encoded by *phaR* [13,14]. For use as a carbon source, PHA is hydrolyzed by PHA depolymerase, encoded by *phaZ*, to yield oligomers or monomers [15,16].

Biochemical and genetic analyses of PHB synthase and other enzymes involved in PHB synthesis have been performed [1,7,8]. However, large-scale microbial PHA production has not been achieved, probably due to unsatisfactory yield and high cost of growth media. In this study, we isolated a purple, non-sulfur bacterium *Rhodobacter sphaeroides* FJ1 that can grow and produce PHB in wastewater. This isolate produced much higher amounts of PHB than the reference strain of *R. sphaeroides*. To understand PHB production in this isolate, we characterized the PHB operon which includes the *phbA, phbB, phbC, phbP, phbR* and *phbZ* genes.

Methods

Bacterial strains and growth conditions

To isolate photosynthetic bacteria, continuously flowing wastewater was sampled and incubated on TSB (10 g of bacto-tryptone, 5 g of bacto-soytone, 5 g of NaCl and 2 g of glucose per liter) agar plates at 28°C for 5 days in an incubator ($100 \times 40 \times 50$ cm^3 in size) illuminated with two 60 W incandescent light bulbs. Individual red colonies were picked and cultured in liquid TSB medium for identification. Bacteria were identified based on microscopic characteristics, results obtained from the MIDI microbial identification system, and nucleotide sequences of the 16S rRNA gene.

Wastewater used for determination of growth requirements of *R. sphaeroides* FJ1 was obtained from the human waste treatment plant of Fu-Jen University, Taiwan. Before adding to the growth medium, the wastewater was filtered through Whatman No. 1 filter and then sterilized by autoclaving at 121°C for 25 min. TSB medium was added to the sterilized wastewater to final concentrations of 0, 25%, 50%, 75%, and 100% (V/V). Growth of *R. sphaeroides* FJ1 in these TSB/wastewater mixtures was determined by colony counts in triplicate for each culture. For PHB production, FJ1 cells were grown at 28°C in wastewater supplemented with 50% TSB to stationary phase and then in modified glutamate-malate yeast extract (GMY) medium [17] for various lengths of time.

GMY medium is composed of the following per liter: KH_2PO_4, 0.5 g; K_2HPO_4, 0.5 g; $MgSO_4$-$7H_2O$, 0.2 g; $CaCl_2$-$2H_2O$, 0.05 g; NaCl, 0.2 g; $(NH_4)_2SO_4$, 0.5 g; yeast extract, 0.1 g; and 1 mL each of trace element and growth factor solution. The trace element solution contained, per liter, disodium ethylenediamine tetra-acetic acid, 2 g; $FeSO_4$-$7H_2O$, 2 g; H_3BO_3, 0.1 g; $CoCl_2$-$6H_2O$, 0.1 g; $ZnCl_2$, 0.1g; $MnCl_2$-$4H_2O$, 0.1 g; $NaMoO_4$-$2H_20$, 0.02 g; $NiCl_2$-$6H_2O$, 0.02g; $CuCl_2$-$2\ H_20$, 0.01g; and Na_2SeO_3, 1 mg. The growth factor solution contained, per liter, thiamine-HCl, 0.5 g; niacin, 0.5 g; *p*-amino benzoic acid, 0.3 g; pyridoxine hydrochloride, 0.1 g; vitamin B_{12}, 0.05 g; and biotin, 0.05 g.

Plasmid construction and molecular cloning techniques

A portion of the *phbC* gene was amplified from the genome of *R. sphaeroides* FJ1 by polymerase chain reaction (PCR) using primers corresponding to conserved regions identified by sequence comparison of *phbC* genes from various bacteria. The forward primer (UHC1, 5'-GGAATTCGTGGGT(C/G)AA(C/T)CC(C/G)GA-3') and reverse primer (LHC1, 5'-CGGGATCCA(C/G)GG(C/G)(A/G)CGATATGGTC-3') were made based on nucleotide sequences corresponding to amino acids 293 to 298 (Ser-Trp-Val-Asn-Pro-Glu) and 498 to 503 (Asp-His-Ile-Ala-Pro-Trp), respectively, of the PhbC protein of *R. sphaeroides*. *Eco*RI and *Bam*HI restriction sites were incorporated at the 5' ends of primers UHC1 and LHC1, respectively, to simplify cloning. PCR was performed in a total volume of 100 mL containing 100 ng of *R. sphaeroides* FJ1 genomic DNA, PCR buffer, 2.5 U of *Taq* DNA polymerase (Invitrogen), 2.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, and 20 pmol of each primer for 35 cycles of 94°C for 1 min, 60°C for 1.5 min, and 72°C for 2 min.

To determine the nucleotide sequence of the *phbC* gene, the entire gene was cloned. Primers UHC2 (5'-GACAAGTCCATATGGCAACCGAAG-3') and LHC2 (5'-AGCGTATACTCGGGGCG-3') were used to amplify the region corresponding to nucleotides –11 to +1812 with respect to the deduced translational starting point of *phbC*. This 1.8-kb PCR fragment was digested with *Nde*I and then ligated into the corresponding restriction sites on pGEM-T (Promega, Madison, WI,

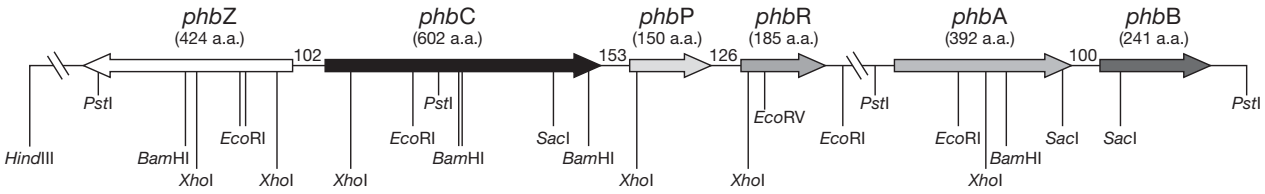

Fig. 1. Restriction map and genetic organization of poly-beta(β)-hydroxybutyrate (PHB) genes of *Rhodobacter sphaeroides* FJ1. The 7.0-kb *Hin*dIII-*Eco*RI fragment containing the *phbZCPR* locus and the 4.7-kb *Pst*I fragment containing the *phbAB* locus are shown. PHB genes include *phbZ* (PHB depolymerase), *phbC* (PHB synthase), *phbP* (phasin), *phbR* (putative regulator protein), *phbA* (β-ketothiolase), *phbB* (acetoacetyl-CoA reductase). The number of amino acids (a.a.) encoded by each ORF is indicated. Numbers shown at intergenic spaces are numbers of nucleotides between the 2 genes.

USA), generating pHBC1. DNA sequences were determined by the dideoxy chain termination method [18] using the *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA). Computer analysis of nucleotide and amino acid sequences was performed with the PC/GENE package version 6.85 (IntelliGenetics, Mountain View, CA, USA). Alignments of deduced sequences of various PhbC proteins were performed with CLUSTAL W [19] (http://www.ebi.ac.uk/clustalw/). The aligned sequence files generated by CLUSTAL W were imported into GeneDoc [20] to determine sequence identities (Fig. 1 and Table 1).

To express the PHB synthase in *Escherichia coli*, the entire coding region of *phbC* was amplified by PCR using primers UHC2 and LHC2 described above. The resulting 1.8-kb PCR product was digested with *Nde*I and then ligated with pTYB4 (New England Biolabs, Beverly, MA, USA), fusing PhbC to the intein tag at the C-terminal end of the PHB synthase. The recombinant *phbC*-intein gene was driven by the T7 promoter and regulated by the *lac* operator. The recombinant plasmid was named pHBC2 and was introduced into the *E. coli* strain ER2566 to express the PhbC protein [21]. The transformants were grown in 1 liter TSB medium to optical density at 600 nm (OD_{600}) of 0.6, and then induced with IPTG at a final concentration of 1 mg/mL. After 60 min of induction, the cells were pelleted, resuspended in 5 mL of column buffer, and then sonicated. The PhbC-intein fusion protein in the cell lysate was purified using the IMPACT (intein-mediated purification with an affinity chitin-binding tag) system (New England Biolabs, Beverly, MA, USA). Since the intein tag contained a chitin-binding domain, cell lysate was load onto a column packed with chitin beads. After washing with column buffer, the PhbC protein was dissociated from intein by eluting the column with 30 μM DTT which caused the intein tag to undergo autocatalytic cleavage [22], releasing the PhbC protein (67 kDa) from chitin beads. The PhbC protein thus purified was electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and a single band of a 67-kDa protein was seen.

Detection of PHB synthase in *R. sphaeroides* FJ1

Cell lysates (each contained 30 μg of protein) prepared from *R. sphaeroides* FJ1 were electrophoresed on a 10%

Table 1. Percent identity of *Rhodobacter sphaeroides* FJ1 *phbA,-B ,-C,-P,-R*, and *-Z* genes with those of various bacteria

Organism	Percent identity of nucleotide and amino acid sequences[a]					
	PhbA	*phbB*	*phbC*	*phb*P	*phb*R	*phbZ*
Rhodobacter sphaeroides	–	–	98 (99)	–	–	–
Rhodobacter capsulatus	–	–	66 (62)	77 (64)	77 (79)	75 (75)
Paracoccus denitrificans	80 (81)	83 (85)	66 (55)	69 (61)	75 (69)	70 (67)
						Z_1 50 (36)
Ralstonia eutropha H16	70 (67)	51 (50)	45 (34)	30 (19)	41 (26)	Z_2 53 (35)
						Z_3 46 (36)
Alcaligenes latus	71 (65)	65 (48)	54 (39)	–	–	–
Rhizobium meliloti 41	75 (76)	78 (80)	48 (34)	–	–	–
Rhizobium etli	74 (74)	76 (79)	48 (36)	–	–	–
Zoogloea ramigera	77 (75)	81 (80)	46 (34)	–	–	–

[a]Numbers in parentheses are percent identity of amino acid sequence; – indicates sequences are absent.

SDS-PAGE. The separated proteins were transferred to a nitrocellulose membrane and subjected to Western blot analysis using polyclonal antibodies against *R. sphaeroides* FJ1 PhbC. The antibody was produced in BALB/c mice by injecting 40 ng each of purified PhbC emulsified in Freund's complete adjuvant 3 times (3 weeks apart). Approximately 10 days after the third injection, the immunized mice were bled and anti-sera were prepared. Immune complexes were detected with a horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) and an enhanced chemiluminescence substrate (EZ-Link kit, Pierce, Rockford, IL, USA). The immunoreactive proteins were quantified by scanning densitometry.

Measurement of PHB production

PHB was extracted from cultured cells with chloroform using a Soxhlet extractor as described previously [23]. Cells from 100 mL of culture were collected by centrifugation (500 *g*, 10 min). Approximately 300 mg of dried cells was mixed with 200 mL chloroform and refluxed for 5 h at 95°C. After the chloroform was evaporated, the resulting white powder containing PHB polymers was dissolved in acetone and then vacuum dried. The total amount of PHB thus purified was weighed, and the ratio of PHB to dry cell weight was determined.

Results

Isolation of PHB-producing bacterium *R. sphaeroides* FJ1

To isolate photosynthetic bacteria, 100 μL of wastewater was added to a screw-capped tube containing 20 mL of TSB medium. After 5-7 days of incubation at 28°C with white light illumination under anaerobic conditions without stirring, significant bacterial growth with red color was observed in most of the cultures. These red-colored cultures were serially diluted, spread onto TSB agar plates, and incubated at 28°C. A total of 142 red colonies appeared. These colonies were analyzed by colony hybridization with an oligonucleotide probe specific for the *phb*C gene, to detect PHB-producing bacteria. Seven of the 142 colonies hybridized with the probe. These 7 isolates, designated FJ1-7, were grown in TSB medium and then assessed for PHB production. Isolate FJ1 was found to have the highest PHB level (36.4 ± 3 mg/g dry weight), 2-fold that produced by the reference strain *R. sphaeroides* O.U.001 [24].

Isolate FJ1 was characterized as a Gram-negative, non-motile, and non-spore-forming rod with red to brown-red pigment when grown under anaerobic light conditions and with pink pigment when grown under aerobic dark conditions. Results of the MIDI Sherlock microbial identification system, which identifies bacteria based on gas chromatographic analysis of cellular fatty acids, indicated that isolate FJ1 belonged to the genus *Rhodobacter*. The 16S rRNA sequence of FJ1 was determined and found to be most homologous to that of *R. sphaeroides* (75% identity). Therefore, isolate FJ1 was identified as *R. sphaeroides* and was designated as *R. sphaeroides* FJ1.

Cloning of genes involved in PHB synthesis in *R. sphaeroides* FJ1

To clone the *phbC* gene, which encodes the PHB synthase, from *R. sphaeroides* FJ1, 2 oligonucleotide PCR primers (UHC1 with *Eco*RI site and LHC1 with *Bam*HI site built in at 5' end) based on conserved sequences of *phbC* genes from various bacterial species were designed. With these 2 primers, a 600-bp DNA fragment was amplified from the genome of *R. sphaeroides* FJ1. After digestion with *Eco*RI and *Bam*HI, this 600-bp fragment was inserted into pBCSK$^+$, generating pHC600. The nucleotide sequence of the entire 600-bp *phbC* gene from *R. sphaeroides* FJ1 was determined and compared with that of *R. sphaeroides* [25]. The 2 sequences were found to share 99% identity, suggesting that this 600-bp PCR fragment indeed contained a portion of the *phbC* gene of *R. sphaeroides* FJ1. The 600-bp fragment was then labeled with digoxigenin and used as a probe to identify a DNA fragment containing the entire *phb* operon of *R. sphaeroides* FJ1. Genomic DNA from isolate FJ1 was digested with restriction enzymes *Apa*I, *Bam*HI, *Cla*I, *Eco*RV, and *Hin*dIII, electrophoresed on a 1% agarose gel, and then subjected to Southern blot analysis with the probe. A *Hin*dIII-*Eco*RV fragment of approximately 6 kb hybridized with the probe. Therefore, 6- to 9-kb *Hin*dIII-*Eco*RV fragments of *R. sphaeroides* FJ1 genomic DNA were isolated and cloned into pBCSK$^+$. This partial genomic library was screened with the digoxigenin-labeled 600-bp probe. Of approximately 1000 colonies examined, 4 reacted with the probe. Restriction enzyme analysis revealed that all of these 4 colonies harbored a 6.5-kb *Hin*dIII-*Eco*RV fragment. A partial restriction map of the insert of the recombinant plasmid pHEV65 containing this 6.5-kb *Hin*dIII-*Eco*RV is shown in Fig. 1.

Sequence analysis of the *phbC* gene of *R. sphaeroides* FJ1

The nucleotide sequence of the entire 6.5-kb *Hin*dIII-*Eco*RV fragment was determined. Analyses of the sequence revealed the presence of 4 open reading frames (ORF) [Fig. 1]. ORF602 contained the sequence of the 600-bp fragment that was initially cloned. The deduced amino acid sequence of this ORF had a 98% homology with that of the PhbC protein of *R. sphaeroides* (GenBank accession no. L17049). Substantial nucleotide sequence homologies also exist between this ORF and those of the *phbC* genes of various bacteria, including *Rhodobacter capsulatus* (GenBank accession no. AJ315564; 66% identity), *Paracoccus denitrificans* (GenBank accession no. AB017045; 66% identity), *Ralstonia eutropha* (GenBank accession no. J05003; 45% identity), *Alcaligenes latus* (GenBank accession no. AF078795; 54% identity), *Rhizobium etli* (GenBank accession no. U30612; 48% identity), *Rhizobium meliloti* (GenBank accession no. AF031938; 48% identity) and *Zoogloea ramigera* (GenBank accession no. U66242; 46% identity) [Table 1].

Alignment of the deduced amino acid sequences of 8 of these PHB synthases revealed a high degree of conservation in the carboxyl-terminal region. The lipase box-like sequence (Gly-X-Cys-X-Gly-Gly), which is believed to be the active site of the PHB synthase [26], is located in this region. In addition, the aspartic acid residues at positions 351, 428 and 480 and histidine residues at positions 481 and 508 of the *R. eutropha* PHB synthase [3] are conserved among the PhbC proteins of all the bacteria compared. These amino acid residues are known to contribute to part of the catalytic activity of the PHB synthase. In the *R. sphaeroides* FJ1 PHB synthase, the lipase box-like sequence is shifted slightly downstream to positions 333-338, and the Gly^{321} residue of the lipase box-like sequence of the *R. eutropha* synthase is changed to aspartic acid at position 337.

The *phbZ, phbP, and phbR* genes of *R. sphaeroides* FJ1

ORF424 located in an opposite transcriptional direction immediately upstream from ORF602 showed nucleotide sequence homology with the *phbZ* gene of *P. denitrificans* (GenBank accession no. AB017045; 74% identity), *R. capsulatus* (GenBank accession no. AJ315564; 75% identity), and *PhbZ1, Z2 and Z3* genes of *R. eutropha* H16 (GenBank accession no. AB017612, AF549808 and AF549809; 50%, 53% and 46% identity, respectively). The amino acid sequence deduced from the nucleotide sequence of ORF424 was similar to that of the intracellular PHB depolymerase of *P. denitrificans* (70% identity) and *R. capsulatus* (75% identity).

Alignment of deduced amino acid sequences of PHB depolymerases from 6 different bacteria revealed that amino acid residues Cys^{183}, Asp^{355} and His^{388}, which make up the catalytic triad of PhaZ1 of *R. eutropha* [27], are perfectly conserved among the PhbZ proteins from all the bacteria compared. In the *R. sphaeroides* FJ1 depolymerase protein, this catalytic triad is shifted to positions 180, 351 and 384, respectively. The $serine^{118}$ and $histidine^{120}$ residues of *R. eutropha* PhaZ1, which may affect the catalytic activity, are also present at positions 114 and 116 of the *R. sphaeroides* FJ1 PhaZ. We therefore concluded that ORF424 encodes the PHB depolymerase of *R. sphaeroides* FJ1.

ORF150 located immediately downstream from ORF602 had a 69% and 77% nucleotide sequence homology with the *phbP* genes of *P. denitrificans* (GenBank accession no. AB017045) *and R. capsulatus* (GenBank accession no. AJ315564), respectively (Table 1). The deduced amino acid sequence of this ORF was found to have a 61% and 64% homology with those of the PhbP proteins of *P. denitrificans and R. capsulatus*, respectively, but only a 19% homology with that of the *phbP* gene of *R. eutropha*. A hydrophilic region (from Val-75 to Ala-123) flanked by 2 hydrophobic regions (from Leu-64 to Gln-73 and from Pro-124 to Ala-141) which form the cytoplasmic face of the *R. eutropha* phasin protein [28] were also found in the PhbP protein from *R. sphaeroides* FJ1. Therefore, ORF150 was considered to encode the phasin protein of *R. sphaeroides* FJ1.

The fourth ORF (ORF185) located downstream from ORF150 showed sequence homology with the *phb*R gene of *P. denitrificans and R. capsulatus*, but was missing the 3'-portion of the gene. To clone the entire *phb*R gene, pHEV65 was digested with *Sac*I and *Eco*RV, and the resulting 900-bp fragment containing the entire ORF150 and a portion of ORF185 was labeled with digoxigenin and used as a probe to identify the putative *phb*R region of *R. sphaeroides* FJ1. Genomic DNA from *R. sphaeroides* FJ1 was digested separately with *Bam*HI, *Eco*RI, and *Hin*dIII, and the resulting fragments were subjected to Southern blot analysis with the probe. An *Eco*RI fragment of approximately 3.0 kb was hybridized with the probe. Therefore, *Eco*RI fragments of *R. sphaeroides* FJ1 genomic DNA of 2 to 4 kb were isolated and ligated with $pBCSK^+$.

The recombinant plasmid designated pERI27 contained a 2.7-kb fragment and was selected by colony hybridization with the same probe. The nucleotide sequence of the entire 2.7-kb fragment was determined, and 3 ORFs were found. Two of the ORFs (ORF602 and ORF150) showed a high degree of sequence homology with the *phbC* and *phbP* genes, respectively, described above. The third ORF had nucleotide sequence homology with the *phbR* gene of *P. denitrificans* (GenBank accession no. AB017045; 75% identity) and *R. capsulatus* (GenBank accession no. AJ315564; 77% identity). The deduced amino acid sequence of this ORF also exhibited a high degree of homology with the PhbR proteins of *P. denitrificans* (69%) *and R. capsulatus* (79%). Multiple sequence alignments of these PhbR proteins showed that the helix-turn-helix motif located at positions 80 to 99 of the PhbR protein of *P. denitrificans* was also present in this ORF (positions 78 to 97). This helix-turn-helix DNA-binding motif is involved in the regulation of *phbP* expression in *P. denitrificans* [11]. We therefore consider this ORF (ORF185) *phbR* of *R. sphaeroides* FJ1. These results indicated that the *phb* locus of *R. sphaeroides* FJ1 is similar to that of *P. denitrificans* [13]. We designated this locus the *phb*ZCPR operon.

The *phbAB* genes of *R. sphaeroides* FJ1

To determine the structure and organization of PHB biosynthesis genes in *R. sphaeroides* FJ1, *phbA* (β-ketothiolase) and *phbB* (acetoacetyl-CoA reductase) were also cloned and sequenced. With 2 oligonucleotide primers (UPA1, 5'-ACTGCAGCCGGCGGCCAGGA AACC-3' and LPA1, CTGCAGGCCGATGGCGATG GCGCC-3') based on conserved sequences of *phbA* genes from various bacteria, a 700-bp DNA fragment was amplified from the *R. sphaeroides* FJ1 genome by PCR. After digestion with *Pst*I, this 700-bp PCR fragment was cloned into pBCSK$^+$, generating pHA700. The nucleotide sequence of this fragment was determined and found to share a 79% homology with that of the *P. denitrificans phbA* gene, suggesting that this 700-bp fragment contains a portion of *R. sphaeroides* FJ1 *phbA*. This 700-bp fragment was labeled with digoxigenin and used to hybridize with genomic DNA from *R. sphaeroides* FJ1 digested with *Pst*I. A fragment of 4.7 kb reacted with the probe. This fragment was isolated and ligated with pBCSK$^+$ to form pPst47. Sequence analysis revealed that this 4.7-kb *Pst*I fragment contained 3 ORFs. A database search revealed that the deduced amino acid sequence of ORF313 shares a 70% identity with that of the 3-methyladenine DNA glycosylase I of *Rhodopseudomonas palustris*. Nucleotide sequences of ORF392 and ORF241 were found to be homologous to those of *phbA* and *phb*B genes, respectively, of *P. denitrificans* (GenBank accession no. D49362; 80 and 89% identity), *R. eutropha* (GenBank accession no. J04987; 70% and 51% identity), *A. latus* (GenBank accession no. AF078795; 75% identity), *Mesorhizobium loti* (GenBank accession no. AJ315564; 75% identity), *R. etli* (GenBank accession no. AF342934; 74 and 76% identity), *R. meliloti* (GenBank accession no. U17226; 75 and 78% identity), and *Z. ramigera* (GenBank accession no. J02631; 77% identity). These results suggest that ORF392 and ORF241 encode the PhbA and PhbB proteins of *R. sphaeroides* FJ1, respectively (Fig. 1).

PHB accumulation and PhbC production from wastewater

To determine the growth requirements of FJ1, FJ1 cells were grown in various concentrations of wastewater (Fig. 2). A culture in which FJI cells were grown in distilled water served as the negative control. Significant growth (OD_{600} = 0.3) was observed 24 h after incubation in 100% wastewater (Fig. 2B), suggesting that the wastewater from the waste treatment plant of Fu-Jen University has the required nutrients for the growth of FJ1 cells. The growth of FJ1 in 100% wastewater stayed at this level for the remaining period of the study up to 48 h. When the wastewater was supplemented with 25% TSB medium, a profound growth (OD_{600} = 0.85) was seen at the 12-h time point. This growth reached the peak (OD_{600} = 1.3) at 36 h of incubation. A better growth was seen when the wastewater was supplemented with 50% TSB medium, in which the cell density reached approximately OD_{600} = 1.1 after 12 h of incubation and up to OD_{600} = 1.5 after 24 h of incubation. The growth stayed at this level (OD_{600} = 1.5) at 36 and 48 h of incubation under this condition. An increase in TSB concentration beyond 50% did not further improve the growth. FJ1 cells did not grow well in TSB diluted with distilled water to a final concentration of 50%; the growth of FJ1 cells in this 50% TSB medium reached a peak at 36 h with a cell density of only OD_{600} = 0.7 and decreased to OD_{600} = 0.35 at the 48 h time point. These results (Fig. 2A) suggest that the optimal TSB concentration is 50% in wastewater for the growth of FJ1.

The production of PHB by isolate FJ1 in wastewater supplemented with 50% TSB was then investigated.

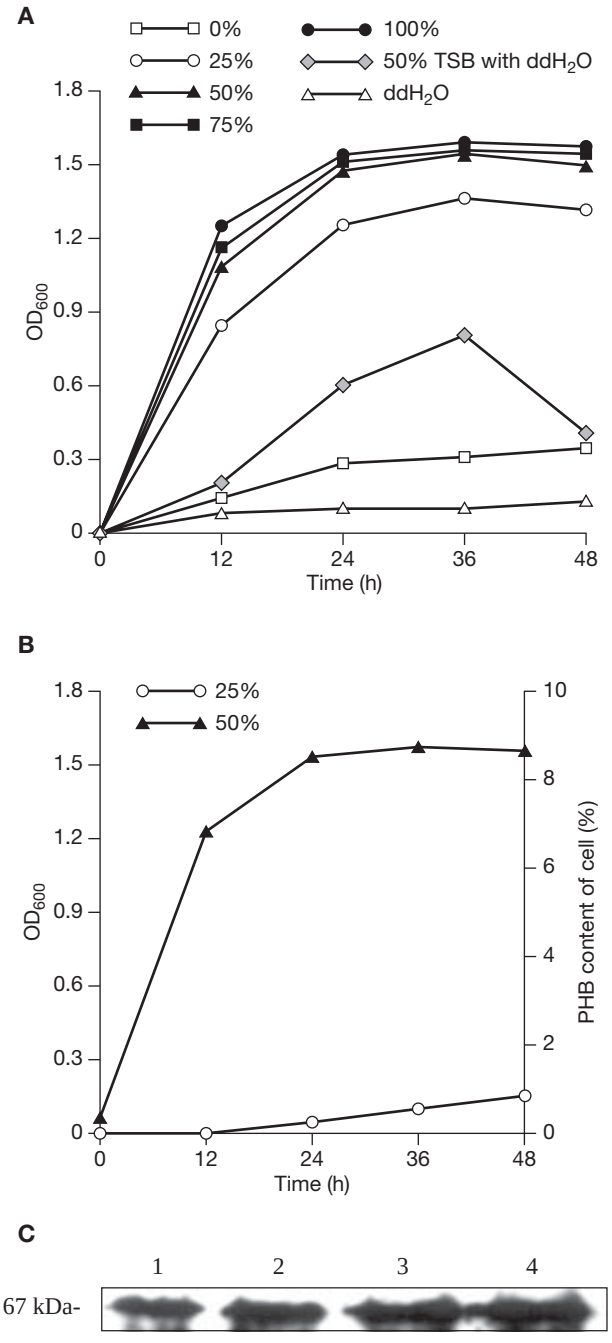

Fig. 2. Growth of *Rhodobacter sphaeroides* FJ1 and its production of poly-beta-hydroxybutyrate (PHB) and PHB synthase in wastewater. (A) Growth curve of *R. sphaeroides* FJ1 cultured in wastewater containing various concentrations of TSB (10 g of bacto-tryptone, 5 g of bacto-soytone, 5 g of NaCl and 2 g of glucose per liter; %, v/v). (B) PHB production in wastewater containing 50% TSB medium. (C) PHB synthase levels in cells grown in wastewater with 50% TSB medium for 12 h (lane 1), 24 h (lane 2), 36 h (lane 3) and 48 h (lane 4) determined by Western blotting. OD_{600} = optical density at 600 nm.

PHB was found to be produced during the stationary phase (12-48 h) of the growth, but the amounts of PHB produced were very low (0.01-0.09 g/L), approximately 8.2% of dry cell weight (Fig. 2B). To rule out the possibility that this low level of PHB production was due to the lack of production of the PHB synthase which is encoded by the *phbC* gene, the expression of the *phbC* gene at different time points (12, 24, 36, and 48 h) was examined by immunoblot analysis. The 67-kDa PHB synthase was found to be produced at a very high level at all time points examined (Fig. 2C).

To determine the best conditions for the production of PHB, FJ1 cells were grown in TSB medium for 48 h at 28°C and then switched to glutamate-yeast extration-mannitol salts (GYM) medium containing different concentrations of sodium acetate with C/N ratios of 20:1, 40:1, and 60:1 adjusted by varying sodium acetate concentrations from 6 to 18 g/L with a fixed $(NH_4)_2SO_4$ concentration of 0.5 g/L. The production of PHB was then accessed at 24, 48, 72, 96, and 120 h of culture (Fig. 3). PHB production in cells grown in TSB medium was found to be minimal, but was greatly increased when grown in GYM medium containing sodium acetate. At a 20:1 C/N ratio, a significant amount (0.3 g/L, 10% PHB w/w) of PHB was produced after 24 h of growth. PHB production reached the peak of 1.2 g/L (40.7% PHB w/w) at the 72-h time point and then gradually decreased to basal level at the 120 h time point. PHB production in cultures with a C/N ratio of 40:1 or 60:1 was similar to that in cultures with a 20:1 C/N ratio during the first 72 h. In media with a 40:1 C/N ratio, PHB production peaked at 96 h of growth (2.3 g/L; 60% PHB w/w) and then decreased slightly at 120 h of growth (2.1 g/L; 56% PHB w/w). In media with a 60:1 C/N ratio, the level of PHB production continued to increase even at the final 120-h time point (2.1 g/L; 56% PHB w/w). The expression of the PHB synthase under different growth conditions was also examined and was found to be relatively constant (Fig. 3B). This result suggests that the *phbC* gene is constitutively expressed.

Discussion

In this study, we isolated a PHA-producing bacterium from wastewater and identified it as *R. sphaeroides* FJ1. We also characterized genes involved in the production of PHA in this bacterium, including *phbZ*, *phbC*, *phbP*, *phbR*, *phbA* and *phbB*. The first 4 genes are clustered in the same locus, whereas the *phbA* and *phbB* cluster is located at a different location. This genetic organization

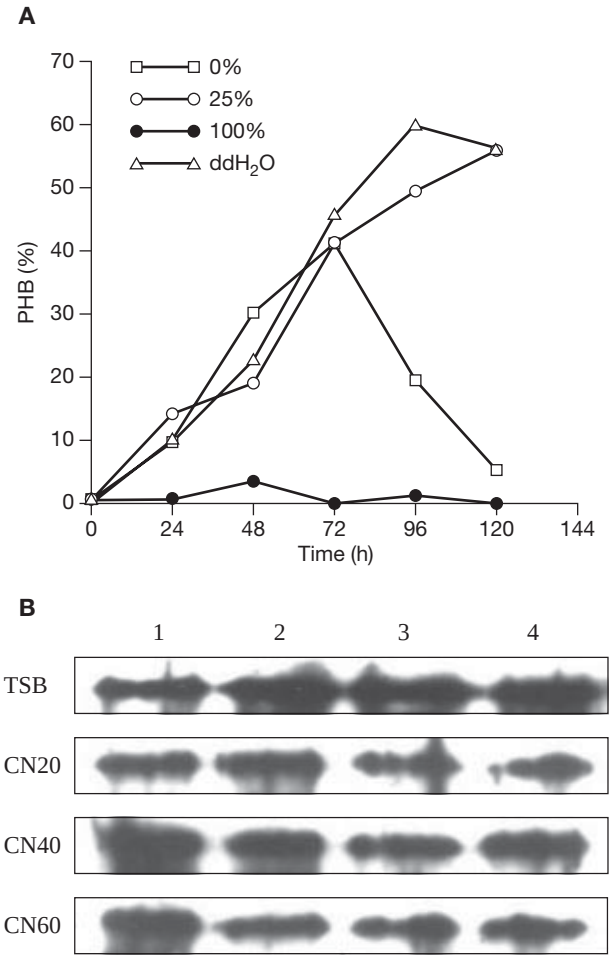

Fig. 3. Poly-beta-hydroxybutyrate (PHB) production and PhbC synthesis in *Rhodobacter sphaeroides* FJ1 grown in media with different C/N ratios. (A) Time course of PHB production of *R. sphaeroides* FJ1 in media with different C/N ratios: —□— , 20; —△—, 40; —○—, 60 (mol/mol). Very little PHB was produced when cells were grown in TSB medium (—●—). (B) PHB synthase levels in cells grown in media with C/N ratio of 20 (CN20), 40 (CN40), and 60 (CN60) at 24 h (lane 1), 48 h (lane 2), 72 h (lane 3) and 96 h (lane 4) determined by Western blotting. TSB = 10 g of bacto-tryptone, 5 g of bacto-soytone, 5 g of NaCl and 2 g of glucose per liter.

is quite different from that of *R. eutropha* in which the 3 genes (*phbC*, *phbA* and *phbB*) involved in PHA synthesis are clustered together to form the *phaCAB* operon. The *phaR* gene is located 450 bp downstream from *phaCAB*, and the *phaP* and *phaZ* genes are clustered in a separate location [29,30]. The *phbC*, *phbA* and *phbB* genes encode the PHA synthase, β-ketothiolase, and the NADP-dependent acetoacetyl-CoA reductase, respectively. The presence of these genes in *R. sphaeroides* FJ1 suggests that the production of PHB in *R. sphaeroides* FJ1 begins with the condensation of 2 acetyl-CoA by β-ketothiolase to yield acetoacetyl-CoA which is then reduced to β-hydroxybutyryl-CoA by acetoacetyl-CoA reductase. β-hydroxybutyryl-CoA is then polymerized by the PHB polymerase.

In addition to *phbABC* genes, *R. sphaeroides* FJ1 also has the *phbZ* gene (Fig. 1) that encodes the intracellular PHB depolymerase (i-PHB depolymerase). In *R. eutropha* [31] and *P. denitrificans* [32], the i-PHB depolymerase has been shown to convert PHB to acetyl-CoA to generate energy for growth and survival. Therefore, there must be a balance between PHB synthesis and degradation, probably by regulating the expression of *phbCBA* and *phbZ* genes in *R. sphaeroides* FJ1. A regulatory gene *phbR* was found to be present in *R. sphaeroides* FJ1. Analysis of the deduced amino acid sequence of the PhbR protein revealed that this protein contains many residues that can form alpha-helical structures. Since most DNA-binding proteins have alpha-helical structures, this observation suggests that PhbR is a DNA-binding protein. The PhbR proteins of *R. eutropha* [33] and *P. denitrificans* [13] have been shown to have DNA-binding activity and bind to the intergenic region of *phbC-phbP*, suppressing the expression of *phaP* [13,33]. Whether this protein also regulates other genes in the *phb* operon remains to be investigated.

The *phbP* gene of *R. sphaeroides* FJ1 has a 77% identity with the phasin gene of *R. capsulatus*. Therefore, we consider *phbP* the phasin gene. Phasin is a granule-associated protein and is the main component on the surface of PHB granules which accumulate in the cytoplasm of PHA-producing bacteria. Many types of phasin proteins have been found, including the GA13 protein of *Acinetobacter* spp. [34], the GA14 protein of *Chromatium vinosum* [35] and the GA24 protein of *R. eutropha* [36]. Phasin is not essential for PHB accumulation but may determine the size and the number of PHB granules in the cell. Phasin proteins have been intensely studied to improve the production of PHB. In this study, the phasin protein of *R. sphaeroides* FJ1 was found to have a high degree of identity to those of *P. denitrificans* [37] and *R. capsulatus* (GenBank accession no. AJ315564) but has a very low degree of homology with those of *R. eutropha* [28,36], *Aeromonas caviae* (GenBank accession no. D88825), *Acinetobacter* RA3849 (GenBank accession no. L37761), and *Chromatium vinosum* ([35] GenBank accession no. A27012) [Table 1]. This observation suggests that phasin proteins are quite diversified although they appear to have similar functions.

In this study, we found that *R. sphaeroides* FJ1 produced very little PHB when grown in rich media such as TSB (Fig. 3). Therefore, we developed a 2-stage cultivation method. The cells were first grown in TSB medium to a high cell mass and then switched to synthetic GYM medium with sodium acetate as the main carbon source to trigger PHB production. Interestingly, wastewater from the human waste treatment plant of Fu-Jen University was found to contain sufficient carbon and nitrogen elements to support the growth of *R. sphaeroides* FJ1. *R. sphaeroides* FJ1 was found to grow equally well in 100% TSB medium and in TSB containing 50% wastewater (Fig. 3). This would reduce the cost of PHB production. In addition, the levels of COD (chemical oxygen demand) and BOD (biochemical oxygen demand) were found to be reduced by 90% and 75%, respectively, by the growth of *R. sphaeroides* FJ1. This observation suggests that *R. sphaeroides* FJ1 can be used to develop a microbial process for environmental detoxification in addition to PHB production.

Acknowledgments

We thank Chao-Hung Lee for valuable discussions and critical editing of the manuscript and Shiu-Huey Chou for preparing antiserum to *R. sphaeroides* PHB synthase. This study was supported by grants (NSC90-2621-Z-030-001 and NSC91-2621-Z-030-001) from the National Science Council, Taiwan, Republic of China.

References

1. Lenz RW, Marchessault RH. Bacterial polyesters: biosynthesis, biodegradable plastics and biotechnology. Biomacromolecules 2005;6:1-8.
2. Reddy CS, Ghai R, Rashmi, Kalia VC. Polyhydroxyalkanoates: an overview. Bioresour Technol 2003;87:137-46.
3. Madison LL, Huisman GW. Metabolic engineering of poly (3-hydroxyalkanoates): from DNA to plastic. Microbiol Mol Biol Rev 1999;63:21-53.
4. Chen GQ, Wu Q. The application of polyhydroxyalkanoates as tissue engineering materials. Biomaterials 2005;26: 6565-78.
5. Young RC, Wiberg M, Terenghi G. Poly-3-hydroxybutyrate (PHB): a resorbable conduit for long-gap repair in peripheral nerves. Br J Plast Surg 2002;55:235-40.
6. Steinbuchel A, Valentin HE. Diversity of bacterial polyhydroxyalkanoic acids. FEMS Microbiol Lett 1995;128: 219-28.
7. Kadouri D, Burdman S, Jurkevitch E, Okon Y. Identification and isolation of genes involved in poly (beta-hydroxybutyrate) biosynthesis in *Azospirillum brasilense* and characterization of a *phbC* mutant. Appl Environ Microbiol 2002;68:2943-9.
8. Steinbuchel A, Hustede E, Liebergesell M, Pieper U, Timm A, Valentin H. Molecular basis for biosynthesis and accumulation of polyhydroxyalkanoic acids in bacteria. FEMS Microbiol Rev 1992;103:217-30.
9. Georgiou G, Jeong KJ. Proteins from PHB granules. Protein Sci 2005;14:1385-6.
10. Pötter M, Müller H, Reinecke F, Wieczorek R, Fricke F, Bowien B, et al. The complex structure of polyhydroxybutyrate (PHB) granules: four orthologous and paralogous phasins occur in *Ralstonia eutropha.* Microbiology 2004;150:2301-11.
11. Seo MC, Shin HD, Lee YH. Functional role of granule-associated genes, *phaP* and *phaR*, in poly-beta-hydroxybutyrate biosynthesis in recombinant *E. coli* harboring *phbCAB* operon. Biotechnol Lett 2003;25:1243-9.
12. York GM, Stubbe J, Sinskey AJ. New insight into the role of the PhaP phasin of *Ralstonia eutropha* in promoting synthesis of polyhydroxybutyrate. J Bacteriol 2001;183:2394-7.
13. Maehara A, Doi Y, Nishiyama T, Takagi Y, Ueda S, Nakano H, et al. PhaR, a protein of unknown function conserved among short-chain-length polyhydroxyalkanoic acids producing bacteria, is a DNA-binding protein and represses *Paracoccus denitrificans phaP* expression in vitro. FEMS Microbiol Lett 2001;200:9-15.
14. Kessler B, Witholt B. Factors involved in the regulatory network of polyhydroxyalkanoate metabolism. J Biotechnol 2001;86: 97-104.
15. Tokiwa Y, Calabia BP. Degradation of microbial polyesters. Biotechnol Lett 2004;26:1181-9.
16. Jendrossek D, Handrick R. Microbial degradation of polyhydroxyalkanoates. Annu Rev Microbiol 2002;56: 403-32.
17. Mohad Ali AH, Shirai Y, Kusubayashi N, Mohad Ismail AK, Nakanishi K, Hashimoto K. Effect of organic acids during anaerobic treatment of palm oil mill effluent on the production of polyhydroxyalkanoate by *Rhodobacter sphaeroides*. J Ferment Bioeng 1996;82:151-6.
18. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 1977; 74:5463-7.
19. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 1994; 22:4673-80.
20. Nicholas KB, Nicholas HB. GeneDoc: a tool for editing and annotating multiple sequence aligments. 1997. Available from: URL: http://www.psc.edu/biomed/genedoc
21. Mount DW. A mutant of *Escherichia coli* showing constitutive

expression of the lysogenic induction and error-prone DNA repair pathways. Proc Natl Acad Sci USA 1977;74:300-4.
22. Yang MK, Wu PI, Yang YC. Identification of a *lexA* gene in, and construction of a *lexA* mutant of, *Xanthomonas campestris* pv citri. Curr Microbiol 2000;40:233-8.
23. Brandl H, Gross RA, Lenz RW, Fuller RC. *Pseudomonas oleovorans* as a source of poly (β-hydroxyalkanoates) for potential applications as biodegradable polyesters. Appl Environ Microbiol 1988;54:1977-82.
24. Yiğit DÖ, Gündüz U, Türker L, Yücel M, Eroğlu . Identification of by-products in hydrogen producing bacteria; *Rhodobacter sphaeroides* O.U. 001 grown in the waste water of a sugar refinery. J Bacteriol 1999;70:125-31.
25. Kim JH, Lee JK. Cloning, nucleotide sequence and expression of gene coding for polyhydroxybutyric acid (PHB) synthase of *Rhodobacter sphaeroides* 2.4.1. J Microbiol Biotechnol 1997;7:229-36.
26. Gerngross TU, Snell KD, Peoples OP, Sinskey AJ, Csuhai E, Masamune S, et al. Overexpression and purification of the soluble polyhydroxyalkanoate synthase from *Alcaligenes eutrophus*: evidence for a required posttranslational modification for catalytic activity. Biochemistry 1994;33: 9311-20.
27. Kobayashi T, Saito T. Catalytic triad of intracellular poly (3-hydroxybutyrate) depolymerase (PhaZ1) in *Ralstonia eutropha* H16. J Biosci Bioeng 2003;96:487-92.
28. Hanley SZ, Pappin DJ, Rahman D, White AJ, Elborough KM, Slabas AR. Reevaluation of the primary structure of *Ralstonia eutropha* phasin and implications for polyhydroxyalkanoic acid granule binding. FEBS Letters 1999;447:99-105.
29. Slater SC, Voige WH, Dennis DE. Cloning and expression in *Escherichia coli* of the *Alcaligenes eutrophus* H16 poly-β-hydroxybutyrate biosynthetic pathway. J Bacteriol 1988;170:4431-6.
30. Peoples OP, Sinskey AJ. Poly-β-hydroxybutyrate biosynthesis in *Alcaligenes eutropha* H16. Characterization of the gene encoding β-ketothiolase and acetoacetyl-CoA reductase. J Biol Chem 1989;264:15293-7.
31. Handrick R, Reinhardt S, Jendrossek D. Mobilization of poly (3-hydroxybutyrate) in *Ralstonia eutropha*. J Bacteriol 2000; 182:5916-8.
32. Gao D, Maehara A, Yamane T, Ueda S. Identification of the intracellular polyhydroxyalkanoate depolymerase gene of *Paracoccus denitrificans* and some properties of the gene product. FEMS Microbiol Lett 2001;196:159-64.
33. York GM, Stubbe J, Sinskey AJ. The *Ralstonia eutropha* PhaP protein couples synthesis of the PhaP phasin to the presence of polyhydroxybutyrate in cells and promotes polyhydroxybutyrate production. J Bacteriol 2002;184:59-66.
34. Schembri MA, Woods AA, Bayly RC, Davies JK. Identification of a 13-kDa protein associated with the polyhydroxyalkanoic acid granules from *Acinetobacter* spp. FEMS Microbiol Lett 1995;133:277-83.
35. Liebergesell M, Steinbuchel A. New knowledge about the PHA-locus and P(3HB) granule-associated proteins in *Chromatium vunosum*. Biotechnol Lett 1996;18:719-24.
36. Wieczorek R, Pries A, Steinbuchel A, Mayer F. Analysis of a 24-kilodalton protein associated with the polyhydroxyalkanoic acid granules in *Alcaligenes eutrophus*. J. Bacteriol 1995;177: 2425-35.
37. Maehara A, Ueda S, Nakano H, Yamane T. Analyses of a polyhydroxyalkanoic acid granule-associated 16-kilodalton protein and its putative regulator in the *pha* locus of *Paracoccus denitrificans*. J Bacteriol 1999;181:2914-21.

