Polyhydroxybutyrate biosynthesis in *Caulobacter crescentus*: molecular characterization of the polyhydroxybutyrate synthase

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Caulobacter crescentus was investigated with respect to polyhydroxybutyrate (PHB) biosynthesis. Polyhydroxyalkanoate (PHA) accumulation contributing to approximately 18% of the cell dry weight was obtained in the presence of glucose. Gas chromatography–mass spectrometry and gel permeation chromatography of the purified PHA showed that this polyester was solely composed of 3-hydroxybutyrate and had a weight average molar mass of $5.5 \times 10^5$ g mol$^{-1}$ and a polydispersity of 1.6. An ORF encoding a conserved, hypothetical protein which shared approximately 47% identity with the PHB synthase from *Azorhizobium caulinodans* was identified within the complete *C. crescentus* genomic sequence. This putative *C. crescentus* PHB synthase gene, *phaC*, consisted of a 2019 nt stretch of DNA (encoding 673 aa residues), which encoded a PHB synthase with a molecular mass of approximately 73 kDa. This is currently the largest PHA synthase identified. The *phaC* coding region was subcloned into vector pBBR1-J02 under lac promoter control. The resulting plasmid, pQQ4, mediated PHB accumulation in the mutant *Ralstonia eutropha* PHB-4 and recombinant *Escherichia coli* JM109(pBHR69), which produced the β-ketothiolase and acetoacetyl-CoA reductase from *R. eutropha*, contributing to approximately 62% and 6% of cell dry weight, respectively. Functional expression of the coding region of *phaC* was confirmed by immunoblotting and *in vitro* PHB synthase activity.

Keywords: polyhydroxyalkanoate, PHA synthase, PHA depolymerase

INTRODUCTION

*Caulobacter crescentus* is a Gram-negative, polarly-flagellated bacterium, which physiologically resembles the aerobic, chemoheterotrophic pseudomonads. The ability of *C. crescentus* to survive in famine conditions suggests that it may produce polyhydroxyalkanoates (PHAs), or other polymers, as carbon and energy reserves. Polyhydroxybutyrate (PHB) has already been shown to be a product of *Caulobacter* spp. metabolism during nitrogen and phosphate starvation conditions (Poindexter, 1981).

PHAs are currently under intensive investigation because of their inherent property as biodegradable thermoplastics. PHA synthases, which use CoA thioesters of (R)-3-hydroxyalkanoates as substrates and catalyse the polymerization of these monomers to PHA with concomitant release of CoA, represent the key enzymes of PHA biosynthesis. More than 40 PHA synthase genes have been assigned and characterized (Rehm & Steinbüchel, 1999, 2001a), and their protein products can be broadly arranged into three different classes based on their subunit composition and substrate specificities. Class I synthases are active towards short-chain-length (R)-3-hydroxyacyl-CoA, consisting of three to five carbon atoms, and are represented by the PHA synthase of *Ralstonia eutropha*. Class II is represented by the PHA synthase of *Pseudomonas aeruginosa* which is active towards medium-chain-length (R)-3-hydroxyacyl-CoA, containing 6 to 14 C atoms. PHA

**Abbreviations:** CDW, cell dry weight; GC–MS, gas chromatography–mass spectrometry; GPC, gel permeation chromatography; PHA, polyhydroxyalkanoate; PHB, polyhydroxybutyrate.
synthases of class I and class II are composed of a single subunit, whereas class III PHA synthases are composed of two non-identical subunits. Class III is represented by the Allochromatium vinosum PHA synthase, consisting of subunits PhaC and PhaE, and exhibiting activity towards short-chain-length (R)-3-hydroxyacyl-CoA. In addition, a few bacteria, such as Aeromonas punctata (Fukui et al., 1998) and Rhodococcus ruber (Haywood et al., 1991), have been reported to have PHA synthases that are composed of one subunit which exhibits specificity for both short- and medium-chain-length (R)-3-hydroxyacyl-CoA. Although numerous PHA synthase genes have been cloned and assigned, only a few PHA synthase genes have been purified and enzymically characterized, e.g. PHA synthases from R. eutropha, A. vinosum and P. aeruginosa (Gerngross et al., 1994; Liebergessell et al., 1994; Qi et al., 2000; Rehm et al., 2001a).

The PHB biosynthesis pathway of R. eutropha has been studied in detail (Peoples & Sinskey, 1989a, b; Schubert et al., 1988). In this bacterium, the biosynthetic process is initiated by the condensation of two acetyl-CoA molecules to produce acetoacetyl-CoA which is catalysed by the enzyme β-ketothiolase (EC 2.3.1.9; gene phbA). Acetoacetyl-CoA is then reduced to (R)-3-hydroxybutyryl-CoA by the NADPH-dependent acetoacetyl-CoA reductase (EC 1.1.1.36; gene phbB). These enzymes have also been found and studied in several other PHB-accumulating bacteria.

The synthesis of medium-chain-length PHAs relies mainly on the β-oxidation pathway (Langenbach et al., 1997; Qi et al., 1997, 1998) when fatty acids are used as carbon source, or on fatty acid de novo synthesis when other non-related carbon sources, such as glaconate, were employed (Rehm et al., 1998; Fiedler et al., 2000; Hoffmann et al., 2000a, 2000b). Meanwhile, various medium-chain-length PHA biosynthetic pathways have been established in recombinant bacteria, which recruit intermediates of fatty acid metabolism (Rehm et al., 2001b; Rehm & Steinbüchel, 2001b).

In this study we describe the capability of C. crescentus DSM 4727T to produce PHB in the presence of excess carbon source. The PHB synthase gene and corresponding enzyme of C. crescentus DSM 4727T were characterized by heterologous functional expression in a PHB-negative mutant of R. eutropha and in Escherichia coli, and by analysis of the in vitro PHB synthase activity. Furthermore, evidence for the re-utilization of PHB by an intracellular PHB depolymerase was also obtained.

**METHODS**

**Bacterial strains, plasmids, and culture conditions.** C. crescentus DSM 4727T was cultivated at 30 °C in Caulobacter medium (CM), which contains 2.0 g Bacto-peptone, 1.0 g yeast extract and 0.2 g MgSO₄*₇H₂O per litre. Glucose (1% w/v) or other carbon sources (0.2% w/v) were added as appropriate. E. coli strains were cultivated at 37 °C in Luria–Bertani broth (LB; Sambrook et al., 1989) or M9 (minimal medium; Sambrook et al., 1989) containing the carbon sources, supplements and antibiotics indicated. R. eutropha strains were cultivated in nutrient broth (Sambrook et al., 1989) medium or mineral salt medium (MM; Schlegel et al., 1970) supplemented with 1% (w/v) gluconate. Kanamycin (500 μg ml⁻¹) or ampicillin (100 μg ml⁻¹) were added if required. The strains and plasmids used in this study are listed in Table 1.

**Isolation and manipulation of DNA.** Chromosomal DNA was prepared from C. crescentus DSM 4727T according to the method described by Mak & Ho (1993). PCR was performed using Vent DNA polymerase (New England Biolabs) and oligonucleotide primers for the N-terminus (5'-GGGATCCGGAGAGAACCCCATGCGCCACG-3') and C-terminus (5'-CGGAATTCCTAGGTTGACTTTGACGAG-5') of the putative phaC coding region. The N-terminus primer contained a BamHI site at its 5' end (underlined); the C-terminus primer contained an EcoRI site at its 5' end (underlined). The resulting PCR product was subcloned into the respective sites of vector pBR321-JO2 (Dr H. Priefert, Institut für Mikrobiologie der Westfälischen Wilhelms-Universität Münster, Germany) to generate pQQ4. New constructs were confirmed by DNA sequencing (MWG Biotech). The DNA sequences of the coding regions of phaC and phaZ of C. crescentus, and their protein sequences, have been deposited in GenBank under the accession numbers AY007313 and AF311864, respectively. Conjugation of R. eutropha PHB 4 with E. coli S17-1 harbouring broad-host-range plasmids was performed as described by Friedrich et al. (1981). All other genetic techniques employed were performed as described by Sambrook et al. (1989).

**Analysis of the PHA in cells.** PHAs were analysed by GC. Cells were harvested by centrifugation at 10000 g for 15 min. The cells were then washed twice in saline solution (0.9%, w/v, NaCl) and lyophilized overnight. About 5–10 mg dry cells were subjected to methanolysis in the presence of 15% (v/v) sulfuric acid. The resulting methyl esters of the respective 3-hydroxyalkanoates were assayed by a GC system (PerkinElmer) equipped with a 0.5 μm Polyspher PEG25M column and a mass standard (Sigma) with a narrow range of polydispersity was used for calibration of the system.

**Gas chromatography–mass spectrometry (GC–MS).** Purified polymer, prepared as described above, was dissolved in chloroform (5 mg PHA ml⁻¹), and 3 μl was injected into a GC–MS instrument (model 6890; Hewlett Packard). The column and temperature profile used for GC analysis were as described by Schubert et al. (1991). The eluted polymer was detected with a differential refractometer (model 410; Waters). A polystyrene molecular mass standard (Sigma) with a narrow range of polydispersity was employed for calibration of the system.

**Gel permeation chromatography (GPC).** Molecular mass analysis was conducted with purified PHA, which was dissolved in chloroform (5–10 mg PHA ml⁻¹) and introduced into a GPC system (Waters). The GPC system was equipped with a Styrage Guard and a Styrage HR 3–6 separation column. The eluted polymer was detected with a differential refractometer (model 410; Waters). A polystyrene molecular mass standard (Sigma) with a narrow range of polydispersity was employed for calibration of the system.

**Protein content.** The protein content was determined by the Bradford method, as described by Laemmli (1970).

**Electrophoresis of proteins.** SDS-PAGE (12.5%, w/v) was performed in a vertical slab gel electrophoresis apparatus, as described by Sambrook et al. (1989).
**Table 1. Bacterial strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strains or strain</th>
<th>Relevant characteristic</th>
<th>Source or reference</th>
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<tr>
<td><strong>Strains</strong></td>
<td></td>
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<tr>
<td><em>C. crescentus</em></td>
<td>Wild-type</td>
<td>DSMZ 4727T</td>
</tr>
<tr>
<td><em>R. eutropha</em> H16</td>
<td>Wild-type</td>
<td>DSMZ 428</td>
</tr>
<tr>
<td><em>R. eutropha</em> PHB-4</td>
<td>PHB-4 mutant of <em>R. eutropha</em> H16</td>
<td>Schlegel et al. (1970)</td>
</tr>
<tr>
<td><em>E. coli</em> JM109</td>
<td>recA1 endA1 gyrA96 thi-1 hisD17(r597 m41) supE44 relA1 Δ(lac-proAB) (F’ proAB lacI prophage) ZAM15</td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> S17-1</td>
<td>proA thi-1 recA, harbouring the tra genes of plasmid RP4 in the chromosome</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBBR1-JO2</td>
<td>pBBR1MCS-2, containing the polylinker from pBluescript SK-</td>
<td>Kovach et al. (1995); Dr H. Priefert</td>
</tr>
<tr>
<td>pPS1</td>
<td>pBBR1MCS-2, containing the coding region of <em>phaC</em> from <em>E. coli</em></td>
<td>This study</td>
</tr>
<tr>
<td>pQQ4</td>
<td>pBBR1-JO2, containing coding region of <em>phaC</em> from <em>C. crescentus</em></td>
<td>This study</td>
</tr>
<tr>
<td>pBHR69</td>
<td>pBluescript SK-, containing <em>phaB</em> and <em>phaA</em> from <em>R. eutropha</em></td>
<td>Spiekermann et al. (1999)</td>
</tr>
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</table>

**Western immunoblot analysis.** Western blots were performed with a semidry Fastblot apparatus (Bio-Rad) as follows. Antiserum against the PHB synthase of *R. eutropha* (anti-PhaC) was raised. This antiserum was applied to a nitrocellulose membrane, and an alkaline phosphatase–antibody conjugate (Sigma) was also applied to the membrane. Bound antibodies were detected using nitro blue tetrazolium chloride and the toluidine salt of 5-bromo-4-chloro-3-indolyl phosphate.

**PHB synthase assay.** The *in vitro* activity of PHB synthase in crude extracts was assayed by the DTNB ([5,5′-dithiobis-(2-nitrobenzoic acid)] method, as described by Valentin & Steinbüchel (1993).

**RESULTS AND DISCUSSION**

**Analysis of PHA production by *C. crescentus***

To investigate the metabolic storage compounds synthesized by *C. crescentus* when it is cultivated in the presence of excess carbon source, we used CM + 1% (w/v) glucose or CM + 0.2% (w/v) decanoate. After cultivation at 30 °C for 24 h we observed granules in the cells under phase-contrast microscopy. The granules were mainly observed in stalked cells; the cell shape was more rod-like than vibrioid at this stage. GC–MS analysis of whole cells showed that when either glucose or decanoate was used as the carbon source, PHA was produced. CM without an additional carbon source did not mediate the formation of PHA. PHA was isolated from *C. crescentus* cells which had been grown in the presence of glucose. The isolated polymer was found to be composed solely of 3-hydroxybutyrate. GPC analysis of the purified polymer revealed a polydispersity of 1.6 with a weight average molar mass of 5.5 × 10⁴ g mol⁻¹ and a number average molar mass of 3.4 × 10⁵ g mol⁻¹. Time-course analysis of PHB production by *C. crescentus* DSM 4727T using glucose as a carbon source indicated growth-associated PHB production (Fig. 1). PHB accumulated simultaneously with cell growth, and reached its maximum level after approximately 60 h (Fig. 1), contributing approximately 18.3% of cell dry weight (CDW) (biomass 2.5 g CDW l⁻¹). A slight decrease in the level of CDW coincided with a small decrease in PHB content, which indicated the presence of an intracellular PHB depolymerase. Further evidence for an intracellular depolymerase was obtained by identification of a conserved hypothetical protein (Locus no. AAK22237; GI no. 13421381) within the genome sequence for *C. crescentus* [GenBank accession no. AE005698 (part 24 of 359); Nierman et al., 2001] whose deduced amino acid sequence showed approximately 45% similarity to the intracellular depolymerase.

![Fig. 1. Time-course analysis of PHB production (□) and growth curve for *C. crescentus* (●). PHB accumulation expressed as a percentage of CDW. Growth rate is expressed in terms of g CDW litre⁻¹ growth medium.](image)
poly- et al. Nierman. 673 aa (73 3356 [Locus no. AAK23361; GI no. 13422735; accession no. genes. In the database we identified a 587 aa protein genome for DNA regions homologous to PHA synthase completely sequenced (www.tigr.com), we searched the Since the C. crescentus; 4. R. eutropha H16 (positive control). Arrows indicate the position of the R. eutropha PHA synthase (PhaCRe) and of the C. crescentus PHA synthase (PhaCce).

Fig. 2. Immunoblot analysis of crude extracts from C. crescentus, E. coli JM109(pQQ4) and R. eutropha PHB 4(pQQ4). Immunoblotting was performed using anti-PhaC antibodies raised against R. eutropha H16. Lanes: M, molecular mass standard; 1, R. eutropha PHB 4(pQQ4); 2, E. coli JM109(pQQ4); 3, C. crescentus; 4, R. eutropha H16 (positive control). Arrows indicate the position of the R. eutropha PHA synthase (PhaCRe) and of the C. crescentus PHA synthase (PhaCce).

Identification of the PHB synthase gene and its expression

Since the C. crescentus genomic DNA has been completely sequenced (www.tigr.com), we searched the genome for DNA regions homologous to PHA synthase genes. In the database we identified a 587 aa protein [Locus no. AAK23361; GI no. 13422735; accession no. AE005313 (part 139 of 339); Nierman et al., 2001] which showed 100% identity to the C-terminal end of our 673 aa (73.6 kDa) phaC protein sequence. Although Nierman et al. (2001) tentatively named their protein poly-β-hydroxybutyrate polymerase they did not functionally characterize the region. Our data also show that they chose an inappropriate start codon for the coding region of their putative protein. A multiple alignment including the protein sequence of phaC revealed that this protein was highly homologous to other PHA synthases, with 25–47% sequence similarity to previously recognized sequences. The strongest homology was obtained with PHA synthases from Azorhizobium caulinodans (47% identity), Rhodospirillum rubrum (46% identity) and R. eutropha (46% identity), suggesting that the putative PHB synthase from C. crescentus belongs to class I PHA synthases.

A putative lipase box GX(C/S)XG was found within the PHB synthase protein sequence, in which the serine essential for the active site of the lipase was replaced by a cysteine. It also contained the conserved amino acids C319, D480 and H508 (positions relative to R. eutropha PHA synthase). Analysis of the adjacent DNA sequence regions (3 kb upstream and downstream of the respective coding region) did not indicate co-localization of PHB biosynthesis genes, such as phbA and phbB. We amplified and cloned the putative PHB-synthase-encoding DNA region as described in Methods. Plasmid pQQ4 was introduced into E. coli JM109 and R. eutropha PHB 4, and the cells were cultivated under the respective PHB accumulation conditions. SDS-PAGE and immunoblot analysis with anti-PhaC antiserum (raised against the R. eutropha protein) revealed that the putative PHB synthase gene was expressed in both E. coli JM109 and R. eutropha PHB 4 (Fig. 2). An immunologically cross-reacting protein of approximately 73 kDa was obtained. A cross-reacting protein with an apparent molecular mass of 73 kDa was also detected, by anti-PhaC antiserum in crude extracts of C. crescentus (Fig. 2).

PHB accumulation in recombinant R. eutropha PHB 4 and E. coli

To confirm that the putative coding region of phaC from C. crescentus encoded an active PHB synthase, phaC was expressed from the lac promoter in R. eutropha PHB 4 and in E. coli JM109. The E. coli strains were grown in 250 ml Erlenmeyer flasks containing 50 ml medium with 1 mM IPTG and 50 μg kanamycin ml⁻¹ at 37 °C for 48 h. R. eutropha was grown in 250 ml Erlenmeyer flasks containing 50 ml medium and 500 μg kanamycin ml⁻¹ at 30 °C for 48 h. PHB content was measured as a percentage of CDW. Wild-type C. crescentus (grown in CM+1% gluconate) demonstrated a PHB content of 18%, while R. eutropha PHB 4(pQQ4) (grown in MM+1% gluconate) appeared to be complemented by the presence of the phaC gene, resulting in PHB accumulation contributing to a PHB content of 62±4%. The negative control R. eutropha PHB 4(pBBR1-JO2) (grown in MM+1% gluconate) did not mediate PHB accumulation (PHB content <1%). E. coli JM109(pQQ4/pBHR69), containing the genes encoding the β-ketothiolase and acetoacetyl-CoA reductase from R. eutropha (pBHR69) as well as the phaC gene (pQQ4) from C. crescentus, resulted in PHB contents of 5–2% and 5–5% when cultivated in LB+1% glucose and M9+1% glucose, respectively. No PHB was detected for E. coli JM109(pBHR69) and E. coli JM109(pQQ4) grown in LB+1% glucose (w/v). E. coli JM109(pPS1/pBHR69), containing the phaC, phaB and phaA genes from R. eutropha and grown in LB+1% glucose, had a PHB content of 5–8%.

Enzymic activity of the putative PHB synthase from C. crescentus

Crude extracts of C. crescentus, recombinant R. eutropha PHB 4(pQQ4) and recombinant E. coli JM109(pQQ4) were investigated with respect to in vitro
Table 2. In vitro PHB synthase activity of C. crescentus and recombinant strains of R. eutropha PHB 4 and E. coli JM109

<table>
<thead>
<tr>
<th>Strain (plasmid)</th>
<th>Crude extract [mg (total protein) ml⁻¹]</th>
<th>PHB synthase activity [mU (mg total protein)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. crescentus DSM 4727</td>
<td>2.56</td>
<td>59.5</td>
</tr>
<tr>
<td>R. eutropha PHB 4(pBBR1-JO2)</td>
<td>2.44</td>
<td>ND</td>
</tr>
<tr>
<td>R. eutropha PHB 4(pQQ4)</td>
<td>2.19</td>
<td>30.4</td>
</tr>
<tr>
<td>E. coli JM109(pBBR1-JO2)</td>
<td>2.75</td>
<td>ND</td>
</tr>
<tr>
<td>E. coli JM109(pQQ4)</td>
<td>2.95</td>
<td>54.5</td>
</tr>
</tbody>
</table>

ND, Not detectable.

* One unit of activity is defined as 1 µmol CoA released min⁻¹ under the assay conditions. (R,S)-3-hydroxybutyryl-CoA was used as the substrate.

PHB synthase activity, employing 3-hydroxybutyryl-CoA as substrate (Table 2). In C. crescentus, a PHB synthase activity of 59.5 mU (mg total protein)⁻¹ was obtained. The PHB synthase activity in crude extracts of recombinant R. eutropha PHB 4(pQQ4) and recombinant E. coli JM109(pQQ4) was 30.4 mU (mg total protein)⁻¹ and 54.5 mU (mg total protein)⁻¹, respectively. As expected, no activity was detected for the control strains, carrying only vector pBBR1-JO2. Hence, the study indicated that the PHB synthase shows in vitro activity in C. crescentus and that the enzyme is functionally produced in R. eutropha PHB 4 and E. coli JM109.

The PHB synthase from C. crescentus represents the largest PHA synthase currently known, with a molecular mass of 73.6 kDa. The multiple alignment of all PHA synthases indicated that the N-terminus of the protein is subject to residue variation. The 85 amino acids from the N-terminus of C. crescentus PHB synthase have almost no homology to other PHA synthases. Conserved residues were identified which might play an important role in the catalytic mechanism, e.g. H390 (H508 of the R. eutropha PHB synthase), a residue which is supposed to be the general base catalyst that activates the nucleophile C406 (C319 of the R. eutropha PHB synthase) (Jia et al., 2000; Jia et al., 2001). The C406 in the lipase-box-like region is supposed to be directly involved in covalent catalysis. D512 (D480 of the R. eutropha PHB synthase) might function as a general base catalyst in activation of the 3-hydroxy group of 3-hydroxybutyryl-CoA for nucleophilic attack on the covalently linked thioester intermediate. The functional assignment of catalytic amino acid residues was based on mutational studies with the R. eutropha and A. vinosum PHA synthase (Hoppensack et al., 1999; Jia et al., 2000, 2001).

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REFERENCES


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