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

Qingsheng Qi and Bernd H. A. Rehm

Author for correspondence: Bernd H. A. Rehm. Tel: +49 251 833 9848. Fax: +49 251 833 8388.
e-mail: rehm@uni-muenster.de

Institut für Mikrobiologie
der Westfälischen Wilhelms-
Universität Münster,
Corrensstrasse 3, 48149
Münster, Germany

***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 $5.5 \times 10^5 \text{ g mol}^{-1}$ 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-JO2 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).

Abbreviations: CDW, cell dry weight; GC–MS, gas chromatography–mass spectrometry; GPC, gel permeation chromatography; PHA, polyhydroxyalkanoate; PHB, polyhydroxybutyrate.

The GenBank accession numbers for the *phaC* and *phaZ* sequences reported in this paper are AY007313 and AF311864, respectively.

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)-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

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 synthases have been purified and enzymically characterized, e.g. PHA synthases from *R. eutropha*, *A. vinosum* and *P. aeruginosa* (Gerngross *et al.*, 1994; Liebergesell *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 gluconate, 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 4727^T to produce PHB in the presence of excess carbon source. The PHB synthase gene and corresponding enzyme of *C. crescentus* DSM 4727^T 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 4727^T 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 4727^T 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'-GCGGATCCGGAGGAAACCCCATGGCCACG-3') and C-terminus (3'-CGGAATTCTCAGGGTTGTGACTTTCACAG-5') of the putative *phaC* coding region. The N-terminus primer contained a *Bam*HI site at its 5' end (underlined); the C-terminus primer contained an *Eco*RI site at its 3' end (underlined). The resulting PCR product was subcloned into the respective sites of vector pBBR1-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 (Perkin-Elmer) equipped with a 0.5 µm Permaphase PEG25 Mx capillary column as described by Brandl *et al.* (1988).

Isolation of the PHA from lyophilized cells. PHA was extracted from lyophilized cells using chloroform extraction in a Soxhlet apparatus and was subsequently precipitated with 10 vols ethanol. The precipitate was dissolved in chloroform and ethanol-precipitated again in order to obtain highly purified PHA.

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).

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 Styragel Guard and a Styragel HR3–6 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 obtained 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

Plasmid or strain	Relevant characteristic	Source or reference
Strains		
<i>C. crescentus</i>	Wild-type	DSMZ 4727 ^T
<i>R. eutropha</i> H16	Wild-type	DSMZ 428
<i>R. eutropha</i> PHB ⁻ 4	PHB ⁻ 4 mutant of <i>R. eutropha</i> H16	Schlegel <i>et al.</i> (1970)
<i>E. coli</i> JM109	<i>recA1 endA1 gyrA96 thi-1 hsdR17</i> (r _k ⁻ m _k ⁻) <i>supE44 relA1</i> Δ (<i>lac-proAB</i>) (F' <i>proAB lacI</i> ^q Δ AM15)	
<i>E. coli</i> S17-1	<i>proA thi-1 recA</i> , harbouring the <i>tra</i> genes of plasmid RP4 in the chromosome	Simon <i>et al.</i> (1983)
Plasmids		
pBBR1-JO2	pBBR1MCS-2, containing the polylinker from pBluescript SK ⁻	Kovach <i>et al.</i> (1995); Dr H. Priefert
pPS1	pBBR1MCS-2, containing the coding region of <i>phaC</i> from <i>R. eutropha</i>	This study
pQQ4	pBBR1-JO2, containing coding region of <i>phaC</i> from <i>C. crescentus</i>	This study
pBHR69	pBluescript SK ⁻ , containing <i>phaB</i> and <i>phaA</i> from <i>R. eutropha</i>	Spiekermann <i>et al.</i> (1999)

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'-dithiobios-(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 5.5×10^5 g mol⁻¹ and a number average molar mass of 3.4×10^5 g mol⁻¹.

Time-course analysis of PHB production by *C. crescentus* DSM 4727^T using glucose as a carbon source

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.

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. 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 (PhaC_{Re}) and of the *C. crescentus* PHA synthase (PhaC_{Cc}).

of *R. eutropha* (Handrick *et al.*, 2000). This putative *phaZ* gene encodes a protein consisting of 375 aa with a molecular mass of 41.8 kDa.

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. AE005813 (part 139 of 359); 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 μ g kanamycin ml⁻¹ at 37 °C for 48 h. *R. eutropha* was grown in 250 ml Erlenmeyer flasks containing 50 ml medium and 500 μ g 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

Strain (plasmid)	Crude extract [mg (total protein) ml ⁻¹]	PHB synthase activity [mU (mg total protein) ⁻¹]*
<i>C. crescentus</i> DSM 4727 ^T	2.56	59.5
<i>R. eutropha</i> PHB ⁻ 4(pBBR1-JO2)	2.44	ND
<i>R. eutropha</i> PHB ⁻ 4(pQQ4)	2.19	304
<i>E. coli</i> JM109(pBBR1-JO2)	2.75	ND
<i>E. coli</i> JM109(pQQ4)	2.95	54.5

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 304 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. H590 (H508 of the *R. eutropha* PHA 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* PHA 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

- Brandl, H., Gross, R. A., Lenz, R. W. & Fuller, R. C. (1988). *Pseudomonas oleovorans* as a source of poly(β-hydroxyalkanoates) for potential applications as biodegradable polyesters. *Appl Environ Microbiol* **54**, 1977–1982.
- Fiedler, S., Steinbüchel, A. & Rehm, B. H. A. (2000). PhaG-mediated synthesis of poly(3-hydroxyalkanoates) consisting of medium-chain-length constituents from nonrelated carbon sources in recombinant *Pseudomonas fragi*. *Appl Environ Microbiol* **66**, 2117–2124.
- Friedrich, B., Hogrefe, C. & Schlegel, H. G. (1981). Naturally-occurring genetic transfer of hydrogen-oxidizing ability between strains of *Alcaligenes eutrophus*. *J Bacteriol* **147**, 198–205.
- Fukui, T., Shiomi, N. & Doi, Y. (1998). Expression and characterization of (R)-specific enoyl coenzyme A hydratase involved in polyhydroxyalkanoate biosynthesis by *Aeromonas caviae*. *J Bacteriol* **180**, 667–673.
- Gerngross, T. U., Snell, K. D., Peoples, O. P., Sinskey, A. J., Csuhai, E., Masamune, M. & Stubbe, J. (1994). Overexpression and purification of the soluble polyhydroxyalkanoate synthase from *Alcaligenes eutrophus* – evidence for a required posttranslational modification for catalytic activity. *Biochemistry* **33**, 9311–9320.
- Handrick, R., Reinhardt, S. & Jendrossek, D. (2000). Mobilization of poly(3-hydroxybutyrate) in *Ralstonia eutropha*. *J Bacteriol* **182**, 5916–5918.
- Haywood, G. W., Anderson, A. J., Williams, G. A., Dawes, E. A. & Ewing, D. F. (1991). Accumulation of a poly(hydroxyalkanoate) copolymer containing primarily 3-hydroxyvalerate from simple carbohydrate substrates by *Rhodococcus* sp. NCIMB 40126. *Int J Biol Macromol* **13**, 83–88.
- Hoffmann, N., Steinbüchel, A. & Rehm, B. H. A. (2000a). The *Pseudomonas aeruginosa* phaG gene product is involved in the synthesis of polyhydroxyalkanoic acid consisting of medium-chain-length constituents from non-related carbon sources. *FEMS Microbiol Lett* **184**, 253–259.
- Hoffmann, N., Steinbüchel, A. & Rehm, B. H. A. (2000b). Homologous functional expression of cryptic phaG from *Pseudomonas oleovorans* establishes the transacylase-mediated polyhydroxyalkanoate biosynthetic pathway. *Appl Microbiol Biotechnol* **54**, 665–670.
- Hoppensack, A., Rehm, B. H. A. & Steinbüchel, A. (1999). Analysis of 4-phosphopantetheinylation of polyhydroxybutyrate synthase from *Ralstonia eutropha*: generation of beta-alanine auxotrophic Tn5 mutants and cloning of the panD gene region. *J Bacteriol* **181**, 1429–1435.

- Jia, Y., Kappock, T. J., Frick, T., Sinskey, A. J. & Stubbe, J. (2000). Lipases provide a new mechanistic model for polyhydroxybutyrate (PHB) synthases: characterization of the functional residues in *Chromatium vinosum* PHB synthase. *Biochemistry* **39**, 3927–3936.
- Jia, Y., Yuan, W., Wodzinska, J., Park, C., Sinskey, A. J. & Stubbe, J. (2001). Mechanistic studies on class I polyhydroxybutyrate (PHB) synthase from *Ralstonia eutropha*: class I and III synthases share a similar catalytic mechanism. *Biochemistry* **40**, 1011–1019.
- Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop, R. M. & Peterson, K. M. (1995). 4 new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* **166**, 175–176.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Langenbach, S., Rehm, B. H. A. & Steinbüchel, A. (1997). Functional expression of the PHA synthase gene *phaC1* from *Pseudomonas aeruginosa* in *Escherichia coli* results in poly(3-hydroxyalkanoate) synthesis. *FEMS Microbiol Lett* **150**, 303–309.
- Liebigesell, M., Sonomoto, I. L., Madkour, M., Mayer, F. & Steinbüchel, A. (1994). Purification and characterization of the poly(hydroxyalkanoic acid) synthase from *Chromatium vinosum* and localization of the enzyme at the surface of poly(hydroxyalkanoic acid) granules. *Eur J Biochem* **226**, 71–80.
- Mak, Y. M. & Ho, K. K. (1993). An improved procedure for direct phenol extraction of single-stranded M13-DNA for sequencing. *Biotechniques* **14**, 732–734.
- Nierman, W. C., Feldblyum, T. V., Laub, M. T. & 34 other authors (2001). Complete genome sequence of *Caulobacter crescentus*. *Proc Natl Acad Sci USA* **98**, 4136–4141.
- Peoples, O. P. & Sinskey, A. J. (1989a). Poly- β -hydroxybutyrate biosynthesis in *Alcaligenes eutrophus* H16 – characterization of the genes encoding β -ketothiolase and acetoacetyl-CoA reductase. *J Biol Chem* **264**, 15293–15297.
- Peoples, O. P. & Sinskey, A. J. (1989b). Poly- β -hydroxybutyrate biosynthesis in *Alcaligenes eutrophus* H16 – identification and characterization of the PHB polymerase gene (*phbC*). *J Biol Chem* **264**, 15298–15303.
- Poindexter, J. S. (1981). The Caulobacters – ubiquitous unusual bacteria. *Microbiol Rev* **45**, 123–179.
- Qi, Q. S., Rehm, B. H. A. & Steinbüchel, A. (1997). Synthesis of poly(3-hydroxyalkanoates) in *Escherichia coli* expressing the PHA synthase gene *phaC2* from *Pseudomonas aeruginosa*: comparison of *PhaC1* and *PhaC2*. *FEMS Microbiol Lett* **157**, 155–162.
- Qi, Q. S., Steinbüchel, A. & Rehm, B. H. A. (1998). Metabolic routing towards polyhydroxyalkanoic acid synthesis in recombinant *Escherichia coli* (*fadR*): inhibition of fatty acid β -oxidation by acrylic acid. *FEMS Microbiol Lett* **167**, 89–94.
- Qi, Q., Steinbüchel, A. & Rehm, B. H. A. (2000). *In vitro* synthesis of poly(3-hydroxydecanoate): purification and enzymatic characterization of type II polyhydroxyalkanoate synthases *PhaC1* and *PhaC2* from *Pseudomonas aeruginosa*. *Appl Microbiol Biotechnol* **54**, 37–43.
- Rehm, B. H. A. & Steinbüchel, A. (1999). Biochemical and genetic analysis of PHA synthases and other proteins required for PHA synthesis. *Int J Biol Macromol* **25**, 3–19.
- Rehm, B. H. A. & Steinbüchel, A. (2001a). Heterologous expression of the acyl–acyl carrier protein thioesterase gene from the plant *Umbellularia californica* mediates polyhydroxyalkanoate biosynthesis in recombinant *Escherichia coli*. *Appl Microbiol Biotechnol* **55**, 205–209.
- Rehm, B. H. A. & Steinbüchel, A. (2001b). PHA synthases – the key enzymes of PHA synthesis. In *Handbook 'Biopolymers'*. Edited by A. Steinbüchel & Y. Doi. Weinheim: Wiley–VCH (in press).
- Rehm, B. H. A., Krüger, N. & Steinbüchel, A. (1998). A new metabolic link between fatty acid *de novo* synthesis and polyhydroxyalkanoic acid synthesis – the *phaG* gene from *Pseudomonas putida* KT2440 encodes a 3-hydroxyacyl–acyl carrier protein coenzyme A transferase. *J Biol Chem* **273**, 24044–24051.
- Rehm, B. H. A., Qi, Q. S., Beermann, B. B., Hinz, H.-J. & Steinbüchel, A. (2001a). Matrix-assisted *in vitro* refolding of *Pseudomonas aeruginosa* class II polyhydroxyalkanoate synthase from inclusion bodies produced in recombinant *Escherichia coli*. *Biochem J* **358**, 263–268.
- Rehm, B. H. A., Hoffmann, N., Qi, Q., Fiedler, S. & Steinbüchel, A. (2001b). Biosynthesis of latex-like polyhydroxyalkanoates. In *Proceedings of the International Symposium on Bioconversion of Renewable Raw Materials*. Braunschweig, Germany: GBF (in press).
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Schlegel, H. G., Lafferty, R. & Krauss, I. (1970). The isolation of mutants not accumulating poly- β -hydroxybutyric acid. *Arch Microbiol* **71**, 283–294.
- Schubert, P., Steinbüchel, A. & Schlegel, H. G. (1988). Cloning of the *Alcaligenes eutrophus* genes for synthesis of poly- β -hydroxybutyric acid (PHB) and synthesis of PHB in *Escherichia coli*. *J Bacteriol* **170**, 5837–5847.
- Schubert, P., Krüger, N. & Steinbüchel, A. (1991). Molecular analysis of the *Alcaligenes eutrophus* poly(3-hydroxybutyrate) biosynthetic operon – identification of the N-terminus of poly(3-hydroxybutyrate) synthase and identification of the promoter. *J Bacteriol* **173**, 168–175.
- Simon, R., Priefer, U. & Pühler, A. (1983). A broad host range mobilization system for *in vivo* genetic engineering – transposon mutagenesis in Gram-negative bacteria. *Bio/Technology* **1**, 784–791.
- Spiekermann, P., Rehm, B. H. A., Kalscheuer, R., Baumeister, D. & Steinbüchel, A. (1999). A sensitive, viable-colony staining method using Nile red for direct screening of bacteria that accumulate polyhydroxyalkanoic acids and other lipid storage compounds. *Arch Microbiol* **171**, 73–80.
- Valentin, H. E. & Steinbüchel, A. (1993). Cloning and characterization of the *Methylobacterium extorquens* polyhydroxyalkanoic-acid-synthase structural gene. *Appl Microbiol Biotechnol* **39**, 309–317.

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