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The complex structure of polyhydroxybutyrate (PHB) granules: four orthologous and paralogous phasins occur in *Ralstonia eutropha*

Markus Pötter,[1] Helena Müller,[1] Frank Reinecke,[1] Roman Wieczorek,[1] Florian Fricke,[2,3] Botho Bowien,[3] Bärbel Friedrich[4] and Alexander Steinbüchel[1]

[1]Institut für Molekulare Mikrobiologie und Biotechnologie, Westfälische Wilhelms-Universität Münster, Corrensstrasse 3, 48149 Münster, Germany

[2,3]Genomik Netzwerk Göttingen[2] and Institut für Mikrobiologie und Genetik[3], Georg-August-Universität Göttingen, Grisebachstrasse 8, 37077 Göttingen, Germany

[4]Institut für Biologie, Humboldt-Universität Berlin, Chausseestrasse 117, 10115 Berlin, Germany

Correspondence
Alexander Steinbüchel
steinbu@uni-muenster.de

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Analysis of the genome sequence of the polyhydroxyalkanoate- (PHA) accumulating bacterium *Ralstonia eutropha* strain H16 revealed three homologues (PhaP2, PhaP3 and PhaP4) of the phasin protein PhaP1. PhaP1 is known to constitute the major component of the layer at the surface of poly(3-hydroxybutyrate), poly(3HB), granules. PhaP2, PhaP3 and PhaP4 exhibited 42, 49 and 45 % identity or 61, 62 and 63 % similarity to PhaP1, respectively. The calculated molecular masses of PhaP1, PhaP2, PhaP3 and PhaP4 were 20·0, 20·2, 19·6 and 20·2 kDa, respectively. RT-PCR analysis showed that *phaP2*, *phaP3* and *phaP4* were transcribed under conditions permissive for accumulation of poly(3HB). 2D PAGE of the poly(3HB) granule proteome and analysis of the detected proteins by MALDI-TOF clearly demonstrated that PhaP1, PhaP3 and PhaP4 are bound to the poly(3HB) granules in the cells. PhaP3 was expressed at a significantly higher level in PhaP1-negative mutants. Occurrence of an unknown protein with an N-terminal amino-acid sequence identical to that of PhaP2 in crude cellular extracts of *R. eutropha* had previously been shown by others. Although PhaP2 could not be localized *in vivo* on poly(3HB) granules, *in vitro* experiments clearly demonstrated binding of PhaP2 to these granules. Further analysis of complete or partial genomes of other poly(3HB)-accumulating bacteria revealed the existence of multiple phasin homologues in *Ralstonia solanacearum*, *Burkholderia fungorum* and *Azotobacter vinelandii*. These new and unexpected findings should affect our current models of PHA-granule structure and may also have a considerable impact on the establishment of heterologous production systems for PHAs.

INTRODUCTION

The Gram-negative facultative chemolithoautotrophic hydrogen-oxidizing bacterium *Ralstonia eutropha* is probably the best-studied micro-organism with regard to the metabolism of polyhydroxyalkanoic acids (PHA). *R. eutropha* belongs to those bacteria which accumulate polyoxoesters of short-carbon-chain-length hydroxyalkanoic acids (PHA_{SCL}), comprising three to five carbon atoms (Schlegel *et al.*, 1961a; Steinbüchel & Valentin, 1995). It also

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Abbreviations: IPG, immobilized pH gradient; MALDI-TOF, Matrix-assisted laser desorption/ionization-time of flight; PHA, polyhydroxyalkanoate; poly(3HB), poly(3-hydroxybutyrate); PTE, polythioester; SCL, short carbon-chain length.

The GenBank accession numbers for the nucleotide sequences reported in this paper are AY489113 (*Ralstonia eutropha* H16 *phaP3*), AY489114 (*phaP4*) and AY489115 (*phaZ5*).

The following are available as supplementary data with the online version of this paper at http://mic.sgmjournals.org: an alignment of the amino-acid sequences of phasin homologues of *R. eutropha* H16 PhaP1 in supplementary Fig. I; the regions adjacent to *phaP1*, *phaP2*, *phaP3* and *phaP4* in the *R. eutropha* H16 genome in supplementary Fig. II; the alignment of multiple Re1052 digestion fragments with the proposed H16 sequence in supplementary Fig. III; the molecular masses of *R. eutropha phaP1* knock-out mutant Re1052 granule proteome fragments by MALDI-TOF in supplementary Table I; the similarities of the five *phaZ* homologues of *R. eutropha* in supplementary Table II; the similarities of the four phaP homologues of *R. eutropha* in supplementary Table III.

synthesizes polythioesters (PTE), which consist of mercaptoalkanoic acids of identical length, and represent a novel class of biopolymers (Lütke-Eversloh *et al.*, 2001a, 2001b, 2002; Lütke-Eversloh & Steinbüchel, 2003). Both PHAs and PTEs are synthesized by PHA synthase. Poly(3-hydroxybutyrate) (poly(3HB)) and, in particular, poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) are currently produced by fermentation of glucose-utilizing mutants of *R. eutropha* H16 in order to obtain biodegradable Biopol from renewable resources for the packaging industry and for medical and pharmaceutical applications (Asrar & Gruys, 2002; Williams & Martin, 2002; Lee & Park, 2002). Moreover, the genes of the *R. eutropha* H16 PHA_{SCL} pathway have been expressed in various other organisms to produce Biopol (for review, see Steinbüchel, 2001). Special emphasis has been given to transgenic plants that produce Biopol directly from carbon dioxide (for review, see Poirier & Gruys, 2002). Therefore, it is important to understand in detail the PHA_{SCL} synthesizing and mobilizing enzymes, as well as the morphogenesis and structure of PHA granules in *R. eutropha*. The ongoing sequencing of the *R. eutropha* H16 genome and the analysis of other bacterial genome sequences have revealed much useful and surprising information in this regard.

Since the PHA operon, comprising the genes for β-ketothiolase (*phaA*), acetoacetyl-CoA reductase (*phaB*) and PHA synthase (*phaC*), was cloned during the 1980s, many other genes involved in PHA metabolism have been identified, and the functions of the encoded proteins at least partially analysed. Whereas the PHA synthase encoded by *phaC* is indispensable for PHA and PTE biosynthesis in *R. eutropha*, PhaA and PhaB can be replaced by isoenzymes. A prominent example is BktB, which is only one of many β-ketothiolases in this bacterium (Slater *et al.*, 1998). In addition to PHA synthase, PHA depolymerases (PhaZ) are also bound to the PHA granule surface. Only recently, three intracellular PHA depolymerases (PhaZ1, PhaZ2 and PhaZ3) and a 3HB-oligomer hydrolase (previously also designated PhaZ2), which hydrolyse PHAs and the cleavage products produced by PhaZs, were cloned and characterized in *R. eutropha* (Saegusa *et al.*, 2001, 2002; Kobayashi *et al.*, 2003; York *et al.*, 2003). However, in contrast to the extracellular degradation of PHAs (Jendrossek *et al.*, 1996, 2002; Jendrossek & Handrick, 2002), relatively little is known about intracellular mobilization and degradation in PHA-accumulating cells (Handrick *et al.*, 2000; Saito & Kobayashi, 2002).

The layer at the surface of the PHA granules contains not only the enzymes mentioned above, but also phospholipids and other proteins, with the phasin PhaP1 as the predominant protein. Phasins occur in any PHA_{SCL}-accumulating bacterium, and are analogues of oleosins, which are bound to the surface of the oleosome in plants (Wieczorek *et al.*, 1995; Steinbüchel *et al.*, 1995; Wieczorek & Steinbüchel, 1996; Mayer & Hoppert, 1997; Hanley *et al.*, 1999). Hitherto, only one phasin (PhaP1) has been known to occur in *R. eutropha*. Recently, a gene encoding a protein exhibiting similarity to PhaP1 was identified on the megaplasmid of *R. eutropha* H16 (Schwartz *et al.*, 2003). In this study, we describe the occurrence of two additional paralogous and orthologous phasin genes in *R. eutropha*. We show that all four genes are expressed, and that the proteins are synthesized when the cells accumulate PHAs. Since all previous models for the structure of PHA granules and metabolism of PHAs in *R. eutropha* were based on the knowledge of only a single phasin, awareness of at least four phasin proteins in this bacterium will be important for PHA research and to adapt the models for the structure of PHA_{SCL} granules (Steinbüchel *et al.*, 1995; Jurasek *et al.*, 2001; Jurasek & Marchessault 2002; Dennis *et al.*, 2003 and references cited therein). Moreover, *R. eutropha* is not exceptional regarding the occurrence of multiple phasins, and this study will show that other PHA_{SCL}-accumulating bacteria also possess multiple copies of phasin genes. Therefore, the data presented in this study are of general relevance.

METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. Cells of *R. eutropha* were grown at 30 °C in mineral salts medium (MM) supplemented with 1·5 % (w/v) filter-sterilized sodium gluconate (Schlegel *et al.*, 1961b). To promote extensive accumulation of PHA, the concentration of ammonium chloride in MM was reduced to 0·02 %, w/v (storage conditions). Cells of *Escherichia coli* were cultivated at 37 °C in Luria–Bertani (LB) medium (Sambrook *et al.*, 1989).

Isolation and manipulation of DNA. Chromosomal DNA of *R. eutropha* H16 was isolated by the method of Marmur (1961). Plasmid DNA was isolated by the method of Birnboim & Doly (1979). DNA restriction fragments were purified with the Nucleotrap kit (Machery-Nagel), as described by the manufacturer. Restriction enzymes, ligases and other DNA-manipulating enzymes were used according to the manufacturer's instructions.

Transfer of DNA. Competent cells of *E. coli* were prepared and transformed by the CaCl_2 procedure (Hanahan, 1983).

Cloning of *phaP2* and purification of recombinant His_6-tagged PhaP2 from recombinant *E. coli*. For cloning of *phaP2* into *E. coli*, PCR was done by using phaP2His_NdeI_fw as sense and phaP2His_*Xba*I_rv as reverse primers (Table 1). They were deduced from the upstream and downstream regions, respectively, of *phaP2* of *R. eutropha* H16 (Schwartz *et al.*, 2003). The phaP2His_6 PCR product obtained was purified and ligated into pMa/c5-914 (Table 1), which harbours the *c*I857ts gene encoding the temperature-sensitive λ repressor. The recombinant His_6-tagged PhaP2 (N-terminal fusion) was purified from *E. coli* TOP10 harbouring pMa/c5-914 : : *phaP2*His_6. Protein purification was carried out under non-denaturing conditions, employing Ni^{2+}-NTA-Spin columns (Qiagen), as described by the manufacturer.

PCR amplification. All PCR amplifications of DNA were carried out as described by Sambrook *et al.* (1989), employing *Pfx*-DNA-polymerase (Invitrogen) and an Omnigene HBTR3CM DNA thermal cycler (Hybaid). All PCR primers used in this study are listed in Table 1.

Isolation of total RNA and RT-PCR. Total RNA was isolated from 1×10^9 cells in the stationary growth phase, which had been

Table 1. Bacterial strains and oligonucleotide primers used for RT-PCR in this study

Strain, plasmid or primer	Description	Reference, source or location
Ralstonia eutropha		
H16	Wild-type	DSM 428
H2275	*phaP1*::Tn*5* mutant derived from *R. eutropha* HF39, Tn*5* insertion 156 bp upstream of *phaP1*	Wieczorek *et al.*, 1995
Re1052	*phaP1* precise-deletion gene replacement strain, derived from *R. eutropha* H16	York *et al.*, 2001
Escherichia coli		
TOP10	F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) *f80lacZ* Δ*M15* Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL endA1 nup*G	Invitrogen
Plasmid		
pMa/c5-914	*amp cat c*I857ts *lacZp*/*o*, TIR, P_L and P_R	SK 6772
pMa/c5-914::*phaP2*His$_6$	*Nde*I–*Xba*I fragment of *R. eutropha* H16 harbouring His$_6$-tagged *phaP2* in pMa/c5-914	This study
Primer		
RT_phaP2_fw	5′-ATGCAGTGGACAGCAGAGC-3′	5′ Region of *phaP2*
RT_phaP2_rv	5′-CTACTTTGCAGCTGCCGGAGAC-3′	3′ Region of *phaP2*
RT_phaP3_fw	5′-ATGTCCCCTTTTATGCCCG-3′	5′ Region of *phaP3*
RT_phaP3_rv	5′-TTATTGCTTGGAAGCGCG-3′	3′ Region of *phaP3*
RT_phaP4_fw	5′-ATGACTCAGTGGTCCCCCG-3′	5′ Region of *phaP4*
RT_phaP4_rv	5′-TTAATTTGCAGCTGCCTTTG-3′	3′ Region of *phaP4*
phaP2His_NdeI_fw	5′-AAAAAACATATGCATCACCACCACCACCACATGACTCAGTG-GACAGCAGAGCAATGCACCAAG-3′	5′ Region of *phaP2*His$_6$
phaP2His_*Xba*I_rv	5′-AAAAAATCTAGAGCGGGCCCAGCGCTGCCGGACTA-3′	3′ Region of *phaP2*His$_6$

cultivated for 72 h in MM under storage conditions (0·02 %, w/v, ammonium chloride). Cells were lysed by lysozyme treatment, and lysates were subjected to the RNeasy RNA purification kit (Qiagen). DNA-free total RNA was obtained after on-column DNase I treatment and elution, as described by the manufacturer. Total RNA was analysed by agarose gel electrophoresis to confirm RNA digestion. In order to qualitatively analyse the *phaP2*-, *phaP3*- and *phaP4*-derived mRNA, RT-PCR was applied, using the oligonucleotides shown in Table 1 as primers. One-step RT-PCR (One Step RT-PCR kit; Qiagen) was carried out according to the manufacturer's protocol, employing 0·5 ng RNA as template. In order to exclude any contaminating DNA that might have served as a template for PCR, template RNA was added in a control experiment, after inactivating the reverse transcriptase and activating *Taq*-polymerase. Absence of PCR products in the control indicated that the RT-PCR products were not derived from contaminating DNA. The PCR products were digested with suitable restriction endonucleases to verify the occurrence of the correct fragments. Amplified PCR products were resolved on a 2 % (w/v) agarose gel and stained with ethidium bromide.

Isolation of native PHA granules. Poly(3HB) granules were isolated by a modification of the method of Preusting *et al.* (1993) from cells of *R. eutropha*, which had been grown in MM under storage conditions. After 72 h incubation, cells of 200 ml cultures were harvested by centrifugation (20 min, 6000 ***g***, 4 °C). The cells were washed and resuspended in 10 ml potassium phosphate (KP) buffer (100 mM, pH 7·5) and, after threefold French press passage (100×10^6 Pa), 5 ml of the lysate was loaded on the top of a linear glycerol gradient. This gradient was obtained from a discontinuous gradient prepared from 1 ml 90 % (v/v) and 4 ml 50 % (v/v) glycerol in KP buffer. After centrifugation (2 h, 210 000 ***g***, 4 °C), a granule layer was obtained at about 90 % (v/v) glycerol. The granules were isolated and washed with KP buffer by centrifugation (10 min, 100 000 ***g***, 4 °C). The granules were resuspended in KP buffer and subsequently loaded on the top of a second linear glycerol gradient, prepared from 1 ml 90 %, (v/v), 2 ml 80 % (v/v), 1 ml 60 % (v/v) and 1 ml 50 % glycerol in KP buffer. After centrifugation (2 h, 210 000 ***g***, 4 °C), the granules again sedimented at about 90 % (v/v) glycerol. These granules were washed twice with KP buffer and then stored at −20 °C.

Preparation of crystalline PHA granules. Crystalline poly(3HB) granules were isolated from *R. eutropha* H16 cells grown in sodium gluconate, by employing the hypochlorite treatment described previously (Jendrossek *et al.*, 1993).

SDS-PAGE, blotting and N-terminal sequence analysis. Protein samples were resuspended in gel loading buffer (0·6 %, w/v, SDS; 1·25 %, w/v, β-mercaptoethanol; 0·25 mM EDTA; 10 %, v/v, glycerol; 0·001 %, w/v, bromophenol blue; 12·5 mM Tris/HCl; pH 6·8) and were separated in 12·5 % (w/v) SDS polyacrylamide gels, as described by Laemmli (1970). Proteins were stained with Coomassie brilliant blue R-250 (Weber & Osborn, 1969) or with silver (Heukeshofen & Dernick, 1985). For N-terminal sequencing, the protein was blotted from an SDS-polyacrylamide gel onto a PVDF membrane, as described by Towbin *et al.* (1979). Sequence analysis was performed by automated Edman degradation.

Analysis of granule-associated proteins by 2D gel electrophoresis and MALDI-TOF. Proteins from native granules (25 mg wet weight) were solubilized in 300 μl solubilization buffer (9 M urea; 4 %, w/v, CHAPS; 50 mM DTT; 2·5 %, w/v, Triton X-114) by stirring for 2 h at room temperature, and the solubilized proteins were separated from the granules by centrifugation (16 100 ***g***, 20 °C). For the first dimension, the sample was mixed with 100 μl of the same buffer, additionally containing carrier ampholytes (covering pH 3–10; Serva) and bromophenol blue. Electrophoresis was performed using immobilized pH gradient (IPG) strips (Bio-Rad

Laboratories). The IPG strips (18 cm, pH 5–8) were rehydrated with the entire granule-protein sample overnight at room temperature under mineral oil. After rehydration, isoelectric focusing in the IPG strip was carried out for a total of 100 kVh (with a maximum of 6000 V) at 20 °C under mineral oil. The focused strip was reduced in 5 ml 50 mM Tris buffer containing 6 M urea, 30 % (v/v) glycerol, 5 % (w/v) SDS and 15 mM DTT for 20 min at room temperature. It was then alkylated in the same Tris buffer containing 150 mM iodoacetamide for 20 min at room temperature. The strip was then run in a 20 cm × 20 cm, 12 % SDS polyacrylamide gel to separate the proteins in the second dimension according to molecular mass. The current was limited to 40 mA per gel. Proteins were detected by Coomassie brilliant blue staining. MALDI–TOF analysis was done by the method of Shevchenko *et al.* (1996).

Analysis of nucleotide and amino-acid sequences. All available sequence data of the National Center for Biotechnology Information (NCBI) at http://www.ncbi.nlm.nih.gov were searched for fragments of high similarity to the translational product of *phaP1* from *R. eutropha* strain H16 using the BLAST program on the BLAST server of the website. The incomplete genome sequence of *R. eutropha* H16 was searched by the ERGO database (Integrated Genomics) for homologous genes of high similarity to *phaP1*. The presented search results represent the data available at the above-mentioned databases as of February 19 2004. Contigs containing fragments of highest similarity were analysed using the evaluation version of DNA Tools 5.1. Sequences were aligned using CLUSTAL X 1.8, and phylogenetic trees were constructed using TREE 1.6.5.

RESULTS

Identification of putative ORFs encoding proteins exhibiting similarity to PhaP1 in *R. eutropha* H16

The currently available genome sequence of *R. eutropha* H16, the ERGO database (Integrated Genomics) and publicly accessible genome sequences of Bacteria and Archaea were analysed for the presence of ORFs whose putative translational products exhibited similarity to the phasin PhaP1 of *R. eutropha* H16. Also, the sequence of the megaplasmid pHG1 was annotated again with regard to phasin similarities (Schwartz *et al.*, 2003). Three additional high-scoring ORFs were identified in the *R. eutropha* H16 genome, and the putative translation products were referred to as PhaP2, PhaP3 and PhaP4, respectively. All four phasins of *R. eutropha* were very similar with respect to size and isoelectric point, with calculated molecular masses ranging from 19·5 to 20·2 kDa and isoelectric points ranging from 5·91 to 6·12 (Table 2). Whereas it was already known that *phaP2* is localized on megaplasmid pHG1 (Schwartz *et al.*, 2003), in this study *phaP1* and *phaP3* were localized on chromosome 1, and *phaP4* on chromosome 2.

In addition, this analysis revealed 19 putative phasin-homologous proteins in other bacteria. Whereas four PhaP1 homologues each were detected in *R. eutropha* JMP134 and *Burkholderia fungorum* LB400, three were detected in *Ralstonia solanacearum*, two in *Azotobacter vinelandii* and one each in *Burkholderia pseudomallei* K96243, *Azotobacter* sp. strain FA8, *Bordetella pertussis*, *Bordetella parapertussis* 12822, *Bordetella bronchiseptica* RB50 and *Rhodospirillum rubrum*. The identities and similarities to *R. eutropha* H16 PhaP1 of the putative phasin proteins detected in the genomes of these bacteria ranged from 25 % to 72 % and from 46 % to 86 %, respectively (Table 2). An alignment of the amino-acid sequences of these phasin homologues with PhaP1 is shown in supplementary Fig. I (available as supplementary data with the online version of this paper at http://mic.sgmjournals.org). Fig. 1 shows a bootstrap neighbour-joining of all confirmed and putative phasins identified so far. The resulting tree was rooted using the granule-associated protein gene *phaF* from *Pseudomonas oleovorans* as outgroup (Prieto *et al.*, 1999). Where more than one phasin is present in a single strain, the tree clearly shows different extents of similarity between homologues. It is worth mentioning that genes encoding PhaP1 homologues have so far only been detected in the β-proteobacterial branch of the Eubacteria, although proteins bound to PHA granules have also been found in other branches of the proteobacteria and in Gram-positive bacteria (Fukui *et al.*, 2001; Vazquez *et al.*, 1996).

The *phaP1* homologues are randomly localized in bacterial genomes, and a regular arrangement is not observed. However, downstream of *phaP1*, and in an antilinear orientation, the gene for a pyruvate formate-lyase activating enzyme (EC 1.97.1.4) is localized. Furthermore, *phaP3* is co-localized with an acetoacetyl-CoA reductase (EC 1.1.1.36) gene, downstream in the same orientation. The regions adjacent to *phaP1*, *phaP2*, *phaP3* and *phaP4* in the genome of *R. eutropha* H16 are shown in supplementary Fig. II (available as supplementary data with the online version of this paper at http://mic.sgmjournals.org).

Comparison of the primary structures of *R. eutropha* PhaP1 and the 20 PhaP1 homologues shown in supplementary Fig. I revealed a few highly conserved positions in these proteins. Leu-38 in *R. eutropha* PhaP1 was the only position which was identical in the 23 proteins, with the exception of PhaP of *Rhodospirillum rubrum*, where this residue was replaced with Val. Several other positions, such as Leu-18, 24, 40, 47, 68, 71, 86, 92; Glu-33, 41, 72, 102, 108, 161; Gln-41, 161; Ile-95, 145; Lys-34, 44, 84; Pro-78, 129; Ala-128; Arg-90; Asn-39; Gly 131; Thr-25 and Val-43, 121 were in only a few phasins replaced by very similar amino acids. The alignment of the PhaP homologues also revealed alanine-rich areas at the C-terminal regions of the phasins (amino acids 120–202).

Analysis of *phaP2*, *phaP3* and *phaP4* transcription in *R. eutropha* H16

The occurrence of four genes for phasin homologues in *R. eutropha* raised the question whether the three additionally detected genes are intact and active, as previously shown for *phaP1* (Wieczorek *et al.*, 1995). Therefore, one-step RT-PCR was employed in order to investigate the transcription of *phaP2*, *phaP3* and *phaP4* in *R. eutropha* H16 qualitatively under conditions permissive for poly(3HB) biosynthesis and accumulation. Cells were cultivated in MM containing 1·5 %

Table 2. Characteristics of known and putative phasins compared to PhaP1 from *R. eutropha* H16

Strain and phasin	Accession no. or ORF no.	Characteristic			
		Amino acid identity (%)	Amino acid similarity (%)	Calculated molecular mass (kDa)	pI
Ralstonia eutropha H16 PhaP1	AAC78327	100	100	20·0	5·96
Ralstonia eutropha H16 PhaP2	AAP85954	42	59	20·2	5·95
Ralstonia eutropha H16 PhaP3	AY489113	51	69	19·5	6·12
Ralstonia eutropha H16 PhaP4	AY489114	45	62	20·2	5·91
Ralstonia solanacearum PhaP1	NP_519726	69	81	20·3	6·92
Ralstonia solanacearum PhaP2	NP_518146	36	54	18·8	6·38
Ralstonia solanacearum PhaP3	NP_520093	30	52	17·6	4·99
Ralstonia eutropha JMP134 PhaP1	RJMP01004*	42	61	19·6	5·84
Ralstonia eutropha JMP134 PhaP2	RJMP06434*	41	66	20·3	5·52
Ralstonia eutropha JMP134 PhaP3	RJMP05795*	35	53	18·3	6·83
Ralstonia eutropha JMP134 PhaP4	RJMP07243*	39	54	20·1	5·95
Burkholderia fungorum LB400 PhaP1	ZP_00035087	67	78	20·1	6·18
Burkholderia fungorum LB400 PhaP2	RBU14151*	36	58	18·4	4·98
Burkholderia fungorum LB400 PhaP3	ZP_00028696	41	62	18·7	6·73
Burkholderia fungorum LB400 PhaP4	ZP_00031614	30	52	16·4	6·12
Bordetella pertussis PhaP†	NP_881967	38	55	19·8	5·26
Burkholderia pseudomallei K96243 PhaP	RBPS03662*	72	86	19·9	5·96
Azotobacter vinelandii PhaP1	AAK72594	36	62	20·3	4·93
Azotobacter vinelandii PhaP2	ZP_00089905	27	46	19·2	5·97
Azotobacter sp. FA8 PhaP	CAD42757	37	61	20·4	4·93
Rhodospirillum rubrum PhaP	ZP_00014946	25	48	17·5	5·37

*ORF identifier in the ERGO database.

†The strains *Bordetella parapertussis* 12822 and *Bordetella bronchiseptica* RB50 exhibit the same amino-acid sequence as *Bordetella pertussis.*

(w/v) sodium gluconate as sole carbon source. Total RNA was isolated from these cells in the stationary growth phase, and RT-PCR with primers specific for the megaplasmid-encoded *phaP2* as well as the chromosomally-encoded *phaP3* and *phaP4* was carried out (Table 1). RT-PCR analysis clearly demonstrated that *phaP2*, *phaP3* and *phaP4* were transcribed under storage conditions (Fig. 2). Control PCR experiments with the isolated RNA as template did not result in PCR-product formation, confirming that DNA contamination did not contribute to RT-PCR product formation.

Identification of PhaP3 in the PhaP1-negative mutant *R. eutropha* H2275

After transcription of all four phasin-homologous genes had been demonstrated, it was necessary to investigate whether all messenger RNAs were translated into proteins and where these proteins were located in *R. eutropha* cells. For this, poly(3HB) granules were isolated from gluconate-grown cells of the wild-type *R. eutropha* H16 by glycerol density gradients, as described in Methods. The granule preparation from *R. eutropha* H16 contained a major 24 kDa protein, PhaP1, as previously shown (Wieczorek *et al.*, 1995), and three other major proteins, one of which appeared in a double band with PhaP1. In addition, some minor weak bands were visible (Fig. 3). PhaP1 is the predominant protein, and contributes to approximately 3–5 % of the total cellular protein, as estimated from the intensity of the stained protein bands (Wieczorek *et al.*, 1995).

From previous studies (Wieczorek *et al.*, 1995), a PhaP1-negative Tn*5*-induced mutant of *R. eutropha* H2275 was available, and poly(3HB) granules were also prepared from cells of this mutant and analysed by SDS-PAGE. An additional protein with an apparent molecular mass of 23 kDa (±1 kDa) was identified (Fig. 3), blotted onto a PVDF membrane, and N-terminal sequencing yielded a sequence of 18 amino acids [MSPFMPEQFAAV(Q)(K)(S)(L)(L)L]. This sequence matched exactly the sequence deduced from the 5′-region of *phaP3*, thus clearly demonstrating that *phaP3* mRNA is translated and that PhaP3 is a granule-bound protein.

Analysis of the *R. eutropha* H16 poly(3HB) granule proteome by 2D PAGE and identification of proteins by MALDI-TOF

One-dimensional SDS-PAGE was for several reasons not suitable to detect all phasin proteins in crude cell extracts or poly(3HB) granules. Firstly, the molecular masses calculated

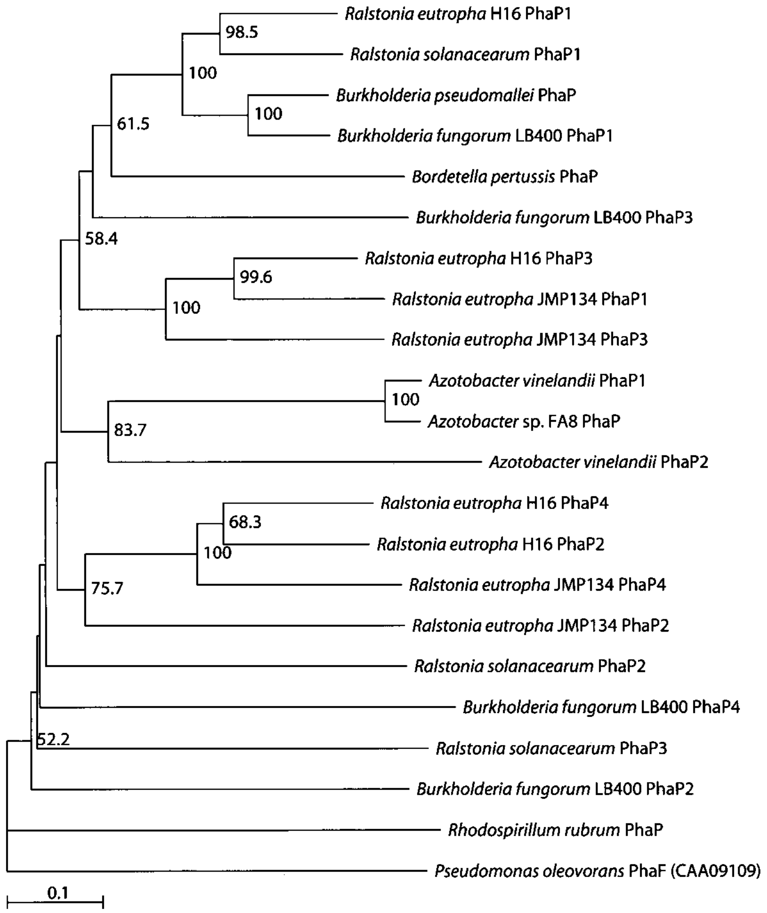

Fig. 1. Phylogenetic tree of known and putative phasins. The tree was constructed using the neighbour-joining method for phasins of *R. eutropha* H16 and related bacteria, based on a comparison of phasin sequences. Bootstrap values for branches are given at the nodes. Bar, 0·1 aa substitutions.

for the four phasin homologues were nearly identical (Table 2). Secondly, large amounts of PhaP1 occurred in the cells and in the granule preparations. Thirdly, PhaP2 and PhaP3 occurred only at low amounts, and even in a *phaP1* mutant, these two phasins could not be clearly identified in one-dimensional SDS-polyacrylamide gels. Therefore, the poly(3HB) granule proteome was analysed by two-dimensional PAGE, and the proteins were subsequently identified by MALDI-TOF.

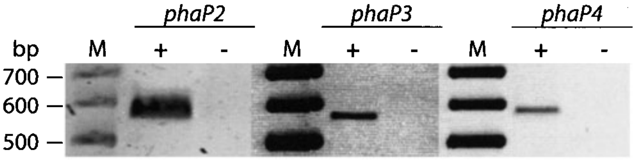

Fig. 2. RT-PCR analysis of *phaP2*, *phaP3* and *phaP4*. Reactions were carried out using the corresponding pairs of specific oligonucleotide primers for the indicated genes. Total RNA was isolated from cells grown to stationary-growth phase in MM under storage conditions. +, Reverse transcriptase present in the reaction; −, reverse transcriptase absent; M, 100 bp DNA ladder as molecular mass standard.

For this, poly(3HB) granules were isolated from the wild-type strain H16 and from the *phaP1* knock-out mutant Re1052. For these experiments, to guarantee the correct phenotype, we used the deletion mutant Re1052 instead of the Tn*5*-induced mutant H2275, which harbours Tn*5* inserted into the promoter region. This procedure was necessary, because PhaP1 is the dominant protein at the surface of wild-type granules, completely or partially masking other phasins occurring in minor amounts. The granule-bound proteins were solubilized as described above, and separated by 2D PAGE, as described in Methods. Three different forms of PhaP1 were observed as predominant granule-associated proteins of *R. eutropha* H16 (Fig. 4A). We also analysed additional polypeptide spots by MALDI-TOF, which were identified as PhaP3 and PhaP4, respectively (Fig. 4B). Veith *et al.* (2001) and Lutter *et al.* (2001) suggested that pI heterogeneity can in some instances be related to conformational equilibria, and not posttranslational modifications.

The spot between GroEL and Bkt, of a similar size to Bkt, could not be clearly identified by MALDI-TOF analysis. This may be due to the incomplete DNA sequence of *R. eutropha*, or posttranslational modification. A very faint spot about 2 mm away from the upper PhaP1 spot in Fig. 4A was at the same position as the upper PhaP3 spot in Fig. 4B (see

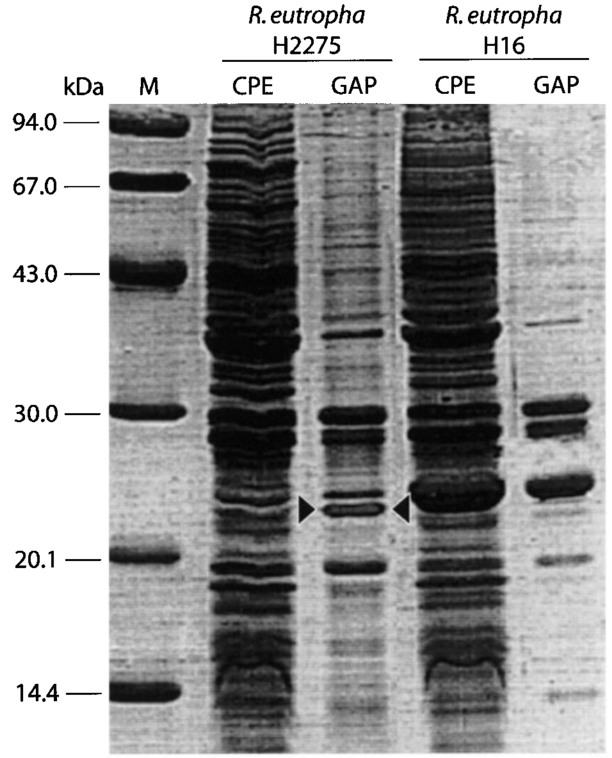

Fig. 3. Analysis of proteins bound to PHA granules in *R. eutropha* H16 and in the *phaP1*-negative Tn*5*-mutant *R. eutropha* H2275. Crude protein extracts (CPE) obtained from cells grown in MM under storage conditions, and proteins solubilized from isolated PHA granules (GAP), were separated by SDS-PAGE and stained with Coomassie brilliant blue. The additional 23 kDa protein in the GAP lane of *R. eutropha* H2275 is indicated by arrows. M, molecular mass standard.

below), and may therefore represent PhaP3 protein. Unfortunately, the amount of protein was too low to allow MALDI-TOF analysis. The PHA synthase could not be detected in the SDS-polyacrylamide gel (Fig. 3) or in the 2D gel (Figs 4A, B), respectively. Previous studies have shown that the PHA synthase constitutes only a minor protein at the granule surface, and that it detaches readily from the surface during the preparation of the granules (Wieczorek and Steinbüchel, 1996).

The granule proteome of the mutant comprised about six different protein species, which were isolated from the gel and digested with trypsin. The molecular masses of the resulting fragments were subsequently determined by MALDI-TOF (supplementary Table I, available as supplementary data with the online version of this paper at http://mic.sgmjournals.org). Definitive evidence of the granule-associated proteins required the alignment of multiple digestion fragments with the proposed sequence (supplementary Fig. III, available as supplementary data with the online version of this paper at http://mic.sgmjournals.org). These masses were in complete agreement with the amino-acid sequences deduced from the putative genes for PhaP1, PhaP3, PhaP4, BktB and GroEL from *R. eutropha* H16. An additional polypeptide spot close to that of BktB could not be identified by MALDI-TOF analysis. Only PhaP2 was not identified in the 2D gels. Additional proteins detected by SDS-PAGE of isolated PHA granules from the Tn*5*-mutant *R. eutropha* H2275 could not be identified in 2D gels, because the IPG strips employed covered a pH range from 5 to 8 only. These studies clearly demonstrated that PhaP1, PhaP3 and PhaP4 are bound to the poly(3HB) granules in *R. eutropha*. However, an N-terminal amino-acid sequence obtained by other workers for a protein occurring in the total proteome of a glucose-utilizing *R. eutropha* mutant has been clearly identified as the translational product of *phaP2* (Srinivasan *et al.*, 2002). Therefore, all four phasin proteins are synthesized in *R. eutropha.*

Studies on the binding of PhaP2 to crystalline poly(3HB) granules

To reveal the capability of PhaP2 to bind to poly(3HB) granules, a His$_6$-PhaP2 fusion protein was isolated from a recombinant strain of *E. coli.* An emulsion of crystalline poly(3HB) granules in water (1·5 %, w/v) was then incubated with the partially purified His$_6$-PhaP2 fusion protein for 90 min on ice (Wieczorek *et al.*, 1995). After this incubation, the granules were collected by centrifugation, washed twice with 1 ml 10 mM Tris/HCl (pH 7·0), and suspended in denaturating buffer. The granule sediments and supernatants of each washing step were analysed by SDS-PAGE (Fig. 5). The His$_6$-PhaP2 fusion protein exhibited a very high affinity for poly(3HB) granules (Fig. 5, lanes 1 and 4). Repeated washing with 1 ml 10 mM Tris/HCl (pH 7·0) did not remove the fusion protein from the poly(3HB) granules (Fig. 5, lanes 2 and 3). To demonstrate that PhaP2 did not precipitate during the assay, PhaP2 was incubated without granules, under the same conditions described above. During the time course of this control experiment, PhaP2 was never detected in the sediment after centrifugation. The results of these experiments indicate that the putative location of PhaP2 is the poly(3HB) granules in *R. eutropha*; however, for several possible reasons it could not be identified during analysis of the native granule proteome.

Search for homologues of further genes relevant to PHA metabolism in *R. eutropha*

The occurrence of multiple phasin-homologous proteins in *R. eutropha* strain H16 prompted us to search the genome sequence of this bacterium for homologues of other genes relevant to PHA metabolism. No homologues to *phaC*, the PHA synthase gene, were detected. Therefore, it is most likely that, in *R. eutropha*, only one PHA synthase protein exists, and that all PHA$_{SCL}$ and PTE$_{SCL}$ known to occur in this bacterium are synthesized by the same PHA synthase. This is consistent with the phenotypes of previously obtained single-insertion (Tn*5*-insertion) and spontaneously occurring point mutants (Schubert *et al.*, 1988).

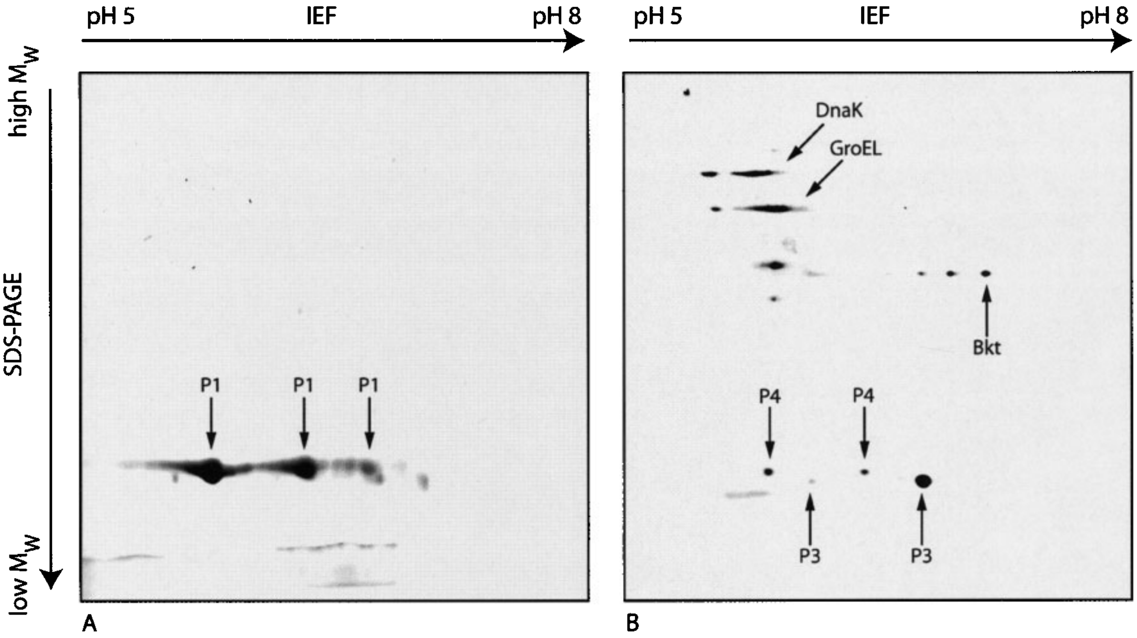

Fig. 4. 2D gel electrophoresis of granule-associated proteins of *R. eutropha* H16 (A) and *R. eutropha* Re1052 (B). Samples shown were collected from cells grown to stationary-growth phase in MM under storage conditions. Samples were resuspended in SDS buffer, and 2D SDS-PAGE was run with each individual sample. The gels were visually analysed, and the spots were analysed by MALDI-TOF. P1, PhaP1; P3, PhaP3; P4, PhaP4.

Furthermore, no homologues to *phaR*, the repressor protein responsible for autoregulation of PhaP1 expression (Pötter *et al.*, 2002), could be detected in the *R. eutropha* genome.

A Search for *phaZ* homologues putatively encoding PHA depolymerases involved in the intracellular mobilization of PHAs revealed five genes. Four of them encoded the recently detected PHA depolymerase genes *phaZ1*, *phaZ2* and *phaZ3* (Saegusa *et al.*, 2001; York *et al.*, 2003), which are localized on chromosome 1 (*phaZ1* and *phaZ2*) and chromosome 2 (*phaZ3*), and also the PHA depolymerase gene *phaZ4*, which is localized on megaplasmid pHG1 (Schwartz *et al.*, 2003), whereas the fifth gene was hitherto unknown and was localized on chromosome 1 (*phaZ5*, Genbank accession no. AY489115). Supplementary Table II (available as supplementary data with the online version of this paper at http://mic.sgmjournals.org) shows the similarities of the five *phaZ* homologues of *R. eutropha.*

DISCUSSION

Analysis of available bacterial genome sequences identified several ORFs putatively encoding proteins of high similarity to the known phasin PhaP1 from *R. eutropha* strain H16 (Wieczorek *et al.*, 1995; Hanley *et al.*, 1999) in various bacteria belonging to the β-proteobacteria. All these bacteria in which PhaP1 homologues occurred have the ability to accumulate large amounts of PHAs of short carbon-chain length (PHA_{SCL}), such as poly(3HB). Very interestingly, many of these bacteria, such as *R. eutropha*, *R. solanacearum*,

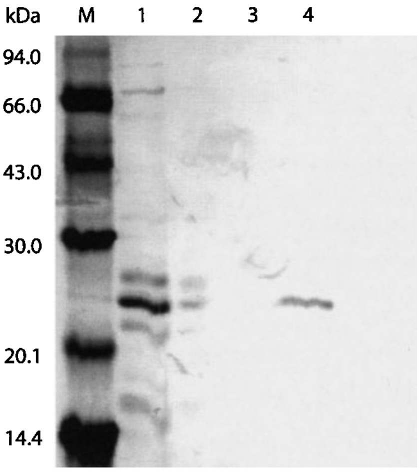

Fig. 5. Binding of His_6-tagged PhaP2 to crystalline granules. Proteins were separated in 12·5 %, w/v, SDS-polyacrylamide gels and stained with silver. The purified His_6-tagged PhaP2 protein (120 µg in 130 µl 100 mM Tris/HCl, pH 7·0) was incubated for 90 min on ice with poly(3HB) granules. The mixture was subsequently separated into sediment and supernatant by centrifugation. Sedimented granules were washed twice with 10 mM Tris/HCl, pH 7·5. Lanes: M, molecular mass protein standard; 1, crystalline poly(3HB) granules incubated with partially purified His_6-tagged PhaP2 protein isolated from *E. coli* TOP10 harbouring pMa/c5-914::*phaP2*His_6; 2, supernatant after first wash step; 3, supernatant after second wash step; 4, pellet with His_6-tagged PhaP2 protein after second wash step.

B. fungorum and *A. vinelandii*, possess two, three or even four of these homologous proteins. It was also shown that *R. eutropha* strain H16, which is the best-studied bacterium with regard to PHA_{SCL} metabolism, expressed any of the four PhaP1, and that the proteins exhibited the capability to bind to poly(3HB) granules.

Since *R. eutropha* strain H16 and mutants derived from this strain are currently employed for the production of PHA_{SCL}, and since the PHA_{SCL} biosynthesis genes of this bacterium are currently used to establish heterologous production systems for PHA_{SCL}, this bacterium serves as a model organism to reveal all aspects of PHA_{SCL} metabolism. This includes PHA biosynthesis and mobilization, as well as the biogenesis and structure of PHA granules and the regulation of these processes. Biosynthesis of the coenzyme A thioesters of the various hydroxyalkanoic acids and mercaptoalkanoic acids, which serve as substrates of the PHA synthase, is achieved by several enzymes: from acetyl-CoA, in the case of poly(3HB) biosynthesis, and by pathways linking the catabolism of carbon sources used as precursor substrates for biosynthesis of all other PHA_{SCL} and PTE_{SCL} in *R. eutropha* (Steinbüchel, 2001; Steinbüchel & Lütke-Eversloh, 2003). Obviously, only one PHA synthase exists in *R. eutropha*, and this enzyme is responsible for the biosynthesis of a wide range of different PHA_{SCL} and PTE_{SCL}.

In contrast to PHA biosynthesis, more than one PHA depolymerase gene is present that may be involved in PHA mobilization. Three different PHA depolymerases were recently detected as functionally active enzymes in *R. eutropha* H16 (Saegusa *et al.*, 2001; York *et al.*, 2003). Furthermore, Schwartz *et al.* (2003) recently detected a fourth PHA depolymerase gene (*phaZ4*) on megaplasmid pHG1. The analysis of the *R. eutropha* H16 genome in this study revealed an additional gene putatively encoding a fifth PHA depolymerase. Why this bacterium has five PHA depolymerase genes, whereas for biosynthesis of PHAs only one PHA synthase is sufficient, is an enigma. At least three of these PHA depolymerase genes are expressed (York *et al.*, 2003). In addition, a putative poly(3HB)-dimer hydrolase was recently detected, which further degrades the cleavage products formed by the PHA depolymerases (Saegusa *et al.*, 2002; Kobayashi *et al.*, 2003).

Another enigma is the presence of four genes encoding highly homologous phasin proteins (Table 3). Whereas PhaP1, PhaP3 and PhaP4 are bound *in vivo* to the poly(3HB) granules, the *in vivo* location of PhaP2, which has the capability to bind crystalline poly(3HB) granules, remains unclear. PhaP2 may be *in vivo* only loosely bound to the granules and washed off during the purification of the granules, or it may *in vivo* only bind to granules under specific cultivation conditions or at a specific stage of PHA accumulation which were not met in this study. PhaP1 is certainly the major phasin protein in *R. eutropha*, and *phaP1* mutants exhibit a distinct phenotype. Such mutants accumulate less poly(3HB) than the wild-type and possess only one single large granule per cell, whereas cells of the wild-type possess several poly(3HB) granules. In a well-regulated process employing the autoregulator and transcriptional repressor protein PhaR, PhaP1 is synthesized in large amounts, contributing 3–5 % of the total cellular protein. PhaP1 is obviously not efficiently compensated by any of the other three phasins (Wieczorek *et al.*, 1995; Pötter *et al.*, 2002). Otherwise, *phaP1* mutants of *R. eutropha* would not exhibit the phenotype PHA-leaky, and the PHA granules would not coalesce to a single granule in the cells of this mutant.

However, the question arises of why are there four phasins in *R. eutropha* if, according to our current view of the structure of PHA granules, one phasin is sufficient. Since the genome of *R. eutropha* H16 consists of three replicons (Schwartz & Friedrich, 2001), it may be speculated whether additional phasin genes have simply arisen through gene duplication and are therefore orthologous. This may be the case with the megaplasmid-encoded PhaP2 and the chromosome

Table 3. Overview of the characteristics of the *pha* homologues

Characteristic	*pha* gene			
	phaP1	***phaP2***	***phaP3***	***phaP4***
Genomics	Sequenced* AAC78327 on chromosome 1	Sequenced† AAP85954 on megaplasmid phg1	Sequenced AY489113 on chromosome 1	Sequenced AY489114 on chromosome 2
Transcriptomics	Translated under storage conditions*	Translated under storage conditions	Translated under storage conditions	Translated under storage conditions
Proteomics	Expressed under storage conditions*	Expressed under storage conditions‡	Expressed under storage conditions	Expressed under storage conditions
Location	Granule associated (*in vivo*)*	Granule associated (*in vitro*)	Granule associated (*in vivo*)	Granule associated (*in vivo*)

*Wieczorek *et al.*, 1995.

†Schwartz *et al.*, 2003.

‡Srinivasan *et al.*, 2002.

2- (2·9 Mb) encoded PhaP4, which are very similar (supplementary Table III). In contrast, PhaP1 and PhaP3 exhibit much less similarity to the other two phasins (supplementary Table III), and the respective genes are localized on the larger chromosome 1 (4·1 Mb). Therefore, PhaP1 and PhaP3 are also homologues, but may represent paralogues to PhaP2 and PhaP4, as is also obvious from Fig. 1. This indicates a different function of PhaP1 and PhaP3 on the one hand and of PhaP2 and PhaP4 on the other. It should be emphasized that the amount of PhaP3 in the granule increased sharply if PhaP1 could not be synthesized, as in the *phaP1* mutants H2275 and Re1052, but with PhaP2 and PhaP4 this was not seen in these mutants.

These findings are new and unexpected, and should affect our current models of the structures of PHA granules (Steinbüchel *et al.*, 1995; Jurasek *et al.*, 2001; Jurasek & Marchessault 2002; Dennis *et al.*, 2003). Furthermore, these findings may also have a considerable impact on the optimization of production strains of *R. eutropha* and on the establishment of heterologous production systems for PHAs, such as genetically modified bacteria and transgenic plants, particularly if the modified organisms rely on *R. eutropha* PHA-biosynthesis genes. Extensive further studies are now required, to reveal the functions of the multiple phasins in *R. eutropha* and other bacteria.

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