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

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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, 20, 19 and 20 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 chemolithooautotrophic 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

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 butyrate) (poly(3HB)) and, in particular, poly(3-hydroxybutyrate-co-3-hydroxyvalerate) are currently produced by fermentation of glucose-utilizing mutants of R. eutropha 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 PHASCL 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; Lu¨ tke-Eversloh & Steinbu¨ chel, 2003). Both PHAs and PTEs 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 PHASCL 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 PHASCL-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 CaCl2 procedure (Hanahan, 1983).

Cloning of phaP2 and purification of recombinant Hiss-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_XbaI_rev as reverse primers (Table 1). They were deduced from the upstream and downstream regions, respectively, of phaP2 of R. eutropha H16 (Schwart et al., 2003). The phaP2His PCR product obtained was purified and ligated into pMA/c5-914 (Table 1), which harbours the c857ts gene encoding the temperature-sensitive λ repressor. The recombinant His6-tagged PhaP2 (N-terminal fusion) was purified from E. coli TOP10 harbouring pMa/c5-914: :phaP2His, Protein purification was carried out under non-denaturing conditions, employing Ni2+ -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 Pfu-DNA-polymerase (Invitrogen) and an OmMigen HBTTR3CM 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 1×109 cells in the stationary growth phase, which had been
cultivated for 72 h in MM under storage conditions (0-0.2%, 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 analysis was performed by automated Edman degradation. After 72 h incubation, cells of 200 ml cultures were harvested by centrifugation (2 h, 210 000 × g). 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 (Heukeshoven & 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

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

<table>
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<tr>
<th>Strain, plasmid or primer</th>
<th>Description</th>
<th>Reference, source or location</th>
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<td>H16</td>
<td>phaP1::Tn5 mutant derived from R. eutropha HF39, Tn5 insertion</td>
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<td>H2275</td>
<td>156 bp upstream of phaP1</td>
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<tr>
<td>Re1052</td>
<td>phaP1 precise-deletion gene replacement strain, derived from R. eutropha H16</td>
<td>York et al., 2001</td>
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<td><strong>Escherichia coli</strong></td>
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<td>Invitrogen</td>
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<td>TOP10</td>
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<td>This study</td>
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<tr>
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<td>5′-AAAAATCTAGACGGGCCGCAGTGCGCTGCGGACTA-3′</td>
<td>3′ Region of phaP2His6</td>
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</table>

**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 (100×10⁶ 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.
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 reloaded for 5 min with 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 with 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. I shows a bootstrap neighbour-joining of all confirmed and putative phasins identified so far. The resulting tree was rooted using the phasin-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; Gly-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 all 23 proteins.

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 and quantitatively. Cells were cultivated in MM containing 1·5 %
(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 demonstrated that DNA contamination did not contribute to RT-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 Tn5-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 [MSPFMPEQQAAV(Q)(K)(S)-(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

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

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<th>Accession no. or ORF no.</th>
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<th>Amino acid similarity (%)</th>
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*ORF identifier in the ERGO database.
†The strains Bordetella parapertussis 12822 and Bordetella bronchiseptica RB50 exhibit the same amino-acid sequence as Bordetella pertussis.
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.

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 Tn5-induced mutant H2275, which harbours Tn5 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 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.

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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

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**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.

**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.
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 Tn5-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 His6-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 His6-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 denaturing buffer. The granule sediments and supernatants of each washing step were analysed by SDS-PAGE (Fig. 5). The His6-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 phaG, the PHA synthase gene, were detected. Therefore, it is most likely that, in R. eutropha, only one PHA synthase protein exists, and that all PHASCL and PTESCL known to occur in this bacterium are synthesized by the same PHA synthase. This is consistent with the phenotypes of previously obtained single-insertion (Tn5-insertion) and spontaneously occurring point mutants (Schubert et al., 1988).
Furthermore, no homologues to \( \text{phaR} \), the repressor protein responsible for autoregulation of \( \text{PhaP1} \) expression (Pöttter et al., 2002), could be detected in the \( R. \text{eutropha} \) genome.

A Search for \( \text{phaZ} \) 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 \( \text{phaZ1}, \text{phaZ2} \) and \( \text{phaZ3} \) (Saegusa et al., 2001; York et al., 2003), which are localized on chromosome 1 (\( \text{phaZ1} \) and \( \text{phaZ2} \)) and chromosome 2 (\( \text{phaZ3} \)), and also the PHA depolymerase gene \( \text{phaZ4} \), which is localized on megaplasmid pHG1 (Schwartz et al., 2003), whereas the fifth gene was hitherto unknown and was localized on chromosome 1 (\( \text{phaZ5} \), 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 \( \text{phaZ} \) homologues of \( R. \text{eutropha} \).

**DISCUSSION**

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

![Fig. 4. 2D gel electrophoresis of granule-associated proteins of \( R. \text{eutropha} \) H16 (A) and \( R. \text{eutropha} \) 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.](image)

![Fig. 5. Binding of \( \text{His}_6 \)-tagged \( \text{PhaP2} \) to crystalline granules. Proteins were separated in 12.5%, w/v, SDS-polyacrylamide gels and stained with silver. The purified \( \text{His}_6 \)-tagged \( \text{PhaP2} \) protein (120 \( \mu \)g in 130 \( \mu \)L 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 \( \text{His}_6 \)-tagged \( \text{PhaP2} \) protein isolated from \( E. \text{coli} \) TOP10 harbouring pMa/c5-914::\( \text{phaP2} \)His\(_6\); 2, supernatant after first wash step; 3, supernatant after second wash step; 4, pellet with \( \text{His}_6 \)-tagged \( \text{PhaP2} \) protein after second wash step.](image)
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 PHASCL 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 PHASCL, and since the PHA biosynthesis genes of this bacterium are currently used to establish heterologous production systems for PHASCL, 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 PHASCL 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 PHASCL 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 large granule per cell, whereas cells of the wild-type possess several poly(3HB) granules. In a well-regulated process employing the autorepressor 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>
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<th>Characteristic</th>
<th>phaP1</th>
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<tr>
<td>Location</td>
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<td>Granule associated (in vitro)</td>
<td>Granule associated (in vivo)</td>
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</tr>
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*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 (41 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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REFERENCES


