Heat-shock protein HspA mimics the function of phasins *sensu stricto* in recombinant strains of *Escherichia coli* accumulating polythioesters or polyhydroxyalkanoates

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Polyhydroxyalkanoic acids (PHAs) are synthesized by unspecific PHA synthases and deposited as energy and carbon storage granules in the cytoplasm of many prokaryotes. The number and size of the granules depend on the presence of phasins which are amphiphilic structural proteins occurring at the granule surface. Recently, it was shown that polythioesters (PTEs) are also synthesized by PHA synthases. To increase the yield of these polymers, the role of recombinant phasins was analysed in an artificial PHA-producing *Escherichia coli* strain. Overexpressed PhaP1 from *Ralstonia eutropha* H16 affected poly(3-mercaptopropionate) [poly(3MP)] and poly(3-hydroxybutyrate) [poly(3HB)] accumulation in recombinant *E. coli*, which expressed the non-natural BPEC pathway consisting of butyrate kinase and phosphotransbutyrylase from *Clostridium acetobutylicum* and PHA synthase from *Thiococcus pfennigii*. For this, BPEC-carrying *E. coli* with and without *phaP1* was cultivated in presence of glucose as carbon source for growth plus 3-mercaptopropionate or 3-hydroxybutyrate as precursor substrates for poly(3MP) or poly(3HB) biosynthesis, respectively. In the presence of PhaP1, the recombinant *E. coli* produced about 50 or 68 % more poly(3MP) or poly(3HB), respectively. Therefore, coexpression of PhaP1 alongside the BPEC pathway is important for optimizing strains towards enhanced PHA or PTE production. Furthermore, in the absence of PhaP1, large amounts of the 16 kDa heat-shock protein HspA were synthesized and bound to the granule surface. Unusual small granules occurred in the cells of the recombinant *E. coli* strains. The diameter of the poly(3MP) granules was only 55 ± 12 nm or 105 ± 12 nm, and of the poly(3HB) granules only 56 ± 10 or 110 ± 22 nm in the presence or absence of PhaP1, respectively. This explains why no single granules capable of accumulating PHAs or PTEs occurred in the recombinant *E. coli*, unlike in PhaP1-negative mutants of *R. eutropha*. Obviously, HspA mimics the phasin, thereby preventing coalescence of granules into one single granule. However, the effect of PhaP1 on granule size and on amounts of accumulated polymers was more severe than that of HspA.

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INTRODUCTION

Polythioesters (PTE), consisting of various 3-mercaptopalkanoic acids, were shown to be a new class of biopolymers by Lütke-Eversloh *et al.* (2001a). PTEs are synthesized by polyhydroxyalkanoate (PHA) synthases, which are the key enzymes for biosynthesis of the polyoxoesters

poly(3-hydroxybutyrate) [poly(3HB)] or other PHAs (Taguchi & Doi, 2004) if bacteria are cultivated in the presence of organic thiochemicals yielding mercaptoacyl-coenzyme A thioesters by cell metabolism, thus indicating the low substrate specificity of these enzymes (Steinbüchel & Valentin, 1995). The Gram-negative bacterium *Ralstonia eutropha* accumulates copolymers of 3-hydroxybutyrate (3HB) and 3-mercaptopropionate (3MP), 3-mercaptopbutyrate (3MB) or 3-mercaptopvalerate (3MV) when the cells are cultivated under conditions permissive for PHA

Abbreviations: 3HB, 3-hydroxybutyric acid; 3MP, 3-mercaptopropionic acid; PHA, polyhydroxyalkanoate; PTE, polythioester.

biosynthesis in the presence of various thiochemicals (Lütke-Eversloh *et al.*, 2001a, b; Lütke-Eversloh & Steinbüchel, 2003). A recombinant strain of *Escherichia coli* expressing the non-natural BPEC pathway, relying on the enzymes butyrate kinase (Buk) and phosphotransbutyrylase (Ptb) from *Clostridium acetobutylicum* plus a two-component PHA synthase (PhaEC) from non-oxygenic photosynthetic bacteria like *Allochrochromatium vinosum* or *Thiococcus pfennigii* (Liu & Steinbüchel, 2000a, b), synthesized poly(3MP), poly(3MB) or poly(3MV) homopolymers when cultivated in the presence of the respective 3-mercaptoalkanoic acids (Lütke-Eversloh *et al.*, 2002a). In addition, PTEs were also synthesized *in vitro*, employing the *Candida antarctica* lipase (Iwata *et al.*, 2003; Kato *et al.*, 2005).

PTEs exhibit interesting physical and biological properties. Thermal properties such as the melting point temperature and glass transition temperatures deviate significantly from those of the corresponding polyoxoester analogues (Lütke-Eversloh *et al.*, 2002a, b; Kawada *et al.*, 2003; Tanaka *et al.*, 2004). For example, the melting point temperature (T_m) increases from 121 °C in poly(3HB) to 170 °C in poly(3MP). The PTE homopolymer poly(3MP) has recently become available in sufficient quantities by up-scaling the BPEC process to the 500 l scale and has been used for biodegradation studies (Thakor *et al.*, 2005). Surprisingly, it turned out that poly(3MP) is non-biodegradable (Elbanna *et al.*, 2004; Kim *et al.*, 2005).

Transmission electron microscopic studies of thin sections of cells of the recombinant *E. coli* strain producing poly(3MP) show a large number of unusually small granules (Lütke-Eversloh *et al.*, 2002a) in comparison to granules occurring, for example, in *R. eutropha*. This was surprising because the PTE biosynthesis pathway was expressed in the absence of a phasin protein. Phasins represent a class of small, amphiphilic structural proteins that bind to the surface of PHA granules and cover most of the surface (Wieczorek *et al.*, 1995). These amphiphilic proteins constitute a boundary layer between the hydrophobic surface of the PHA granules owing to the amorphous hydrophobic polyester molecules and the mostly hydrophilic constituents of the cytoplasm. Thus they stabilize the 'PHA in water dispersion' in the cytoplasm, thereby yielding distinct, non-coalescing granules (Steinbüchel *et al.*, 1995; Pötter & Steinbüchel, 2005). *R. eutropha* synthesizes four homologous phasin proteins (PhaP1, PhaP2, PhaP3 and PhaP4) with PhaP1 being the most abundant protein constituting about 3–5 % (w/w) of the total cell protein if the cells contain large amounts of PHAs (Wieczorek *et al.*, 1995; Pötter *et al.*, 2004). The number and size of PHA granules in cells depend very much on the presence of phasin proteins. In most cells of a *phaP1* mutant of *R. eutropha*, only one single large and oval granule (length up to 2 µm) is seen, which occupies almost the entire cytoplasm, whereas wild-type cells accumulate several round granules of medium size (0.2–0.5 µm diam.). Furthermore, *R. eutropha* cells

harbouring several copies of the *phaP1* gene contain a much larger number of granules of small size. These findings provide evidence for the hypothesis of the 'PHA in water emulsion'-stabilizing effect of phasins.

Since PTE and PHA molecules are not very different with regard to the hydrophobicity of the polymer molecules, the extraordinary small size of PTE granules in recombinant *E. coli* cells is not consistent with the hypothesis mentioned above. It indicates that other proteins could override the function of phasins in *E. coli*. Therefore, we investigated the proteins associated with PTE granules in recombinant *E. coli* strains expressing the BPEC pathway in the absence and presence of PhaP1 and of other *R. eutropha* phasins under conditions permissive for poly(3MP) or poly(3HB) biosynthesis and accumulation.

METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. *E. coli* was cultivated at 37 °C in 500 ml M9 mineral salts medium (Sambrook *et al.*, 1989) supplemented with 0.01 % (w/v) yeast extract in 2 l Erlenmeyer flasks with baffles on a rotary shaker at 125 r.p.m. As carbon source, 1.0 % (w/v) glucose was added from a 20 % (w/v) filter-sterilized stock solution. After 12 h, 3-mercaptopropionic acid (99 %; Acros Organics) or sodium 3-hydroxybutyrate (Sigma-Aldrich Chemie) was added to the medium as the precursor substrate for poly(3MP) or poly(3HB) synthesis, respectively, to a concentration of 0.2 % (v/v or w/v, respectively). In addition, 1 mM IPTG was added to induce transcription of *phaP1* on pCDFDuet-1. To maintain the plasmids, 75 µg ampicillin ml⁻¹ was added to *E. coli* BL21 (DE3)/pBPP1 and 75 µg ampicillin ml⁻¹ plus 50 µg streptomycin ml⁻¹ to *E. coli* BL21 (DE3)/pBPP1 + pCDFDuet-1::*phaP1*.

Cloning of *phaP1*. For cloning of *phaP1* into *E. coli*, PCR was done by using *phaP1*_*NcoI*_IV (5'-CCAGCCATGGTCCTCAC-CCCGGAACAAGTTGC-3') as sense and *phaP1*(rev)_C_*HindIII* (5'-GCAGAAGCTTATCAGGCAGCCGTCGTCTTC-3') as reverse primers, thereby introducing an *NcoI* restriction site in the upstream region and a *HindIII* restriction site in the downstream region of the gene, respectively. The PCR amplification of DNA was carried out as described by Sambrook *et al.* (1989), employing *Pfx* DNA polymerase (Invitrogen) and an Omnigene HBTR3CM DNA thermal cycler (Hybaid). By using the Perfectprep Gel Cleanup Kit (Eppendorf), following the instructions described in the enclosed manual, the 579 bp fragment was purified from an agarose gel. The fragment was then digested with *NcoI* and *HindIII*, and was ligated into *NcoI*- and *HindIII*-digested pCDFDuet-1 vector DNA (Novagen), yielding pCDFDuet-1::*phaP1*.

Transfer of DNA. Competent *E. coli* cells were prepared and transformed by the CaCl₂ procedure as described by Hanahan (1983).

Analysis of PHAs and PTEs. The polymer contents of the cells were determined upon methanolysis of 5–10 mg lyophilized cells in the presence of 85 % (v/v) methanol and 15 % (v/v) sulfuric acid. The resulting methyl esters of 3-HB and 3-MP were analysed by GC as described by Brandl *et al.* (1988) and Timm & Steinbüchel (1990).

Isolation of native PHA and PTE granules. For the examination of granule-associated proteins, poly(3HB) and poly(3MP) granules were isolated by a modification of the method of Preusting *et al.* (1993) from *E. coli* cells which had been grown in M9 medium.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
<i>Ralstonia eutropha</i> H16	Wild-type	DSM 428
<i>Escherichia coli</i> BL21 (DE3)	F [−] <i>ompT hsdSB</i> (r _B [−] , m _B [−]) <i>gal dcm</i> (DE3)	Novagen
Plasmids		
pBPP1	Ap ^r <i>buk</i> ⁺ <i>ptb</i> ⁺ <i>phaEC</i>	Liu & Steinbüchel (2000b)
pCDFDuet-1	Sm ^r	Novagen
pCDFDuet-1:: <i>phaP1</i>	Sm ^r <i>phaP1</i> ⁺	This study

Samples (50 ml) were withdrawn from the medium after 18, 24, 30 and 36 h incubation. The cells were harvested by centrifugation (20 min, 6000 g, 4 °C), washed in 100 mM Tris/HCl buffer (pH 8.0) and then suspended in 2 ml 100 mM Tris/HCl buffer (pH 8.0). After a threefold passage through a French press (100 × 10⁶ Pa), the lysate was loaded on the top of a glycerol gradient. The gradient used for isolation of poly(3HB) granules was obtained from a discontinuous gradient prepared from 4 ml 90 % (v/v) plus 4 ml 60 % (v/v) glycerol in 100 mM Tris/HCl buffer (pH 8.0). The gradient for isolation of poly(3MP) granules was prepared from 3 ml 90 % (v/v), 3 ml 80 % (v/v) plus 3 ml 60 % (v/v) in 100 mM Tris/HCl buffer (pH 8.0). After centrifugation (1 h, 100 000 g, 4 °C), a granule layer of poly(3HB) was obtained at about 90 % (v/v) glycerol and a layer of poly(3MP) at about 80 % (v/v) glycerol. The granules were isolated from the gradients and then washed three times with Tris/HCl buffer (pH 8.0) by centrifugation (15 min, 16 100 g, 4 °C). The granules were stored at −20 °C for further analyses.

One-dimensional PAGE. Protein samples were resuspended in gel loading buffer (0.6 %, w/v, SDS; 1.25 %, v/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). The proteins were stained with Coomassie brilliant blue R-250 (Weber & Osborn, 1969). Samples of crude extracts and of the native isolated granules were examined by this method.

Analysis of granule-associated proteins by MALDI-TOF MS. Spots were excised from the PAGE gels, destained and washed using a slightly modified procedure to that described by Koltzschner *et al.* (2003). Proteins were typically digested in the gel, and peptides were extracted and C18-purified for MALDI-TOF MS. Peptide masses were measured using ToFSpec-2E (Waters/Micromass). Database searches were performed with the Mascot engine in-house (Matrix Science) on Swiss-Prot, specifying *E. coli* proteins.

Electron microscopy studies. To obtain transmission electron micrographs (TEM), cells were fixed with 2.5 % (v/v) glutaraldehyde in 0.1 M PBS (pH 7.3) immediately after they were withdrawn from the cultivation vessels. After three washing steps with 0.1 M PBS each for 20 min, the cells were post-fixed in 1 % (w/v) osmium tetroxide in 0.1 M PBS (pH 7.3) and washed once with the same PBS for 20 min. Then water was removed by a graded water/ethanol series (30, 50, 70, 90, 96 %, v/v, ethanol in water and absolute ethanol as final step), each step lasting for about 15 min. The following preparation steps were made according to the specific requirements of the microscopic method used. For thin sectioning, the samples were embedded in SPURR resin (without propylene oxide) (Spurr, 1969). Sections with a thickness of 70–80 nm were made with an Ultracut apparatus (Leica Mikroskopie und Systeme) using a diamond knife and were then positioned on a 200 mesh copper grid.

Imaging was performed with an H-500 transmission electron microscope (Hitachi) in the bright-field mode at 75 kV acceleration voltage and at room temperature. Photographs were taken on Agfa-Gevaert 23 D 56 films.

RESULTS AND DISSCUSION

Construction of plasmids for coexpression of the BPEC pathway and phasin PhaP1

The major phasin PhaP1 influences the accumulation of poly(3HB) in *R. eutropha* H16 (Pötter *et al.*, 2004). To analyse the impact of PhaP1 on the accumulation of poly(3MP) in *E. coli*, we combined the genes encoding the non-natural BPEC pathway with *phaP1*. Moreover, the BPEC pathway was used to produce poly(3HB) in the presence and absence of PhaP1. For this, *phaP1* was inserted into pCDFDuet-1, as described in Methods, yielding pCDFDuet-1::*phaP1* (Fig. 1a). The genes for the BPEC pathway are encoded on the 11.96 kbp vector pBPP1 (Fig. 1b). Because of the compatibility of the two plasmids, they could both be transferred to and maintained in *E. coli* BL21 (DE3). In addition, *E. coli* BL21 (DE3) was transformed with pBPP1 only as a negative control.

Influence of PhaP1 on accumulation of poly(3MP) and poly(3HB)

R. eutropha H16 cells, defective in *phaP1*, produce less poly(3HB) than the wild-type (Wieczorek *et al.*, 1995) and exhibit a PHA-leaky phenotype. Therefore, coexpression of PhaP1 in recombinant strains of *E. coli* capable of accumulating PTEs due to the expression of a PHA biosynthesis pathway might yield greater PTE content in the cells. The effect of *phaP1* on poly(3HB) and poly(3MP) accumulation in *E. coli* cells expressing the non-natural BPEC pathway has not been investigated yet. To analyse the influence of PhaP1 from *R. eutropha* H16 on the accumulation of poly(3MP) and poly(3HB) in recombinant strains of *E. coli* expressing the non-natural BPEC pathway, *E. coli* BL21 (DE3)/pBPP1 and *E. coli* BL21 (DE3)/pBPP1+pCDFDuet-1::*phaP1* were cultivated in M9 medium containing glucose. After 12 h cultivation, 3MP or 3HB was added and served as precursor substrates for

poly(3MP) or poly(3HB) biosynthesis, respectively. After induction of expression of *phaP1* by adding IPTG after 12 h, the cells were then further cultivated for 24 h. To obtain reliable results, all cultivations were done in duplicate. The growth behaviour of phasin-positive and -negative cultures was similar (Figs 2 and 3) and after about 18 h of cultivation, the cells reached stationary phase. Samples were withdrawn every 6 h and the poly(3HB) and poly(3MP) contents of the cells were analysed.

Coexpression of PhaP1 exerted a clear positive effect on poly(3MP) and poly(3HB) accumulation in *E. coli* BL21 (DE3)/pBPP1 + pCDFDuet-1::*phaP1* when the cells were cultivated in the presence of 3MP or 3HB, respectively. It should be noted that the cells only accumulated the respective homopolymer as shown in previous studies (Lütke-Eversloh *et al.*, 2002a; Liu & Steinbüchel, 2000b). In

Fig. 2. Growth of *E. coli* BL21 (DE3)/pBPP1 and *E. coli* BL21 (DE3)/pBPP1 + pCDFDuet-1::*phaP1* in the presence of glucose plus 3-mercaptopropionic acid and accumulation of poly(3MP) in the cells. The composition of the medium and the cultivation conditions were as described in Methods. The arrow marks the time of IPTG (1 mM) and 3MP (0.2 %, v/v) addition. After 18, 24, 30 and 36 h, samples were withdrawn and poly(3MP) contents were analysed by GC. Squares and black bars represent growth behaviour and poly(3MP) content, respectively, of *E. coli* BL21 (DE3)/pBPP1 + pCDFDuet-1::*phaP1*. Triangles and white bars represent growth behaviour and poly(3MP) content, respectively, of *E. coli* BL21 (DE3)/pBPP1.

Fig. 1. Physical maps of plasmids pCDFDuet-1::*phaP1* (a) and pBPP1 (b).

E. coli BL21 (DE3)/pBPP1 + pCDFDuet-1::*phaP1* cells, poly(3MP) contributed up to about 30 % (w/w) of the cell dry matter, whereas the poly(3MP) content of *E. coli* BL21 (DE3)/pBPP1 cells was only about 20 % (w/w) of the cell dry matter (Fig. 2). The situation was similar when the cells were cultivated in the presence of 3HB. Whereas *E. coli* BL21 (DE3)/pBPP1 + pCDFDuet-1::*phaP1* cells accumulated poly(3HB) up to 27 % (w/w) of cell dry matter, this polymer amounted to only 17 % (w/w) of cell dry matter in *E. coli* BL21 (DE3)/pBPP1 cells (Fig. 3).

The results of these experiments clearly demonstrated that the major phasin PhaP1 of *R. eutropha* H16 exerts not only a positive effect on PHA accumulation in its own cells, but also if a PHA biosynthesis pathway is expressed in *E. coli*. The amounts of poly(3HB) synthesized by *R. eutropha* strains lacking intact PhaP by deletion of the gene or by Tn5 insertion are decreased by about 50 % compared to the wild-type (Wieczorek *et al.*, 1995; York *et al.*, 2002). It may be expected that similar positive effects also occur in other recombinant organisms expressing a PHA biosynthesis pathway. Moreover, this effect of PhaP1 is not restricted to biosynthesis and accumulation of polyoxoesters like poly(3HB), but also occurs with PTEs like poly(3MP) as clearly shown in this study. The positive effect of PhaP1 on polymer accumulation is significant, and the poly(3MP) and poly(3HB) contents of the cells could be increased by about 50 or 68 %, respectively. Both findings will be important for the optimization of strains suitable for biotechnological production of PHAs and PTEs.

Fig. 3. Growth of *E. coli* BL21 (DE3)/pBPP1 and *E. coli* BL21 (DE3)/pBPP1+pCDFDuet-1::phaP1 in the presence of glucose plus sodium 3-hydroxybutyrate and accumulation of poly(3HB) in the cells. The composition of the medium and the cultivation conditions were as described in Methods. The arrow marks the time of IPTG (1 mM) and 3HB (0.2%, w/v) addition. After 18, 24, 30 and 36 h, samples were withdrawn and poly(3HB) contents were analysed by GC. Squares and black bars represent growth behaviour and poly(3HB) content, respectively, of *E. coli* BL21 (DE3)/pBPP1+pCDFDuet-1::phaP1. Triangles and white bars represent growth behaviour and poly(3HB) content, respectively, of *E. coli* BL21 (DE3)/pBPP1.

These results are in line with other studies, in which a positive effect of phasins on PHA biosynthesis and accumulation was also observed. Studies with the purified PhaEC of *Allochrodatum vinosum* have demonstrated that the amount of *in vitro*-synthesized poly(3HB) can be significantly increased by the addition of purified PhaP1 (Jossek *et al.*, 1998). Studies with the purified PhaC2 of *Pseudomonas aeruginosa* have shown that PhaP1 from *R. eutropha* increases the activity of the PHA synthase by about 50% (Qi *et al.*, 2000). It is possible that PhaP1 has the same influence on the activity of the PHA synthase PhaEC during poly(3MP) accumulation. Experiments with a recombinant strain of *E. coli* harbouring a plasmid encoding the *phbCAB* operon have shown that the accumulation of poly(3HB) can be increased from about 16 to 57% of the cell dry matter when PhaP1 is also expressed (Seo *et al.*, 2003). So far, unfortunately, a positive effect of phasins on PHA accumulation has not been demonstrated in transgenic plants. In transgenic *Arabidopsis thaliana*, coexpression of PhaP1 alongside PHB biosynthesis genes neither increases the polymer content nor alleviates the negative effect of expression of PHB biosynthesis on growth and development (Bohmert *et al.*, 2002). To our knowledge, similar coexpression experiments have so far not been done in other transgenic plants.

Analysis of poly(3HB) and poly(3MP) granule-associated proteins

The protein patterns of crude extracts as well as those of isolated granules were analysed by PAGE in the same

samples of *E. coli* BL21 (DE3)/pBPP1 and *E. coli* BL21 (DE3)/pBPP1+pCDFDuet-1::phaP1 cells cultivated in the presence of 3MP or 3HB that were analysed for their polymer contents (see above). The expression of PhaP1 could be demonstrated in cells of *E. coli* BL21 (DE3)/pBPP1+pCDFDuet-1::phaP1. The electropherograms of crude extracts of cells of *E. coli* BL21 (DE3)/pBPP1+pCDFDuet-1::phaP1, which were cultivated in the presence of 3MP, showed abundant amounts of a protein with an apparent molecular mass of 22 kDa representing PhaP1 (Fig. 4a). Similarly, crude extracts from cells of the same strain, which were cultivated in the presence of 3HB, showed a protein of identical size (Fig. 5a). This protein band was absent in crude extracts prepared from cells of *E. coli* BL21 (DE3)/pBPP1. Surprisingly, however, a protein exhibiting an apparent molecular mass of about 16 kDa occurred in the electropherograms of all crude extracts prepared from this *E. coli* strain which expressed the BPEC pathway but lacked phaP1, irrespective of whether the cells were cultivated in the presence of 3MP (Fig. 4a) or 3HB (Fig. 5a), respectively.

When the proteins from isolated poly(3MP) or poly(3HB) granules were separated by PAGE, either the phasin protein or the 16 kDa protein occurred in the electropherograms depending on whether the granules were isolated from cells of *E. coli* BL21 (DE3)/pBPP1+pCDFDuet-1::phaP1 or *E. coli* BL21 (DE3)/pBPP1 as shown in Figs 4(b) and 5(b), respectively. This demonstrated that, in the absence of phasin PhaP1, this 16 kDa protein is expressed in *E. coli* and that it binds to the granules in the cytoplasm. MALDI-TOF analysis identified this 16 kDa protein unequivocally as the small heat-shock protein HspA (accession no. NP_756468) of *E. coli* CFT073. HspA is identical with the small heat-shock protein IbpA (accession no. P0C054) from *E. coli*. IbpA is expressed in *E. coli* in combination with another heat-shock protein, IbpB (accession no. P0C058), in situations where the cells are exposed to unfavourable conditions, for example during the overproduction of recombinant protein when it binds to inclusion bodies produced from recombinant proteins (Allen *et al.*, 1992). The genes *ibpA* and *ibpB* constitute an operon, and the encoded proteins occur in complexes (Kuczyńska-Wisnik *et al.*, 2002). IbpB is stimulated by IbpA to associate with a substrate (Matuszewska *et al.*, 2005). While expressing recombinant proteins, *E. coli* synthesizes large amounts of IbpA and IbpB, and the proteins are bound to the inclusion bodies (Han *et al.*, 2004). Interestingly, expression of these proteins to a detectable level and binding to the granules did not occur in *E. coli* expressing PhaP1. Obviously, HspA seems to act like a phasin and binds to the PHA and PTE granules yielding increased amounts of either polymer in the cells. The electropherograms indicate that, irrespective of whether *E. coli* BL21 (DE3)/pBPP1 cells were cultivated in the presence of 3MP or 3HB, they produce larger quantities of HspA than *E. coli* BL21 (DE3)/pBPP1+pCDFDuet-1::phaP1 cells produce PhaP1. Therefore, HspA is very abundant in the phasin-negative strains of *E. coli*. In recent

Fig. 4. Crude extracts (a) and isolated granules (b) of *E. coli* BL21 (DE3)/pBPP1 and *E. coli* BL21 (DE3)/pBPP1+pCDFDuet-1::*phaP1* cultivated with glucose as carbon source and 3MP as precursor substrate. IPTG (1 mM) and 3MP (0.2%, v/v) were added after 12 h of growth. Samples were taken before and after induction at different times. Proteins were separated in 12.5% (w/v) SDS-polyacrylamide gels and stained with Coomassie brilliant blue. (a) Lanes: C1 and C2, negative controls of *E. coli* BL21 (DE3)/pBPP1 and *E. coli* BL21 (DE3)/pBPP1+pCDFDuet-1::*phaP1*, respectively, before induction of PhaP1; subsequent lanes, *E. coli* BL21 (DE3)/pBPP1 (-) and *E. coli* BL21 (DE3)/pBPP1+pCDFDuet-1::*phaP1* (+) withdrawn at the times indicated (18, 24, 30 or 36 h, respectively). (b) Poly(3MP) granules from cells of *E. coli* BL21 (DE3)/pBPP1 (-) or *E. coli* BL21 (DE3)/pBPP1+pCDFDuet-1::*phaP1* (+) withdrawn at the times indicated (18, 24, 30 or 36 h, respectively).

studies, other larger heat-shock proteins, DnaK and GroEL/ES, have been detected in *R. eutropha* as granule-associated proteins when *phaP1* was deleted (Pötter *et al.*, 2004). These heat-shock proteins were also found in recombinant *E. coli* expressing the *phbCAB* operon (Han *et al.*, 2001).

Electron microscopy studies

It is assumed that phasins bind to PHA granules not only to stabilize the dispersion of the hydrophobic polymer in the hydrophilic cytoplasm, thereby preventing individual granules from coalescing to a few granules or even a single large granule as observed in a *phaP1* mutant in *R. eutropha* (Wieczorek *et al.*, 1995), but probably also to prevent the unspecific binding of other proteins to the large surface of the granules, thereby avoiding misrouting of proteins. This will protect the cells from various forms of stress. Previously, it was shown, surprisingly, that recombinant *E. coli* cells which accumulated poly(3MP), deposit the accumulated polymer in a large number of very small granules, although the cells lack a phasin (Lütke-Eversloh *et al.*, 2002a; Lütke-Eversloh & Steinbüchel, 2004). TEM images obtained in this study show that poly(3MP) granules isolated from *E. coli* BL21 (DE3)/pBPP1+pCDFDuet-1::*phaP1* were even smaller (Fig. 6c, d) than those isolated from cells of the phasin-negative strain (Fig. 6a, b). The mean size of the poly(3MP) granules was only 55 ± 12 nm in cells of *E. coli* BL21 (DE3)/pBPP1+pCDFDuet-1::*phaP1* in comparison to the mean size of 105 ± 12 nm in cells of *E. coli* BL21 (DE3)/pBPP1. Similarly, the mean size of poly(3HB) granules that accumulated in *E. coli* BL21 (DE3)/pBPP1+pCDFDuet-1::*phaP1* was 56 ± 10 nm (Fig. 7a, b), whereas the mean size of the poly(3HB) granules in the phasin-negative strain was 110 ± 22 nm (Fig. 7c, d). This indicates that HspA could act like a phasin and, due to the large amounts of this heat-shock protein, coalescence of individual granules is widely, although not completely, prevented in the absence of PhaP1. Mutants with a defect in HspA will probably accumulate much larger granules or even just one single granule if, instead of HspA, the formation of a further protein which compensates for the loss of HspA is not induced.

Conclusions

It was previously shown that proteins not related to PHA metabolism or PHA granule structure bind to the surface of PHA granules. Examples are lysozyme (Liebergesell & Steinbüchel, 1992) and bovine serum albumin (Horowitz & Sanders, 1995). Analysis of the PHA granule proteome by two-dimensional gel electrophoresis and MALDI-TOF analysis revealed the presence of homologues to the heat-shock proteins DnaK and GroEL, and also the β -ketothiolase Bkt in PHA granules isolated from cells of a phasin-negative mutant of *R. eutropha* (Pötter *et al.*, 2004). However, the latter proteins were not bound to the granules of the wild-type, thus indicating that their binding is prevented by the phasin. In a study by Han *et al.* (2001), it was shown that the expression of the heat-shock proteins

Fig. 5. Crude extracts (a) and isolated granules (b) of *E. coli* BL21 (DE3)/pBPP1 and *E. coli* BL21 (DE3)/pBPP1+pCDFDuet-1::phaP1 cultivated with glucose as carbon source and 3HB as precursor substrate. IPTG (1 mM) and 3HB (0.2%, w/v) were added after 12 h of growth. Samples were taken before and after induction at different times. Proteins were separated in 12.5% (w/v) SDS-polyacrylamide gels and stained with Coomassie brilliant blue. (a) Lanes: C1 and C2, negative controls of *E. coli* BL21 (DE3)/pBPP1 and *E. coli* BL21 (DE3)/pBPP1+pCDFDuet-1::phaP1, respectively, before induction of PhaP1; subsequent lanes, *E. coli* BL21 (DE3)/pBPP1 (–) and *E. coli* BL21 (DE3)/pBPP1+pCDFDuet-1::phaP1 (+) withdrawn at the times indicated (18, 24, 30 or 36 h, respectively). (b) Poly(3HB) granules from cells of *E. coli* BL21 (DE3)/pBPP1 (–) and *E. coli* BL21 (DE3)/pBPP1+pCDFDuet-1::phaP1 (+) withdrawn at the times indicated in the figure (18, 24 or 30 h, respectively).

small heat-shock proteins to reduce stress by binding these proteins to recombinant expressed inclusion bodies. However, the influence of HspA is not equal to the influence of PhaP1 on poly(3HB) accumulation in *R. eutropha* H16, which can be observed in the different amounts of accumulated polymer. Because of the physical properties of PTEs and with regard to their industrial use, for example in medical or technical applications, these

Fig. 6. TEM of poly(3MP)-accumulating cells of recombinant strains of *E. coli* BL21 (DE3) expressing the BPEC pathway in the absence or presence of PhaP1. *E. coli* BL21 (DE3)/pBPP1 cells are shown in (a) and (b), whereas *E. coli* BL21 (DE3)/pBPP1+pCDFDuet-1::phaP1 cells are shown in (c) and (d). Cells were cultivated in M9 medium containing 1% glucose (w/v) plus 0.2% 3MP (v/v) and were harvested in the stationary phase after 24 h growth. Thin sections were prepared and electron micrographs were obtained as described in Methods. Bars, 0.2 μm.

GroEL, GroES and DnaK was significantly up-regulated in a recombinant strain of *E. coli* expressing the *Alcaligenes latus* PHA biosynthesis genes; however, these proteins were detected only in crude extracts and no efforts were undertaken to analyse the PHA granule proteome in this *E. coli* strain. It has also been shown that the presence of these unspecifically bound proteins enhances the specific activity of the PHA synthase *in vitro* (Jossek *et al.*, 1998). This and other studies indicate that the expression of PHA biosynthesis in *E. coli* causes stress to the cells and that significant changes occur in the proteome. *E. coli* synthesizes

Fig. 7. TEM of poly(3HB)-accumulating cells of recombinant strains of *E. coli* BL21 (DE3) expressing the BPEC pathway in the absence or presence of PhaP1 after cultivation in the presence of glucose and 3HB. *E. coli* BL21 (DE3)/pBPP1 cells are shown in (a) and (b), whereas *E. coli* BL21 (DE3)/pBPP1+pCDFDuet-1::*phaP1* cells are shown in (c) and (d). Cells were cultivated in M9 medium containing 1% glucose (w/v) plus 0.2% 3HB (w/v) and were harvested in the stationary phase after 24 h growth. Thin sections were prepared and electron micrographs were obtained as described in Methods. Bars, 0.2 μ m.

findings will be important for optimization of the biotechnological production of PTEs.

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