Impact of *Ralstonia eutropha*’s Poly(3-Hydroxybutyrate) (PHB) Depolymerases and Phasins on PHB Storage in Recombinant *Escherichia coli*

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The model organism for polyhydroxybutyrate (PHB) biosynthesis, *Ralstonia eutropha* H16, possesses multiple isoenzymes of granules coating phasins as well as of PHB depolymerases, which degrade accumulated PHB under conditions of carbon limitation. In this study, recombinant *Escherichia coli* BL21(DE3) strains were used to study the impact of selected PHB depolymerases of *R. eutropha* H16 on the growth behavior and on the amount of accumulated PHB in the absence or presence of phasins. For this purpose, 20 recombinant *E. coli* BL21(DE3) strains were constructed, which harbored a plasmid carrying the phaCAB operon from *R. eutropha* H16 to ensure PHB synthesis and a second plasmid carrying different combinations of the genes encoding a phasin and a PHB depolymerase from *R. eutropha* H16. It is shown in this study that the growth behavior of the respective recombinant *E. coli* strains was barely affected by the overexpression of the phasin and PHB depolymerase genes. However, the impact on the PHB contents was significantly greater. The strains expressing the genes of the PHB depolymerases PhaZ1, PhaZ2, PhaZ3, and PhaZ7 showed 35% to 94% lower PHB contents after 30 h of cultivation than the control strain. The strain harboring phaZ7 reached by far the lowest content of accumulated PHB (only 2.0% [wt/wt] PHB of cell dry weight). Furthermore, coexpression of phasins in addition to the PHB depolymerases influenced the amount of PHB stored in cells of the respective strains. It was shown that the phasins PhaP1, PhaP2, and PhaP4 are not substitutable without an impact on the amount of stored PHB. In particular, the phasins PhaP2 and PhaP4 seemed to limit the degradation of PHB by the PHB depolymerases PhaZ2, PhaZ3, and PhaZ7, whereas almost no influence of the different phasins was observed if phaZ1 was coexpressed. This study represents an extensive analysis of the impact of PHB depolymerases and phasins on PHB accumulation and provides a deeper insight into the complex interplay of these enzymes.

Polyhydroxyalkanoates (PHAs) are storage compounds that are accumulated by a diverse range of microorganisms under unbalanced growth conditions (excess of a carbon source with simultaneous depletion of an essential growth element) (1). PHAs are of great interest for industry due to their physical properties, which are similar to those of industrially produced plastics, and also due to their biodegradability. They have been classified by the length of the carbon chain of the monomers as short-chain-length PHAs (PHA<sub>accl</sub>S) (2 to 5 carbon atoms), medium-chain-length PHAs (PHA<sub>accl</sub>S) (6 to 16 carbon atoms), and long-chain-length PHAs (PHA<sub>accl</sub>S) (>16 carbon atoms) (2). The most prominent PHA-accumulating organism is the Gram-negative, facultative chemolithoautotrophic bacterium *Ralstonia eutropha* H16. It was isolated and characterized in 1961, and it was found to accumulate up to 90% PHA<sub>accl</sub> poly(3-hydroxybutyrate) (PHB) of cell dry weight (CDW) (3–5).

For the synthesis of PHB in *R. eutropha* H16, three enzymes are necessary: the β-ketothiolase (PhaA), the NADPH-dependent acetoacetyl coenzyme A (acetoacetyl-CoA) reductase (PhaB1), and the PHB synthase (PhaC1) (6–8). PhaA and PhaB1 catalyze the condensation of two molecules of acetyl-CoA to acetoacetyl-CoA and the reduction of acetoacetyl-CoA to 3-hydroxybutyryl-CoA (3HB-CoA), respectively. PhaC1 polymerizes the 3HB-CoA monomers to PHB, whereas one molecule of CoA per monomer is released. The resulting PHB is stored as water-insoluble granules in the cytoplasm of the cells.

The surface of these granules is coated with phospholipids and many proteins, which are referred to as PHB granule-associated proteins (PGAPs) (9, 10). The phasins represent the predominant protein class of the PGAPs, with PhaP1 accounting for the greatest share of the protein amount. For a long time, only four different phasins were known (11, 12), but recently, the existence of three additional phasins (PhaP5 to PhaP7) was discovered (10, 13). A catalytic function of the seven phasins has remained unknown so far, although a strong influence of PhaP1 on the size and amount of PHB granules (11, 14, 15) and of PhaP5 on the location of the granules in *R. eutropha* H16 has been observed (16).

The PHB synthase PhaC1 (16), the regulator PhaR (17), and the recently discovered activator PhaM of the PHB synthase (18) were also bound to the surface of PHB granules. Another class of PGAPs is represented by the intracellular PHB depolymerases. *R. eutropha* H16 possesses seven intracellular PHB depolymerase isoenzymes (PhaZ1 to PhaZ7) and two PHB oligomer hydrolases (PhaY1 and PhaY2) for the intracellular degradation of PHB (19–24). Only the PHB depolymerases PhaZ1 and PhaZ6 were found

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to be attached to the surface of PHB granules (25, 26). Binding of the other isoenzymes is predicted but has not yet been shown. The mechanism of intracellular PHB degradation and the precise function of all PHB depolymerase isoenzymes or a putative influence of other PGAPs are not yet fully understood. A study of the interaction of PGAPs utilizing in vitro two-hybrid assay revealed strong interactions of the phasins PhaP1 to PhaP5. Weak interactions between the PHB depolymerase PhaZ1 and the phasins, in particular PhaP2 and PhaP3, were demonstrated (10). The other PHB depolymerase isoenzymes were not investigated in that study.

In the past years, several PHB depolymerase deletion strains of R. eutropha H16 were generated to elucidate the importance of the respective intracellular PHB depolymerases for PHB degradation (20, 22, 23, 26, 27). All single mutants of R. eutropha H16 generated and investigated so far were still able to degrade PHB granules, although some of them showed a significant reduction of PHB utilization. The other isoenzymes are capable of replacing the mutated and investigated so far were still able to degrade PHB granules, although some of them showed a significant reduction of PHB utilization. The other isoenzymes are capable of replacing the missing PHB depolymerase at least partly. Only the deletion of PHB utilization. The other isoenzymes are capable of replacing the

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** The bacterial strains used in this study are listed in Table 1. R. eutropha H16 was grown in nutrient broth (NB) at 30°C. E. coli strains were grown in lysogeny broth (LB) at 37°C (30). For growth analysis, E. coli strains were cultivated in 600 ml mineral salts medium (MSM) (4) with a reduced ammonium chloride concentration (0.05%, wt/vol), providing conditions permissive for PHB accumulation, and supplemented with 1% (wt/vol) glucose at 30°C for 3 h in 2-liter Erlenmeyer flasks with baffles on a rotary shaker with agitation at 120 rpm. Growth of cells was measured photometrically in a Klett-Summerson photometer (Manostat Corporation, NY, USA), using filter no. 54120 rpm. Growth of cells was measured photometrically in a Klett-Summerson photometer (Manostat Corporation, NY, USA), using filter no. 54.

**DNA sequencing.** The correct insertion of the phasin and PHB depolymerase genes into pETDuet-1 was verified by DNA sequencing of MCS-1 and MCS-2 of all constructed plasmids. Sequencing reactions were carried out by Seqlab (Sequence Laboratories Göttingen, Göttingen, Germany) according to standard procedures and analyzed with Chromas software (ver. 1.43; School of Health Science, Griffith University, Queensland, Australia).

**SDS-polyacrylamide gel electrophoresis (PAGE).** Samples were taken after induction of gene expression during the exponential growth phase. E. coli cells were disrupted by sonication and centrifuged for 10 min at 16,000 × g to separate the soluble from the insoluble protein fraction. The protein samples were resuspended in loading buffer (0.6% [wt/vol] SDS, 1.25% [wt/vol] β-mercaptoethanol, 0.25 mM EDTA, 10% [vol/vol] glycerol, 0.001% [wt/vol] bromophenol blue, 12.5 mM Tris-HCl [pH 6.8]) and separated in 12.5% (wt/vol) SDS-polyacrylamide gels, as described previously by Laemmli (36). Proteins were stained with Coomasie brilliant blue R-250 (37). Protein concentrations were determined with Bradford reagent (38).

**RESULTS**

**Expression of the phasin and PHB depolymerase genes.** In order to analyze the influence of selected intracellular PHB depolymerases of R. eutropha H16 on growth performance and on PHB accumulation in the presence or absence of different phasins, 20 recombinant strains of E. coli BL21(DE3) were constructed as described in Materials and Methods. All recombinant strains harbor a plasmid containing the phaCAB operon of R. eutropha H16, including its natural promoter, to enable synthesis of PHB. In addition, the phasin- and depolymerase-encoding genes to be examined were provided on a second plasmid. For simultaneous expression of these genes, the pETDuet-1 vector was chosen. Due
TABLE 1 Bacterial strains, plasmids, and oligonucleotides

<table>
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<th>Strain, plasmid, or oligonucleotide</th>
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<td>5′-GACAGGACGCCATATGCCCCTCCTAC-3′</td>
<td>5′ region of phaZ7</td>
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a phaCABRe, *R. eutropha* phaCAB.

to the two multiple-cloning sites (MCSs), which were both preceded by a T7 promoter/lac operator and identical ribosome binding sites, the vector allows the simultaneous expression of two genes at nearly the same expression levels. In this study, the genes of the following phasins and PHB depolymerases were selected to examine their influence on PHB accumulation: PhaP1, PhaP2, PhaP4, PhaZ1, PhaZ2, PhaZ3, and PhaZ7. Eight of the constructed strains contained either the empty vector (control) or one phasin gene in MCS-1 or one PHB depolymerase gene in MCS-2 of pETDuet-1. The other 12 strains contained pETDuet-1 with different combinations of one of the three phasin genes in MCS-1 and one of the four PHB depolymerase genes mentioned above in MCS-2. The correct insertion of the genes was verified by sequence analyses of the constructed plasmids.

In addition, the expression of the three selected genes encoding the phasins PhaP1, PhaP2, and PhaP4 and the four selected genes encoding the PHB depolymerases PhaZ1, PhaZ2, PhaZ3, and PhaZ7 was examined. *E. coli* BL21(DE3) strains harboring the pETDuet-1 vector including a phasin gene in MCS-1 or a PHB depolymerase gene in MCS-2 were cultivated in LB. Expression was induced during the early exponential growth phase by the addition of IPTG, and the cells were harvested as described in Materials and Methods. To obtain protein samples, the cells were disrupted by sonication, and the soluble fraction was separated from the insoluble fraction by centrifugation. The protein samples of the soluble and insoluble fractions were then separated by an SDS-polyacrylamide gel. Discrete protein bands corresponding to the phasins and the PHB depolymerases were observed in the soluble, insoluble, or both fractions for all tested strains (Fig. 1).

Analysis of growth behavior. The 20 recombinant strains of *E. coli* BL21(DE3) harboring the plasmid-borne phaCAB operon of *R. eutropha* H16 and additionally the genes of phasins and PHB
depolymerases as single genes alone or in different combinations were cultivated in ammonium chloride-reduced mineral salts medium (MSM). MSM is generally used for the cultivation of R. eutropha strains and is not optimized for the growth of E. coli. M9 medium represents typically the standard minimal growth medium for E. coli strains (34). However, ammonium chloride-reduced MSM supplemented with 1% (wt/vol) glucose was chosen for the cultivation of the recombinant E. coli strains because the strains used accumulated PHB to a greater extent in ammonium chloride-reduced MSM medium than in M9 medium (data not shown).

The growth of the recombinant E. coli strains was determined photometrically in a Klett-Sumner photometer over a time period of 30 h. The resulting growth curves are divided into five groups for better clarity. The first group comprises the recombinant E. coli strains that possess only the phasin genes (Fig. 2A). The other recombinant E. coli strains are divided into four groups with regard to the PHB depolymerase that is present in the strains (Fig. 2B to E). The growth curve of the control strain harboring the empty pETDuet-1 vector is provided for all five groups. Below, the short-form designations of the recombinant E. coli strains, as described in Materials and Methods, are used.

Figure 2 shows that the growth behaviors of all recombinant E. coli strains were very similar regardless of the combination of phasin and PHB depolymerase genes used. After a lag phase of ~2.5 h, all strains entered the exponential growth phase, which was followed by the stationary growth phase after ~12 h of cultivation. During the stationary growth phase of the recombinant E. coli cells, the determined Klett units (KU) increased by an average value of ~90 KU. This was due to the increasing content of PHB, which influenced the turbidity of the cell culture.

The maximum optical densities that were reached varied between 485 KU in the case of E. coli PHB Z1 and 567 KU in the case of E. coli PHB P2, which was even slightly higher than the maximum KU value reached by the control (E. coli PHB).

**Determination of the PHB content in the recombinant E. coli strains.** In order to explore the effect of the different PHB depolymerases on accumulation of PHB and the influence of the presence of phasins, the PHB contents of the 20 different recombinant E. coli strains were determined during the cultivation described above. Samples were taken every 3 h starting at 9 h of cultivation and were prepared for, and analyzed by, gas chromatography, as described in Materials and Methods. The complete results are presented in Table S1 in the supplemental material; in addition, a graphic summary of the results is shown in Fig. 3.

The control strain harboring the plasmid-borne phaCAB operon of R. eutropha H16 and the empty expression vector pETDuet-1 accumulated up to 31% (wt/wt) PHB of cell dry weight (CDW) after 30 h of cultivation. This PHB content was also achieved by cells of the strain that additionally expressed the gene of the phasin PhaP2 (E. coli PHB P2) (Fig. 3). The expression of the genes encoding the phasin PhaP1 or PhaP4 resulted in lower PHB contents after 30 h of cultivation (19.8% and 26.5% [wt/wt] of CDW for E. coli PHB P1 and E. coli PHB P4, respectively) (Fig. 3).

If the gene of the PHB depolymerase PhaZ1 was expressed, the respective strain accumulated ~50% of the amount of PHB accumulated by the control strain after 30 h of cultivation (14.3% [wt/wt] of CDW). It is apparent that in this strain, the PHB content increased from ~5.9% (wt/wt) of CDW at 9 h of cultivation to 12.6% (wt/wt) of CDW after 12 h of cultivation but remained almost constant during the next 18 h (14.3% [wt/wt] of CDW). The maximum optical densities that were reached varied between 485 KU in the case of E. coli PHB Z1) (Fig. 3). If phaZ1 and one of the phasins PhaP1, PhaP2, and PhaP4 genes were expressed, the PHB contents of the respective strains were higher after 30 h of cultivation than without the expression of a phasin gene (17.0%, 16.0%, and 15.7% [wt/wt] of CDW for E. coli PHB P1Z1, E. coli PHB P2Z1, and E. coli PHB P4Z1, respectively) (Fig. 3).

The recombinant E. coli strain that harbored the gene of the PHB depolymerase PhaZ2 accumulated up to 20.3% (wt/wt) PHB of CDW and thereby reached 65% of the PHB content of the control strain. The strains that harbored phaZ2 and, in addition, one gene of the phasins PhaP2 or PhaP4 accumulated PHB to approximately the same fraction (18.9% and 21.8% [wt/wt] of CDW for E. coli PHB P2Z2 and E. coli PHB P4Z2, respectively) (Fig. 3). Expression of phaZ2 and phaP1 resulted in a lower PHB content after 30 h of cultivation (13.4% [wt/wt] of CDW for E. coli PHB P2Z2) (Fig. 3).

The PHB contents of cells of the recombinant strains harboring phaZ3 with or without a phasin gene were very similar to the PHB contents of cells of the respective strains harboring phaZ2 after 30 h of cultivation (18.2% [wt/wt] of CDW for E. coli PHB Z3, 10.9% [wt/wt] of CDW for E. coli PHB PZ3, 20.1% [wt/wt] of CDW for E. coli PHB P2Z3, and 21.5% [wt/wt] of CDW for E. coli PHB P4Z3) (Fig. 3).

The recombinant strain that harbored the PHB depolymerase PhaZ7 gene accumulated PHB to a significantly lesser extent than all other recombinant strains that were investigated in this study (2.0% [wt/wt] of CDW after 30 h for E. coli PHB Z7) (Fig. 3). If the strain harbored the gene encoding the phasin PhaP1 or
PhaP4 in addition to phaZ7, PHB contents of 10.2% and 15.1% (wt/wt) of CDW, respectively, were achieved (E. coli PHB P1Z7 and E. coli PHB P4Z7, respectively) (Fig. 3). Expression of phaZ7 in combination with phaP2 resulted in a final PHB content of 21.5% (wt/wt) of CDW (E. coli PHB P2Z7) (Fig. 3).

**DISCUSSION**

This study shows the impact of intracellular PHB depolymerase and phasin isoenzymes of *Ralstonia eutropha* H16 on the growth performance and PHB accumulation of recombinant *Escherichia coli* strains. Heterologous expression of selected *phaP* or *phaZ* genes or coexpression of *phaP* and *phaZ* had almost no effect on the growth behavior of the recombinant *E. coli* strains. Only the expression of *phaZ1* or of *phaZ2* in combination with *phaP1* led to slightly reduced optical densities of the respective strains in the stationary growth phase. However, in these strains, growth during the exponential phase was also not affected. The insignificantly reduced cell growth of some recombinant *E. coli* strains did not correlate with a reduced PHB content of these strains and may result from a difference in the strength of overexpression of the heterologous genes.

On the other hand, the analysis of the PHB contents of the recombinant *E. coli* strains revealed differences in the amounts of accumulated PHB. It was noted that the overexpression of *phaZ7* resulted in by far the lowest PHB content among the analyzed *E. coli* strains (2.0% [wt/wt] of CDW after 30 h for *E. coli* PHB Z7) (Fig. 3). Recently, a study of the physiological functions of the PHB depolymerases PhaZ6 and PhaZ7 of *R. eutropha* was reported (26). That study demonstrated that the overexpression of both PHB depolymerase genes prevented the accumulation of PHB during cultivation in NB medium supplemented with glucose. *In vitro* assays showed that PhaZ7 had a significantly lower specific PHB depolymerase activity against native granules than did PhaZ6. However, the activity of PhaZ7 was increased 15-fold when trypsin-treated granules (protein surface of the granules is removed) were used, in comparison to the native granules (26). This is consistent with our observation that the overexpression of *phaZ7* in *E. coli* BL21 (DE3) resulted in a low PHB content, whereas coexpression of *phaZ7* in combination with *phaP1*, *phaP2*, or *phaP4* led to significantly higher PHB contents (Fig. 3; see also Table S1 in the supplemental material).

In 2012, the influence of overexpressed PHB depolymerases in *R. eutropha* H16 as a host strain under different growth conditions was investigated (27). The authors of that study monitored the PHB content of *R. eutropha* H16 harboring plasmids encoding PhaZ1, PhaZ2, PhaZ3, or PhaZ5. Reduction of the PHB content during growth in tryptic soy broth (TSB) medium or minimal

FIG 2 Growth behavior of recombinant *E. coli* strains under PHB storage conditions (MSM containing 0.05% [wt/vol] ammonium chloride and 1.0% [wt/vol] glucose). (A) Growth of *E. coli* PHB (×), *E. coli* PHB P1 (○), *E. coli* PHB P2 (△), and *E. coli* PHB P4 (□). (B) Growth of *E. coli* PHB (×), *E. coli* PHB Z1 (○), *E. coli* PHB P1Z1 (○), *E. coli* PHB P2Z1 (△), and *E. coli* PHB P4Z1 (□). (C) Growth of *E. coli* PHB (×), *E. coli* PHB Z2 (□), *E. coli* PHB P1Z2 (○), *E. coli* PHB P2Z2 (△), and *E. coli* PHB P4Z2 (□). (D) Growth of *E. coli* PHB (×), *E. coli* PHB Z3 (□), *E. coli* PHB P1Z3 (○), *E. coli* PHB P2Z3 (△), and *E. coli* PHB P4Z3 (□). (E) Growth of *E. coli* PHB (×), *E. coli* PHB Z7 (□), *E. coli* PHB P1Z7 (○), *E. coli* PHB P2Z7 (△), and *E. coli* PHB P4Z7 (□). The arrows indicate the time points of sample drawing for analysis of the PHB content by gas chromatography. KU values in these graphs are the averages from duplicate experiments. Standard deviations are indicated as error bars.
medium (for PHB production) was not demonstrated for the strains overexpressing a PHB depolymerase in comparison to the wild type. In PHB consumption medium, the strains harboring the plasmid-borne gene phaZ1 or phaZ2 showed a faster reduction of the PHB content than the other strains. However, after 24 h, the PHB content was almost the same for all plasmid-harboring strains and the wild type. These results differ from our data obtained for recombinant E. coli strains grown under conditions optimized for PHB production. E. coli strains expressing the PHB depolymerase gene phaZ1, phaZ2, or phaZ3 contained 30 to 65% less PHB than the control strain (Fig. 3; see also Table S1 in the supplemental material).

Another approach to identifying enzymes that may be involved in certain parts of metabolism is analysis of the transcriptome. Microarray and transcriptome sequencing (RNA-seq) studies of the whole genome of R. eutropha H16 identified expression data from different growth phases (41) and under different growth conditions (growth, PHB production, and PHB consumption) were reported previously (42, 43). Increasing concentrations of the transcripts of the phasin PhaP1 and of the depolymerases PhaZ2 and PhaZ6 under growth conditions permissive for PHB accumulation were shown in both microarray studies (41, 42). In addition, Brigham et al. (42) monitored the upregulation of the phaP4, phaZ3, and phaZ5 genes in the PHB production phase of R. eutropha H16. RNA-seq analyses revealed increasing expression levels of the phaP1, phaP2, phaZ1, and phaZ6 genes during the PHB accumulation phase (43). Interestingly, none of those transcriptome studies detected significant upregulation of phaZ7. Furthermore, an assay for the detection of PhaZ7 at the translational level did not indicate any expression of phaZ7 in R. eutropha H16 when grown on solid or liquid NB medium supplemented with gluconate (26). The reason why the PHB depolymerase PhaZ7 gene is not upregulated or even not expressed in representable amounts, although the enzyme is highly active in vitro and in vivo, remained obscure.

In this study, it was noted that the replacement of PhaP1 by PhaP2 or PhaP4 resulted in higher PHB contents of E. coli strains that coexpressed phaZ2, phaZ3, or phaZ7 (Fig. 3; see also Table S1 in the supplemental material). Previously, two studies analyzing the impact of homologous phasins on PHB synthesis and degradation in R. eutropha were reported (44, 45). R. eutropha strains lacking one or multiple phasin genes were examined for their ability to store and mobilize PHB in comparison to the wild type. In contrast to our results obtained with the recombinant E. coli strains, those two studies demonstrated reduced PHB storage in R. eutropha when the phasin PhaP1 was missing. In addition, Kuchta et al. monitored a faster degradation of PHB when phaP1 alone or in combination with one or more of the other phasin genes was deleted (45). However, as those studies represent top-down approaches, the impact of the recently discovered phasins PhaP5, PhaP6, and PhaP7, in addition to PhaM, on storage and degradation of PHB is difficult to assess. In general, studies of the homologous phasins led to the hypothesis that the phasins on the granule surface protect the polymer against random degradation by regulating the access of the PHB depolymerases to the polymer (46).

A previous described bottom-up approach showed that the surface of PHB granules was coated with the heat shock protein HspA when PHB was synthesized heterologously in the absence of phasins in an E. coli strain (47). Regarding the PHB contents of the recombinant E. coli strains in this study, HspA did not seem to substitute for the protective function of the phasins against degradation of the polymer by PhaZ7. However, in the case of the PHB depolymerases PhaZ1, PhaZ2, and PhaZ3, the degradation of the polymer was, at least in part, prevented, although the phasins were absent. In R. eutropha H16, the phasin PhaP1 represents the predominant protein on the surface of PHB granules (11, 12). Our study showed that the coexpression of phaP1 with the PHB depolymerase gene phaZ2, phaZ3, or phaZ7 resulted in notably lower PHB contents (~10% to 13% [wt/wt] of CDW) (Fig. 3) than the coexpression of phaP2 or phaP4 (~15% to 22% [wt/wt] of CDW) (Fig. 3). Potentially, PhaP1 facilitates the access of the PHB depolymerases to the polymer, whereas PhaP2 and PhaP4 limit the access. If this were the case, the mobilization of PHB by these PHB depolymerases could be regulated by changing the relative amounts of the respective phasins on the granule surface. The microarray and RNA-seq studies mentioned above indeed showed a slight upregulation of phaP2 and phaP4 in the PHB production phase, whereas the expression of phaP1 remained at approximately the same high level under all conditions.

FIG 3 Amount of PHB accumulated by recombinant E. coli strains grown under PHB storage conditions (MSM containing 0.05% [wt/vol] ammonium chloride and 1.0% [wt/vol] glucose). PHB contents of cells of recombinant E. coli strains are indicated. Samples were withdrawn during cultivation after 12 h (white bars), 18 h (light gray bars), 24 h (dark gray bars), and 30 h (black bars). The values in this graph represent the averages from duplicate experiments (for standard deviations, see Table S1 in the supplemental material).
analyzed conditions (growth, PHB production, and PHB consumption).

However, this assumption contradicts the results obtained with the phaP deletion strains of *R. eutropha*. In these strains, as mentioned above, PHB storage was decreased and PHB degradation was increased when PhaP1 was missing. In addition, no influence on the PHB contents was observed when PhaP2 or PhaP4 was deleted. Furthermore, our results from heterologous overexpression of phaZ1 in *E. coli* also did not support the assumption that PhaP2 and PhaP4 could limit the access of the PHB depolymerase to the polymer. Almost no difference in the final PHB content was obtained for the strains expressing phaZ1 irrespective of whether or not a phasin was coexpressed or which phasin was coexpressed (~15% [wt/wt] of CDW after 30 h of cultivation) (Fig. 3; see also Table S1 in the supplemental material). We presume that the degradation of PHB by PhaZ1 might be regulated by an as-yet-unknown mechanism, which is possibly independent of a certain phasin. As PhaZ1 was shown to have the greatest impact on PHB degradation (22, 27), an effect of PhaP2 and PhaP4 on the other PHB depolymerases during PHB degradation could have been superimposed in the experiments with *R. eutropha*.

In the past, degradation of PHB granules by intracellular PHB depolymerases was also examined by using different types of *in vitro* enzyme assays. Due to the complex structure of the PHB granules and their protein surface layer, these assays are difficult to perform, and the results are difficult to interpret. The state of the granules (native, amorphous, or denatured) is preprogrammed by the method that is chosen for their preparation and significantly influences the ability of PHB depolymerases to degrade the granules (for a review, see reference 48). In addition, although thiolytic degradation of native PHB granules was measured some years ago (25, 35), hydrolysis of PHB by intracellular PHB depolymerases has also been shown (23, 25, 49). To avoid taking all these things into account, we decided to analyze the impact of selected PHB depolymerases and phasins *in vivo* with a host strain naturally lacking the genes for these enzymes.

The disadvantage of this approach is that it cannot reproduce the native conditions and the regulatory patterns in *R. eutropha* during PHB metabolism. The influence of the phasins and PHB depolymerases on PHB accumulation, which was observed in this study, may differ from their impact on PHB metabolism in *R. eutropha*. In addition, it is necessary to mention that differences in the expression levels of the phasin and PHB depolymerase genes (Fig. 1) may also have an impact on the final PHB contents of the recombinant *E. coli* strains. However, this putative impact seemed to be less significant, as phaZ7 was expressed at a relatively low level, whereas the impact of PhaZ7 on the PHB content of the respective *E. coli* strain was extremely great. In this study, the obtained amounts of accumulated PHB did not considerably differ among the investigated strains, except for the strain expressing phaZ7. We could not identify the specific role of each analyzed phasin or PHB depolymerase during PHB accumulation and mobilization.

Nevertheless, it was shown that the analyzed phasins PhaP1, PhaP2, and PhaP4 are not substitutable without an impact on the amount of stored PHB in recombinant *E. coli*. Furthermore, the phasins PhaP2 and PhaP4 seemed to limit the degradation of PHB by the PHB depolymerases PhaZ2, PhaZ3, and PhaZ7. However, degradation of PHB by the PHB depolymerase PhaZ1 was not prevented by any of the analyzed phasins. The greatest effect on PHB storage was achieved by overexpressing the PHB depolymerase PhaZ7. The respective recombinant *E. coli* strain showed a ~94% lower PHB content after 30 h of cultivation than the control strain.

Further work is required to understand the complex interplay of the phasin and PHB depolymerase isoenzymes during PHB metabolism in *R. eutropha* H16. This study provides some interesting hints on enzymes that would be promising to investigate in the future.

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