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.
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 an 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 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 more than one PHB depolymerase in *R. eutropha* H16 in some cases resulted in an almost complete prevention of PHB degradation (22, 27). Defining the certain role of the individual PHB depolymerases in *R. eutropha* H16 would require the construction of diverse multidetide strains, which is not feasible.

For this study, we decided to carry out in vivo analyses to examine the impact of some PHB depolymerases and phasins of *R. eutropha* H16 on cell growth and PHB accumulation by using *Escherichia coli* BL21 as a host strain. *E. coli* is able to accumulate PHB when the phaCAB operon of *R. eutropha* H16 is provided (28, 29). To unravel the influence of the different PHB depolymerases on PHB accumulation in the absence or presence of phasins, we constructed 19 plasmids with different combinations of the genes encoding phasin and PHB depolymerase isoenzymes.

### 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 cultured 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 30 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-Summers photometer (Manostat Corporation, NY, USA), using filter no. 54 (520 to 580 nm). The expression of heterologous genes was induced by the addition of 200 μM isopropyl-β-D-thiogalactopyranoside (IPTG) in the early exponential growth phase at ~150 Klett units (KU). For expression analysis, *E. coli* strains were cultured in LB medium at 37°C. Gene expression was induced in the early exponential growth phase by the addition of 200 μM IPTG, and the cells were harvested after cultivation for 4 h at 30°C. To maintain the utilized plasmids, antibiotics were added to the medium at the following concentrations: ampicillin at 100 μg/ml and kanamycin at 50 μg/ml.

**Transfer of DNA.** Chemically competent cells of *E. coli* were prepared and transformed with DNA by using the CaCl₂ method (31).

**Construction of recombinant *E. coli* strains.** All plasmids and oligonucleotides used in this study are listed in Table 1. Genomic DNA of *R. eutropha* H16 was isolated according to the method of Marmur (32), and plasmids were isolated according to the method of Birnboim and Doly (33). Restriction enzymes used in this study were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The phasin genes *phaP1*, *phaP2*, and *phaP4* and the PHB depolymerase genes *phaZ1*, *phaZ2*, *phaZ3*, and *phaZ7* were amplified from genomic DNA of *R. eutropha* H16 according to the method of Maniatis et al. (34), using Taq DNA polymerase (Life Technologies, Carlsbad, CA, USA) and the oligonucleotides listed in Table 1 (synthesized by Eurofins MWG Operon, Ebersberg, Germany). PCR fragments were subcloned into pCR2.1-TOPO (Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions. Construction of the expression plasmids containing the phasin genes *phaP1*, *phaP2*, and *phaP4* and the PHB depolymerase gene *phaZ1* was described previously by Eggers and Steinbüchel (35). The PHB depolymerase genes *phaZ2*, *phaZ3*, and *phaZ7* were cut with Ndel and Kpn1 and ligated with pETDuet-1 (multiple-cloning site 2 [MCS-2]; Merck Millipore, Darmstadt, Germany) or with those plasmids that already contained one of the three phasin genes. The resulting plasmids were used for the recombinant expression of the phasins and PHB depolymerases in *E. coli* BL21(DE3), each as a single gene alone or in different combinations. For reasons of simplicity, short-form designations are used for the recombinant *E. coli* strains. These short forms were composed of the strain (*E. coli*), a designation of the plasmid carrying the PHB synthesis genes (PHB), and a short form of the phasin and/or PHB depolymerase genes provided by pETDuet-1 (e.g., P1 or P1Z1). For example, *E. coli* strain (DE3) (pBBR1MCS-2::phaCAB/pETDuet-1::phaP2::phaZ7) is designated *E. coli* PHB P2Z7.

**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. 1.45; 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 Coomassie brilliant blue R-250 (37). Protein concentrations were determined with Bradford reagent (38).

**Determination of PHB content by gas chromatography.** For determination of PHB contents of the recombinant *E. coli* strains, samples of 50 ml of culture medium during growth analysis were taken 9, 12, 15, 18, 21, 24, 27, and 30 h after inoculation. Cells were harvested, washed with 50 mM Tris-HCl (pH 7.5), and lyophilized for 24 h. For methanolysis of PHB, 5 to 10 mg of lyophilized cells was incubated at 100°C with 1 ml of 85% (vol/vol) methanol and 15% (vol/vol) sulfuric acid and in the presence of 1 ml of chloroform. The reaction was stopped after 4 h by the addition of 1 ml H₂O, and the solution was mixed thoroughly. The chloroform phase containing the resulting methyl esters of 3HB was analyzed by gas chromatography, as described previously (39, 40).

### 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
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 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 phasin and 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

**TABLE 1** Bacterial strains, plasmids, and oligonucleotides

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<th>Strain, plasmid, or oligonucleotide</th>
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<td>5′-GAGACAGAGCCATATGCCTGGCACTCCT-3′</td>
<td>3′ region of phaZ7</td>
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\*phaCAB<sub>Δ</sub>, *R. eutropha* phaCAB.
The 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-Summerson 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 phasins 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 PHB of CDW at 30 h for 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 P2Z1, E. coli PHB P2Z2, 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 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 P1Z2) (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 P1Z3, 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 the 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 gluconate. 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](http://aem.asm.org/) 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 P1Z1 (○), *E. coli* PHB P2Z1 (△), and *E. coli* PHB P4Z1 (□). (C) Growth of *E. coli* PHB (○), *E. coli* PHB P1Z2 (□), *E. coli* PHB P2Z2 (△), and *E. coli* PHB P4Z2 (□). (D) Growth of *E. coli* PHB (○), *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 the 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). However, in contrast to our study, the host strain of that study was R. eutropha H16. Although selected PHB depolymerases were overexpressed, the genes of all seven PHB depolymerases and of the seven phasins were present in the analyzed strains. Basal activity and an influence of the other isoenzymes in addition to the influence of the phasins cannot be excluded.

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 compared the transcriptomic 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 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). However, in contrast to our study, the host strain of that study was R. eutropha H16. Although selected PHB depolymerases were overexpressed, the genes of all seven PHB depolymerases and of the seven phasins were present in the analyzed strains. Basal activity and an influence of the other isoenzymes in addition to the influence of the phasins cannot be excluded.

Impact of PHB Depolymerases and Phasins on PHB Storage

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 compared the transcriptomic 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 phaZ4, 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 previously 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 PhaZ2, PhaZ3, and PhaZ7 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 (growth, PHB production, and PHB consumption) of recombinant E. coli strains with a single copy number of the corresponding plasmid.
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 pre-

sumed 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 PHB polymerase gene (phaP). 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, asphaZ7 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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