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

Jessica Eggers,^a Alexander Steinbüchel^{a,b}

Institut für Molekulare Mikrobiologie und Biotechnologie, Westfälische Wilhelms Universität Münster, Münster, Germany^a; Environmental Sciences Department, King Abdulaziz University, Jeddah, Saudi Arabia^b

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_{SCL}) (2 to 5 carbon atoms), medium-chain-length PHAs (PHA_{MCL}) (6 to 16 carbon atoms), and long-chain-length PHAs (PHA_{LCL}) (>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_{SCL} 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 (10).

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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Address correspondence to Alexander Steinbüchel, steinbu@uni-muenster.de.

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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 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, 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 multideletion 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 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 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-Summerson 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 μ M isopropyl- β -D-thiogalactopyranoside (IPTG) in the early exponential growth phase at ~150 Klett units (KU). For expression analysis, *E. coli* strains were cultivated in LB medium at 37°C. Gene expression was induced in the early exponential growth phase by the addition of 200 μ M 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 μ g/ml and kanamycin at 50 μ g/ml.

Transfer of DNA. Chemically competent cells of *E. coli* were prepared and transformed with DNA by using the CaCl_2 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 *Nde*I and *Kpn*I 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 $\times$ 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 H_2O , 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

TABLE 1 Bacterial strains, plasmids, and oligonucleotides

Strain, plasmid, or oligonucleotide	Description or sequence ^a	Source, reference, or location
Strains		
<i>R. eutropha</i> H16	Wild type; Gm ^r	DSM 428
<i>E. coli</i> TOP10	Cloning strain; F [−] <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) <i>galK rpsL</i> φ80 <i>lacZ</i> ΔM15 Δ <i>lacX74 recA1 araD139</i> Δ(<i>ara-leu</i>)7697 <i>galU</i> (Str ^r) <i>endA1 nupG</i>	Life Technologies
<i>E. coli</i> BL21(DE3)	Expression strain; <i>E. coli</i> B F [−] <i>dcm ompT hsdS</i> (r _B [−] m _B [−]) <i>gal</i> (DE3)	Stratagene
Plasmids		
pCR2.1-TOPO	Cloning vector; Ap ^r Km ^r	Life Technologies
pBBR1MCS-2	Broad-host-range vector; Km ^r <i>lacPOZ'</i> <i>mobRP4</i>	Life Technologies
pETDuet-1	Expression vector with two MCSs, each preceded by a T7 promoter/ <i>lac</i> operator and <i>rbs</i> ; ColE1 <i>ori lacI</i> Ap ^r	Merck Millipore
pBHR68	pBluescriptSK(−) harboring the <i>phaCAB</i> _{Re} operon including the promoter; Ap ^r	50
pBBR1MCS-2:: <i>phaCAB</i>	pBBR1MCS-2 carrying the BamHI-EcoRI fragment of pBHR68 harboring the <i>phaCAB</i> operon including the promoter; Km ^r	35
pETDuet-1:: <i>phaP1</i>	pETDuet carrying <i>phaP1</i> _{Re} in MCS-1; Ap ^r	35
pETDuet-1:: <i>phaP2</i>	pETDuet carrying <i>phaP2</i> _{Re} in MCS-1; Ap ^r	35
pETDuet-1:: <i>phaP4</i>	pETDuet carrying <i>phaP4</i> _{Re} in MCS-1; Ap ^r	35
pETDuet-1:: <i>phaZ1</i>	pETDuet carrying <i>phaZ1</i> _{Re} in MCS-2; Ap ^r	35
pETDuet-1:: <i>phaZ2</i>	pETDuet carrying <i>phaZ2</i> _{Re} in MCS-2; Ap ^r	This study
pETDuet-1:: <i>phaZ3</i>	pETDuet carrying <i>phaZ3</i> _{Re} in MCS-2; Ap ^r	This study
pETDuet-1:: <i>phaZ7</i>	pETDuet carrying <i>phaZ7</i> _{Re} in MCS-2; Ap ^r	This study
pETDuet-1:: <i>phaP1</i> :: <i>phaZ1</i>	pETDuet carrying <i>phaP1</i> _{Re} in MCS-1 and <i>phaZ1</i> _{Re} in MCS-2; Ap ^r	35
pETDuet-1:: <i>phaP1</i> :: <i>phaZ2</i>	pETDuet carrying <i>phaP1</i> _{Re} in MCS-1 and <i>phaZ2</i> _{Re} in MCS-2; Ap ^r	This study
pETDuet-1:: <i>phaP1</i> :: <i>phaZ3</i>	pETDuet carrying <i>phaP1</i> _{Re} in MCS-1 and <i>phaZ3</i> _{Re} in MCS-2; Ap ^r	This study
pETDuet-1:: <i>phaP1</i> :: <i>phaZ7</i>	pETDuet carrying <i>phaP1</i> _{Re} in MCS-1 and <i>phaZ7</i> _{Re} in MCS-2; Ap ^r	This study
pETDuet-1:: <i>phaP2</i> :: <i>phaZ1</i>	pETDuet carrying <i>phaP2</i> _{Re} in MCS-1 and <i>phaZ1</i> _{Re} in MCS-2; Ap ^r	35
pETDuet-1:: <i>phaP2</i> :: <i>phaZ2</i>	pETDuet carrying <i>phaP2</i> _{Re} in MCS-1 and <i>phaZ2</i> _{Re} in MCS-2; Ap ^r	This study
pETDuet-1:: <i>phaP2</i> :: <i>phaZ3</i>	pETDuet carrying <i>phaP2</i> _{Re} in MCS-1 and <i>phaZ3</i> _{Re} in MCS-2; Ap ^r	This study
pETDuet-1:: <i>phaP2</i> :: <i>phaZ7</i>	pETDuet carrying <i>phaP2</i> _{Re} in MCS-1 and <i>phaZ7</i> _{Re} in MCS-2; Ap ^r	This study
pETDuet-1:: <i>phaP4</i> :: <i>phaZ1</i>	pETDuet carrying <i>phaP4</i> _{Re} in MCS-1 and <i>phaZ1</i> _{Re} in MCS-2; Ap ^r	35
pETDuet-1:: <i>phaP4</i> :: <i>phaZ2</i>	pETDuet carrying <i>phaP4</i> _{Re} in MCS-1 and <i>phaZ2</i> _{Re} in MCS-2; Ap ^r	This study
pETDuet-1:: <i>phaP4</i> :: <i>phaZ3</i>	pETDuet carrying <i>phaP4</i> _{Re} in MCS-1 and <i>phaZ3</i> _{Re} in MCS-2; Ap ^r	This study
pETDuet-1:: <i>phaP4</i> :: <i>phaZ7</i>	pETDuet carrying <i>phaP4</i> _{Re} in MCS-1 and <i>phaZ7</i> _{Re} in MCS-2; Ap ^r	This study
Oligonucleotides		
<i>phaZ2</i> _NdeI	5'-GCGGGGACCGAGCCATATGCTGTACCAC-3'	5' region of <i>phaZ2</i>
<i>phaZ2</i> _KpnI	5'-GCGCGCTGGCGGGTACCGGGTTAGCTGCTGG-3'	3' region of <i>phaZ2</i>
<i>phaZ3</i> _NdeI	5'-GGCGGGAATCGCACATATGGCGCTCTATGCTCTGCG-3'	5' region of <i>phaZ3</i>
<i>phaZ3</i> _KpnI	5'-CCTCGCCAAGGCGACGGAGCGCGGTACCTCAGTCGGCGCACC-3'	3' region of <i>phaZ3</i>
<i>phaZ7</i> _NdeI	5'-GACAGGACGCCCATATGCCCGCTCATC-3'	5' region of <i>phaZ7</i>
<i>phaZ7</i> _KpnI	5'-CAAGCCTGCCGTACCTCACCCCCAAC-3'	3' region of <i>phaZ7</i>

^a *phaCAB*_{Re}, *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

FIG 1 Analysis of the expression of the phasin and PHB depolymerase genes in recombinant *E. coli* strains. Recombinant strains of *E. coli* BL21(DE3) were grown in LB, and protein expression was induced by the addition of 200 μ M IPTG. Cells were broken as described in Materials and Methods, and the proteins of the soluble fraction (SF) and insoluble fraction (IF) of the crude extracts of the following strains were separated by SDS-PAGE and stained with Coomassie brilliant blue: *E. coli* BL21(DE3)(pETDuet-1) (C), *E. coli* BL21(DE3)(pETDuet-1::phaP1) (P1), *E. coli* BL21(DE3)(pETDuet-1::phaP2) (P2), *E. coli* BL21(DE3)(pETDuet-1::phaP4) (P4), *E. coli* BL21(DE3)(pETDuet-1::phaZ1) (Z1), *E. coli* BL21(DE3)(pETDuet-1::phaZ2) (Z2), *E. coli* BL21(DE3)(pETDuet-1::phaZ3) (Z3), and *E. coli* BL21(DE3)(pETDuet-1::phaZ7) (Z7). The arrows indicate the respective protein bands of the phasins and the PHB depolymerases. M, molecular mass standard.

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 $\sim$ 2.5 h, all strains entered the exponential growth phase, which was followed by the stationary growth phase after $\sim$ 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 $\sim$ 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 $\sim$ 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 $\sim$ 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] 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 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 phasin 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 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 (x), *E. coli* PHB P1 (diamond), *E. coli* PHB P2 (triangle), and *E. coli* PHB P4 (circle). (B) Growth of *E. coli* PHB (x), *E. coli* PHB Z1 (square), *E. coli* PHB P1Z1 (diamond), *E. coli* PHB P2Z1 (triangle), and *E. coli* PHB P4Z1 (circle). (C) Growth of *E. coli* PHB (x), *E. coli* PHB Z2 (square), *E. coli* PHB P1Z2 (diamond), *E. coli* PHB P2Z2 (triangle), and *E. coli* PHB P4Z2 (circle). (D) Growth of *E. coli* PHB (x), *E. coli* PHB Z3 (square), *E. coli* PHB P1Z3 (diamond), *E. coli* PHB P2Z3 (triangle), and *E. coli* PHB P4Z3 (circle). (E) Growth of *E. coli* PHB (x), *E. coli* PHB Z7 (square), *E. coli* PHB P1Z7 (diamond), *E. coli* PHB P2Z7 (triangle), and *E. coli* PHB P4Z7 (circle). 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.

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).

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). 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 comparing 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 *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 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

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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