Poly(3-Hydroxybutyrate) (PHB) Depolymerase PhaZa1 Is Involved in Mobilization of Accumulated PHB in *Ralstonia eutropha* H16[▽]

Keiichi Uchino,[1,2] Terumi Saito,[2] and Dieter Jendrossek[1]*

Institut für Mikrobiologie, Universität Stuttgart, 70550 Stuttgart, Germany,[1] and Laboratory of Molecular Microbiology, Kanagawa University, Kanagawa 259-1293, Japan[2]

The recently finished genome sequence of *Ralstonia eutropha* H16 harbors nine genes that are thought to encode functions for intracellular depolymerization (mobilization) of storage poly(3-hydroxybutyrate) (PHB). Based on amino acid similarities, the gene products belong to four classes (PhaZa1 to PhaZa5, PhaZb, PhaZc, and PhaZd1/PhaZd2). However, convincing direct evidence for the in vivo roles of the gene products is poor. In this study, we selected four candidate genes (*phaZa1*, *phaZb*, *phaZc*, and *phaZd1*) representing the four classes and investigated the physiological function of the gene products (i) with recombinant *Escherichia coli* strains and (ii) with *R. eutropha* null mutants. Evidence for weak but significant PHB depolymerase activity was obtained only for PhaZa1. The physiological roles of the other potential PHB depolymerases remain uncertain.

Polyhydroxyalkanoates (PHA) are typical storage compounds of carbon and energy and are found widely in prokaryotes. The most common PHA is poly(3-hydroxybutyrate) (PHB), and this polymer can accumulate up to 90% of the cellular dry weight of some bacteria. PHA are thermoplasts, and despite their relatively high production costs, biologically produced PHA have entered the industrial market.

PHA can be degraded extracellularly by many types of bacteria that are able to secrete specific extracellular PHA depolymerases into the environment or by the intracellular mobilization of PHA in the accumulating strain itself. Considerable knowledge of the biochemical properties of the respective extracellular PHA depolymerases has accumulated (14, 17, 18). Intracellular mobilization of PHA differs from extracellular degradation because of the differences between the biophysical conformations of extracellular (denatured) PHA and those of intracellular (native) PHA. (For definitions of the terms "denatured" and "native PHA," see references 15, 25, and 26.) Several groups have reported on the identification of potential intracellular PHB depolymerases (1, 5, 8, 19, 20, 33, 40, 43; for an overview see references 15, 17, and 34). These data, together with the genome sequence of *Ralstonia eutropha*, suggested that *R. eutropha* H16 might have as many as nine PHB depolymerases/oligomer hydrolases (29). Five putative PHB depolymerase isoenzymes (PhaZa1 to PhaZa5), two 3-hydroxybutyric acid (3HB) oligomer hydrolases (PhaZb and PhaZc [PhaY1 and PhaY2]), and two isoenzymes of a recently found new type of putative intracellular PHB depolymerase (PhaZd1 and PhaZd2) have been described (1). The last two have amino acid sequences that are significantly similar to those of the catalytic domain of extracellular PHB depolymerases (16, 36). We were surprised to find evidence for so many PHB depolymerases in one organism, and we wondered whether all of these proteins were physiologically important for intracellular PHB mobilization. Clarification of this point appeared necessary because independent evidence for the function of the above-mentioned proteins such as the physiological PHB depolymerases exists only for one of them, namely PhaZa1 (8, 32, 43). Unfortunately, an in vitro assay of intracellular PHB depolymerase activity is difficult to perform, and to our knowledge no publication shows high in vitro activity of an intracellular PHB depolymerase by using the natural substrate native PHB (nPHB) granules. Abe et al. reported on a new type of intracellular PHB (iPHB) depolymerase (PhaZd) with high activity toward artificial PHB but with low specific activity toward nPHB granules (0.43 U/mg) (1).

Interestingly, the production of large amounts of 3HB from glucose-grown cells of recombinant *Escherichia coli* by coexpression of the PHB biosynthetic operon *phaCAB* of *R. eutropha* together with the putative PHB depolymerase gene *phaZa1* (all genes present on pSYL105red) was reported (23, 28), and a patent claiming the production of ~14 g/liter 3HB from 20 g/liter glucose by the use of this method was granted (Fig. 4 and 5 of reference 21). Inspired by these successful in vivo data, we thought that the expression of other PHB depolymerases/oligomer hydrolase genes, such as *phaZb*, *phaZc*, and *phaZd1*, together with PHB biosynthetic genes from recombinant *E. coli* could be a valuable tool with which to check other genes for their potential roles in PHB mobilization, and we intended to use the same principle for the detection of PHB depolymerase activity in vivo by the determination of the amount of 3HB and/or 3HB oligomers released. In a first attempt to elucidate the roles of the respective depolymerase proteins, we chose (i) PhaZa1 as the representative of gene products PhaZa1 to PhaZa5, (ii) PhaZb, (iii) PhaZc, and (iv) PhaZd1 as the representative of PhaZd1/PhaZd2 for further investigation.

* Corresponding author. Mailing address: Institut für Mikrobiologie, Universität Stuttgart, Allmandring 31, 70550 Stuttgart, Germany. Phone: 49-711-685-65483. Fax: 49-711-685-65725. E-mail: dieter.jendrossek@imb .uni-stuttgart.de.
▽ Published ahead of print on 21 December 2007.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids used in this study are given in Table 1. Bacteria were grown on NB (*R. eutropha* strains) or LB medium (*E. coli*) at 30 or 37°C, respectively. For some

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
Strains		
E. coli BL21(DE3)	Expression strain	Novagen
E. coli XL1-Blue	Host for pSYL105red	
R. eutropha H16	Wild type	Accession no. DSMZ428
R. eutropha D1	PhaZa1 null mutant	32
R. eutropha OH1	PhaZb null mutant	19
R. eutropha TK0130	PhaZc null mutant	20
R. eutropha ReDZd1	PhaZd null mutant	1
Plasmids		
pSYL105red	Plasmid carrying *phaCAB* and *phaZa1*	23
pSYL105	pSYL105red derivative without *phaZa1*	23; this study
pSYL105redNEW	Plasmid carrying *phaCAB* and *phaZa1*	This study
pBHR68	Source of *phaCAB* operon	39
pAE171	*R. eutropha* pUC18 carrying *phaZa1*	32
pHWG640	Rhamnose-inducible vector	J. Altenbuchner
pHWG640::*phaCAB*	pHWG640 harboring *phaCAB* under rhamnose promoter control	This study
pET23b	Expression vector for T7 promoter	Novagen
pET171H	pET23b harboring *phaZa1* under T7 promoter	32
pETOH	pET23b harboring *phaZb* under T7 promoter	19
pE3ReZc	pET23b harboring *phaZc* under T7 promoter	20
pE3ReZd1	pET23b harboring *phaZd* under T7 promoter	1

experiments, a mineral salt-based medium (R medium [22]) supplemented with thiamine (20 μg/ml) and glucose (20 g/liter) was used according to the composition reported by Lee and Lee (23), as follows (per liter): KH$_2$PO$_4$, 13.5 g; (NH$_4$)$_2$HPO$_4$, 4.0 g; MgSO$_4 \cdot$ 7H$_2$O, 1.4 g; citric acid, 1.7 g (pH 6.8 [NaOH]); trace metal solution, 10.0 ml trace metal solution per liter of 0.5 N HCl [FeSO$_4 \cdot$ 7H$_2$O, 10.0 g; CaCl$_2 \cdot$ 2H$_2$O, 2.0 g; ZnSO$_4 \cdot$ 7H$_2$O, 2.2 g; MnSO$_4 \cdot$ 4H$_2$O, 0.5 g; CuSO$_4 \cdot$ 5H$_2$O, 1.0 g; (NH$_4$)$_6$Mo$_7$O$_{24} \cdot$ 4H$_2$O, 0.1 g; Na$_2$B$_4$O$_7 \cdot$ 10H$_2$O, 0.02 g]. Antibiotics were added according to the presence of plasmid-borne resistance genes. Five milliliters of seed culture (LB) or 20 ml of R medium culture was used for inoculation. When strains harboring the pHGW640 plasmid or derivates of this plasmid were cultivated, 2% (wt/vol) rhamnose was added to the LB medium. Alternatively, a combination of 0.2% (wt/vol) rhamnose and 0.4% (wt/vol) acetate was used to induce the expression of PHB biosynthetic genes and to promote PHB formation. Plasmid-encoded PHB depolymerase expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG, 0.2 mM) during exponential growth phase. IPTG was added at about 1 h after the addition of rhamnose. Bacterial cells were used for nPHB granule isolation or for the determination of the release of 3HB. For the latter, bacteria were resuspended in 1 volume of 0.1 M potassium phosphate buffer (pH 7). Cells were incubated at 30°C (*R. eutropha*) or at 37°C (*E. coli*) without shaking.

Construction of recombinant *E. coli* strains. A 1.7-kbp HindIII-SalI DNA fragment of pAE171 harboring *phaZa1* was cloned into the major DNA fragment of the HindIII/XhoI-digested pSYL105red plasmid to give pSYL105redNEW. DNA sequencing confirmed the presence of one *phaZa1* copy plus 129 bp of the 5′ upstream region and 302 bp of the downstream region of *phaZa1*. The plasmid pBHR68 (39) was used as a source for the *R. eutropha* H16 *phaCAB* operon: the 5′ end of *phaC* was cloned into pHWG640 as an NdeI-BglII DNA fragment by PCR. The 3′ end of *phaC* including *phaAB* was cloned into the former construct as a BglII-HindIII fragment that was obtained from pBHR68, resulting in pHWG640, with *phaCAB* under the control of the rhamnose promoter. *E. coli* strains harboring pHWG640::*phaCAB* produced PHB granules, as revealed by the appearance of fluorescent globular structures after cells were stained with Nile red. For details of fluorescence microscopy see reference 12.

In vivo hydrolysis of PHB and secretion of 3HB. Based on reports that the in vivo hydrolysis of PHA and the secretion of 3-hydroxyalkanoic acids by different bacteria can be efficient at a pH level of 10 to 11 (30, 42) or at a pH level of 3 to 4 (24), cells were washed to limit the source of secreted 3HB to intracellularly accumulated PHB and resuspended in the same volume of 0.1 M of potassium phosphate buffer (pH 7), morpholineethanesulfonic acid-NaOH buffer (pH 4), or Tris-HCl buffer (pH 10). After cell-free samples were incubated at 30°C, they were analyzed for secreted 3HB. In some experiments, culture supernatants were treated with alkali to hydrolyze 3HB oligomers to monomeric 3HB, as described previously (23), as follows: 500 μl of supernatant was alkalized with 500 μl of 10 N NaOH and incubated at 95°C for 2 h. After the supernatant was cooled to

room temperature, 500 μl of 10 N HCl was added, and the content of 3HB was determined enzymatically.

Other methods. The concentration of 3HB was determined using a NAD$^+$-dependent 3HB dehydrogenase assay at an E value at 340 nm (38). In brief, the reaction mixture contained 3.3 mM NAD$^+$, 1 mM MgCl$_2$ in 100 mM Tris-HCl (pH 8.0). The reaction was started by the addition of 2.5 μl of 3HB dehydrogenase (10 mg/ml). If the ΔE_{340} value was above 0.2, the assay was repeated with a diluted sample to ensure that complete conversion of the substrate had occurred. 3HB content was also determined by high-performance liquid chromatography (HPLC) after the samples underwent derivatization with bromophenacylbromide, as described previously (6). This method allowed the detection of 3HB oligomers. Glucose was determined enzymatically with hexokinase and glucose-6-phosphate dehydrogenase. Western blotting analysis of the expression of PhaZa1, PhaZb, PhaZc, and PhaZd1 was performed by the standard procedure using polyclonal antisera, as described previously (1, 20, 32, 33). nPHB granules were isolated from cells broken by French press and two subsequent steps of glycerol density gradient centrifugation (6, 15). The numbers and sizes of PHB granules in PHB-accumulating bacteria were determined by fluorescence microscopy after the samples were stained with Nile red (12). PHB content was also determined according to the method described in reference 2.

RESULTS

Inspired by the detection of large amounts of secreted 3HB (23) obtained after the coexpression of *phaZa1* together with *phaCAB* in recombinant *E. coli*, we selected this in vivo system for the detection of potential PHB depolymerase/hydrolase activities of the candidate PHB depolymerase genes (*phaZb*, *phaZc*, and *phaZd1*). To test this concept, *E. coli* XL1-Blue (control), *E. coli* XL1-Blue(pSYL105) (carrying *phaCAB* only), *E. coli* XL1-Blue(pSYL105red) (carrying *phaCAB*/*phaZa1*), and *E. coli* XL1-Blue(pSYL105redNEW) (the new version of pSYL105red, carrying *phaCAB*/*phaZa1*) were grown in R medium with 2% glucose for 48 h at 37°C under the conditions described in reference 23. The pSLY105redNEW plasmid was constructed because preliminary experiments with pSYL105red failed to reproduce the data for 3HB production. Cell growth (Klett units), cellular dry weight, pH, glucose concentration, the concentration of 3HB by 3HB dehydrogenase,
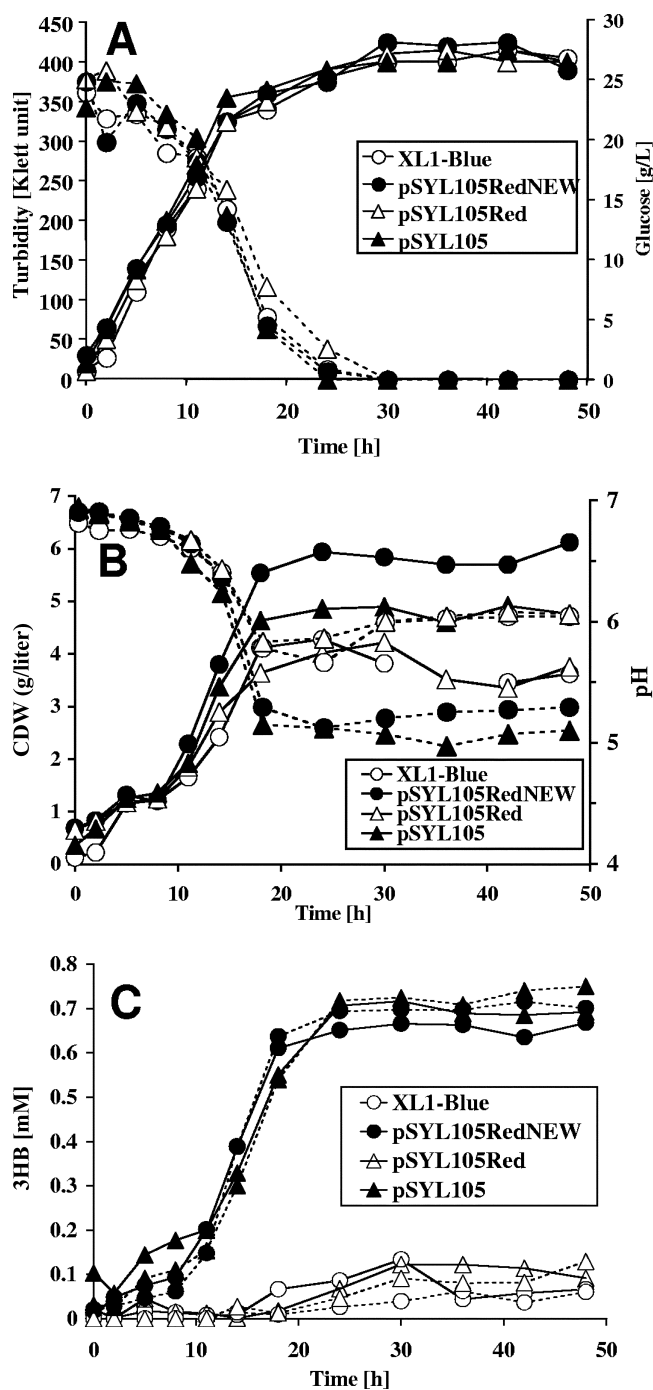
FIG. 1. Growth (turbidity) and glucose consumption (A); cellular dry weight (CDW)/liter and pH profile (B); and secretion of 3HB (C) with NaOH treatment for the determination of total 3HB concentration (dotted lines) or without NaOH treatment (solid lines) by *E. coli* XL1-Blue harboring different combinations of *R. eutropha pha* genes (Table 1). Growth was measured in R medium with 20 g/liter glucose without pH control as described in references 21 and 23. Three independent experiments have been performed. A typical result is shown.

and the concentration levels of 3HB, 3HB oligomers, and acetate were determined by HPLC analysis (Fig. 1). The presence of PHB was determined by fluorescence microscopy after samples were stained with Nile red (12) and by gas chroma-

tography after methanolysis. All four strains showed almost the same growth behavior in terms of Klett units (final Klett unit density, ~400 Klett units) and complete glucose consumption within 30 h (Fig. 1A). The concentration levels of cellular dry weight ranged from less than 4 g/liter for the control strain and for the *E. coli* strain harboring pSYL105red to about 6 g/liter for the *E. coli* strain harboring pSYL105redNEW or *E. coli* with pSYL105 (Fig. 1B). The latter two strains produced the most PHB, as revealed by the appearance of one to a few cell pole-located PHB granules. *E. coli* harboring pSYL105red produced only a little PHB, resulting in a relatively low concentration of cellular dry matter, comparable to that of the XL1-Blue control strain. The *E. coli* XL1-Blue control strain produced no PHB, as expected. All strains secreted acids, as indicated by the decrease in pH from a neutral value to a pH range of 5.2 to 6.2 (Fig. 1B). Enzymatic determination of the 3HB concentration in a cell-free supernatant by using NAD^+-dependent 3HB dehydrogenase showed that the XL1-Blue control strain and the *E. coli* strain harboring pSYL105red produced traces of 3HB ($\leq$0.1 mM). Both *E. coli* XL1-Blue (pSYL105) (carrying only *phaCAB*) and *E. coli* XL1-Blue(pSYL105redNEW) (carrying *phaCAB* and *phaZa1*) produced little more 3HB (Fig. 1C, 0.6 to 0.7 mM 3HB), but a correlation between 3HB secretion and the presence of *phaZa1* was not obvious. Microscopical inspection of Nile red-stained cells did not indicate a significant decrease in PHB content between 24 and 48 h in any of the strains investigated. Treating the samples with alkali before the 3HB dehydrogenase assay resulted in a marginal or no increase in 3HB concentration, suggesting that only small amounts of 3HB oligomers were produced (Fig. 1C). The concentration of 3HB produced (after alkali treatment) by *phaZa1*-containing strains varied between 0.5 and 1.5 mM (0.05 to 0.16 g/liter) in different experiments. This result was confirmed by HPLC analysis after the derivatization of the sample with bromophenacylbromide: only small amounts of 3HB and no significant amounts of 3HB oligomers were detected. Acetate concentration varied between 0.05 and 0.35% (not shown). Western blotting analysis using antibodies raised against PhaZa1 confirmed the fact that PhaZa1 was present in the cell extracts of *phaZa1*-harboring strains and excluded the possibility that the absence of PHB depolymerase expression could be the reason for low 3HB production (not shown). Apparently, PhaZa1 has only a low level of PHB depolymerase activity under the conditions used. In conclusion, we were not able to reproduce the production of 3HB to a level as high as ~9.6 g/liter or even to 14 g/liter (21, 23), and our results do not support the finding that *phaZa1* can be used to promote the secretion of large amounts of 3HB with recombinant *E. coli* under the conditions described.

The "pSYL105red system," in which the *pha* genes are transcribed from their own promoters, did not work well in our hands; therefore, another system was chosen to evaluate candidate depolymerases: a two-plasmid system with inducible promoters was constructed as described in Materials and Methods. The PHB synthesizing ability was supplied by one plasmid harboring the *R. eutropha phaCAB* operon under rhamnose promoter control, and the candidate PHB depolymerases to be evaluated were introduced by the other plasmid under *lac* promoter control. The growth of *E. coli* was performed in LB medium supplemented with 2% (wt/vol) rham-
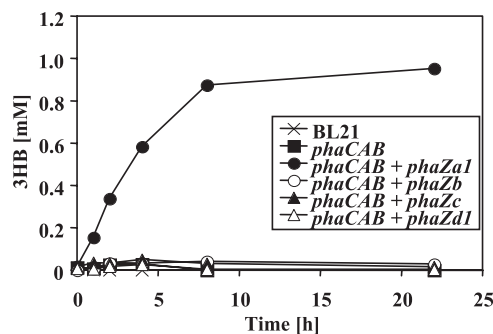
FIG. 2. Secretion of 3HB by washed cells of recombinant *E. coli* BL21 harboring different combination of *pha* genes (Table 1). Bacteria were grown in the presence of rhamnose and IPTG as described in the text. Washed cells were incubated in buffer (100 mM potassium phosphate [pH 7]) at 37°C. Cell-free samples taken at the time points shown were assayed for amounts of 3HB released, by 3HB dehydrogenase assay. Three independent experiments have been performed. A typical result is shown.
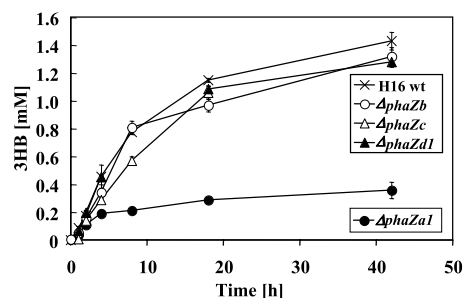

FIG. 3. Secretion of 3HB by washed cells of *R. eutropha* H16 and *phaZ* null mutants as indicated. Bacteria were grown in mineral salts medium (35) with gluconate. Washed cells were incubated in buffer (100 mM potassium phosphate [pH 7]) at 30°C. Cell-free samples taken at the time points shown were assayed for the amounts of 3HB released, by 3HB dehydrogenase assay.

nose and IPTG (0.2 mM) for the induction of PHB accumulation and PHB depolymerase expression, respectively. Cells harboring *phaCAB* with or without the depolymerase (*phaZa1*, *phaZb*, *phaZc*, or *phaZd1*) accumulated roughly 15 to 30% of PHB after 18 h of growth. The expression of depolymerase protein was confirmed by Western blotting analysis using depolymerase-specific antibodies. Similar amounts of PHB content were observed when rhamnose concentration was reduced to 0.2%, and 0.4% acetate was added as a second carbon source to provide precursor molecules for PHB synthesis. No significant reduction in the numbers and sizes of PHB granules and PHB content was detectable with any of the strains during incubation prolonged for up to 48 h (not shown). The cells were centrifuged, washed with buffer, and incubated in buffers of different pH levels. None of the strains investigated secreted significant amounts of 3HB at low pH (pH 4 [3HB], ≤0.05 mM). At an alkaline pH level (pH 10), only *E. coli* strains containing *phaZa1* and *phaCAB* secreted significant amounts of 3HB (0.4 mM). No 3HB was secreted by cells expressing *phaZb*, *phaZc*, or *phaZd1* at pH 10 (≤0.05 mM; data not shown). Significant amounts of 3HB were detected in *E. coli* strains expressing *phaCAB* and *phaZa1* incubated at pH 7: the secretion of 3HB increased almost continuously and reached 0.88 mM within 8 h, after which the concentration increased only marginally to 0.95 mM at 22 h (Fig. 2). Only a small amount of 3HB was detected (≤0.05 mM) in the *E. coli* cells incubated at pH 7 that contained only the PHB biosynthetic operon or in those in which *phaZa1* was replaced by any of the other three putative PHB depolymerase/hydrolase genes (*phaZb*, *phaZc*, or *phaZd*) (Fig. 2). These data suggested that only *phaZa1* can function as an intracellular PHB depolymerase, resulting in the release of 3HB to the culture fluid in recombinant *E. coli*; however, in our hands, the amount was about 2 orders of magnitudes lower than that reported in references 21 and 23.

To find independent evidence for the function of PhaZa1 as an intracellular PHB depolymerase, the *R. eutropha* wild-type strain and the *phaZa1*, *phaZb*, *phaZc*, or *phaZd1* null mutant were constructed and investigated for the production of 3HB.

PHB-accumulated cells of the corresponding mutant strains were incubated in 0.1 M potassium phosphate buffer (pH 7) at 30°C, and the concentration of the 3HB released was determined by enzymatic assay and by HPLC after derivatization in cell-free samples. The presence of significant amounts of 3HB oligomers could be excluded for all strains by using HPLC analysis after strains underwent derivatization with bromophenacylbromide. The *R. eutropha* wild-type strain and the Δ*phaZb*, Δ*phaZc*, and Δ*phaZd1* mutant strains all secreted significant amounts of monomeric 3HB (1.2 to 1.5 mM) into the culture medium. However, the Δ*phaZa1* mutant secreted significantly less 3HB (≤0.3 mM; Fig. 3), confirming that of the four genes tested, only *phaZa1* contributed significantly to 3HB secretion in vivo. When the *R. eutropha* H16 wild type and the four null mutants were cultivated in NB medium and inspected for the numbers and sizes of PHB granules by fluorescence microscopy, only the Δ*phaZa1* strains showed significantly more and bigger PHB granules between 12 and 18 h of incubation (B. Gebauer and D. Jendrossek, unpublished results). However, after 30 h of incubation, all strains, including the Δ*phaZa1* mutant, had completely reutilized the intermediately accumulated PHB. These results are in agreement with the depolymerase function of PhaZa1 and suggest the presence of at least one additional physiologically important PHB depolymerase.

DISCUSSION

The biochemical mechanism by which intracellularly accumulated PHB can be reutilized (mobilized) is poorly understood. The literature contains frequent reports of putative iPHB depolymerases (iPhaZs) (1, 5, 8, 32, 40, 43) or medium-chain-length iPHA (iPHA$_{MCL}$) depolymerases (3, 4, 13). However, a convincing in vitro assay system for iPhaZs does not exist, and unfortunately, researchers have used differently prepared substrates for assays (6, 15). Artificial PHB granules most often have been used as substrates. Artificial PHB granules, unlike nPHB granules, do not contain phasin proteins or any other proteins but surfactants (e.g., sodium dodecyl sulfate, cholate, or others) at the polymer surface. Some confusion also exists about the term "native" for describing nPHB granules. We have suggested restricting the term "native" to

those natural PHB granules that have been purified by density gradient centrifugation only (15, 26). It is difficult to judge the physiological importance of the in vitro activity observed with PhaZa1 (32) or PhaZd1 (1) with artificial PHB granules because both enzymes have only very little or no (0.43 U/mg) depolymerase activity with the natural substrate nPHB. A bacterial cell has many esterases/hydrolases, some of which might have the ability to hydrolyze ester bonds of artificial PHB in an in vitro system, but such an activity would not indicate whether this enzyme is also able to hydrolyze the densely protein-covered polymer of the native PHB granules in vivo. The importance of the proteinaceous PHB surface layer to the susceptibility of PHB granules for in vitro hydrolysis has been described for a PHB depolymerase purified from *Rhodospirillum rubrum* (9–11, 26, 27). The intracellular expression of the PHB depolymerase PhaZ7 of *Paucimonas lemoignei*, an extracellular PHB depolymerase with unusual specificity for proteinaceous and amorphous nPHB granules (7), in a PHB-accumulating background resulted in an increase of secreted 3HB (37). Therefore, the high depolymerase activity of cautiously isolated nPHB granules could be indicative of a physiological function of the respective protein. In this context, the in vivo system for PHB hydrolysis in recombinant *E. coli* strains harboring the PHB biosynthetic pathway (*phaCAB*) together with the iPhaZ (*phaZa1*) described by Lee and Lee (21, 23, 28) appeared very attractive, and we thought that this in vivo system might be a valuable tool for the detection of potential physiological PHB depolymerase activity of other putative iPhaZs. However, we could not reproduce the data. In our hands, only marginal amounts of 3HB, together with other acids such as acetate, were detected in the culture supernatant of PhaZa1- and PhaCAB-expressing strains of *E. coli*.

The production of 9.6 g/liter 3HB equivalents (92 mM) from 20 g/liter glucose (111 mM) according to reference 23 is a high value: the biochemical pathway allows conversion of one glucose molecule into one molecule of 3HB. The maximum theoretical value of 3HB produced from 111 mM of glucose is, therefore, 111 mM. However, a significant portion of glucose is used for the synthesis of cellular biomass, for the maintenance of metabolism, and for overflow metabolism (acetate production). Therefore, the production of 92 mM of 3HB (83% of the theoretical maximum) is a surprisingly high value. The production of 14 g/liter 3HB (135 mM) from 20 g/liter glucose (111 mM), as shown in Fig. 5 and 6 of the patent for 3HB production (21), is not possible based on theoretical considerations, even if the 9 mM of citrate of the R medium is additionally taken into consideration.

Although the expression of PhaZa1 did not enable recombinant *E. coli* strains to hydrolyze the accumulated PHB completely to 3HB, washed cells were able to secrete significantly more 3HB (range of 1 mM 3HB) than cells of control strains. The finding that strains expressing *phaCAB* also secreted more 3HB than the background (*E. coli* without plasmid) indicated that elevated levels of 3HB-coenzyme A can be used partially for the secretion of 3HB. Only very small amounts of 3HB were secreted when *phaZa1* was replaced with other putative iPhaZs or oligomer hydrolases (*phaZb*, *phaZc*, or *phaZd1*) (Fig. 2), indicating that PhaZa1 is the most likely candidate for a physiological iPHB depolymerase. Recent in vitro data obtained from our laboratory suggest that intracellular PHB de-

polymerase PhaZa1 can cleave accumulated PHB by thiolysis, resulting in the formation of 3HB-coenzyme A instead of the free acid (41). The secretion of free 3HB would therefore require high thioesterase activity.

Recently, several reports were published showing that PHA-accumulating bacteria can rapidly hydrolyze accumulated PHA and secrete 3HB or $3HA_{MCL}$ (3, 24, 30, 31) simply by incubating PHA-rich bacteria in buffers. Mutants of *Pseudomonas putida* defective in intracellular PHA depolymerase secreted considerably less $3HA_{MCL}$, suggesting that iPHA depolymerase activity is responsible for $3HA_{MCL}$ secretion (42). In *R. eutropha*, the Δ*phaZa1* mutant was still able to secrete ~0.3 mM 3HB. Since no soluble exogenous carbon sources were available, *R. eutropha* apparently also has at least one other physiological iPHB depolymerase. This conclusion is in agreement with earlier reports (8, 32, 43). The results with *R. eutropha* cells are complementary to and in agreement with the results obtained with recombinant *E. coli* strains (Fig. 2). However, the concentration of secreted 3HB was rather low (1 to 2 mM) and significantly lower than the secretion of $3HA_{MCL}$ by *P. putida* (3, 30, 31, 42). In conclusion, PhaZa1 is a physiologically important iPHB depolymerase in *R. eutropha* but apparently is only little suited for the efficient biotechnological production of 3HB.

REFERENCES

1. **Abe, T., T. Kobayashi, and T. Saito.** 2005. Properties of a novel intracellular poly(3-hydroxybutyrate) depolymerase with high specific activity (PhaZd) in *Wautersia eutropha* H16. J. Bacteriol. **187:**6982–6990.
2. **Brandl, H., R. A. Gross, R. W. Lenz, and R. C. Fuller.** 1988. *Pseudomonas oleovorans* as a source of poly(β-hydroxyalkanoates) for potential applications as biodegradable polyesters. Appl. Environ. Microbiol. **54:**1977–1982.
3. **de Eugenio, L. I., P. Garcia, J. M. Luengo, J. M. Sanz, J. S. Roman, J. L. Garcia, and M. A. Prieto.** 2007. Biochemical evidence that *phaZ* gene encodes a specific intracellular medium chain length polyhydroxyalkanoate depolymerase in *Pseudomonas putida* KT2442: characterization of a paradigmatic enzyme. J. Biol. Chem. **282:**4951–4962.
4. **Foster, L. J. R., R. W. Lenz, and R. C. Fuller.** 1999. Intracellular depolymerase activity in isolated inclusion bodies containing polyhydroxyalkanoates with long alkyl and functional substituents in the side chain. Int. J. Biol. Macromol. **26:**187–192.
5. **Gao, D., A. Maehara, T. Yamane, and S. Ueda.** 2001. Identification of the intracellular polyhydroxyalkanoate depolymerase gene of *Paracoccus denitrificans* and some properties of the gene product. FEMS Microbiol. Lett. **196:**159–164.
6. **Gebauer, B., and D. Jendrossek.** 2006. Assay of poly(3-hydroxybutyrate) depolymerase activity and product determination. Appl. Environ. Microbiol. **72:**6094–6100.
7. **Handrick, R., S. Reinhardt, M. L. Focarete, M. Scandola, G. Adamus, M. Kowalczuk, and D. Jendrossek.** 2001. A new type of thermoalkalophilic hydrolase of *Paucimonas lemoignei* with high specificity for amorphous polyesters of short chain-length hydroxyalkanoic acids. J. Biol. Chem. **276:**36215–36224.
8. **Handrick, R., S. Reinhardt, and D. Jendrossek.** 2000. Mobilization of poly(3-hydroxybutyrate) in *Ralstonia eutropha*. J. Bacteriol. **182:**5916–5918.
9. **Handrick, R., S. Reinhardt, P. Kimmig, and D. Jendrossek.** 2004. The "intracellular" poly(3-hydroxybutyrate) (PHB) depolymerase of *Rhodospirillum rubrum* is a periplasm-located protein with specificity for native PHB and with structural similarity to extracellular PHB depolymerases. J. Bacteriol. **186:**7243–7253.
10. **Handrick, R., S. Reinhardt, D. Schultheiss, T. Reichart, D. Schuler, V. Jendrossek, and D. Jendrossek.** 2004. Unraveling the function of the *Rhodospirillum rubrum* activator of polyhydroxybutyrate (PHB) degradation: the activator is a PHB-granule-bound protein (phasin). J. Bacteriol. **186:**2466–2475.

11. **Handrick, R., U. Technow, T. Reichart, S. Reinhardt, T. Sander, and D. Jendrossek.** 2004. The activator of the *Rhodospirillum rubrum* PHB depolymerase is a polypeptide that is extremely resistant to high temperature (121 degrees C) and other physical or chemical stresses. FEMS Microbiol. Lett. **230:**265–274.

12. **Hermawan, S., and D. Jendrossek.** 2007. Microscopical investigation of poly(3-hydroxybutyrate) granule formation in *Azotobacter vinelandii*. FEMS Microbiol. Lett. **266:**60–64.

13. **Huisman, G. W., E. Wonink, R. Meima, B. Kazemier, P. Terpstra, and B. Witholt.** 1991. Metabolism of poly(3-hydroxyalkanoates) (PHAs) by *Pseudomonas oleovorans*. Identification and sequences of genes and function of the encoded proteins in the synthesis and degradation of PHA. J. Biol. Chem. **266:**2191–2198.

14. **Jendrossek, D.** 2002. Extracellular polyhydroxyalkanoate depolymerases: the key enzymes of PHA degradation, p. 41–83. *In* Y. Doi and A. Steinbüchel (ed.), Biopolymers, vol. 3b. Polyesters II. Wiley-VCH, Hoboken, NJ.

15. **Jendrossek, D.** 2007. Peculiarities of PHA granules preparation and PHA depolymerase activity determination. Appl. Microbiol. Biotechnol. **74:**1186–1196.

16. **Jendrossek, D., A. Frisse, A. Behrends, M. Andermann, H. D. Kratzin, T. Stanislawski, and H. G. Schlegel.** 1995. Biochemical and molecular characterization of the *Pseudomonas lemoignei* polyhydroxyalkanoate depolymerase system. J. Bacteriol. **177:**596–607.

17. **Jendrossek, D., and R. Handrick.** 2002. Microbial degradation of polyhydroxyalkanoates. Annu. Rev. Microbiol. **56:**403–432.

18. **Jendrossek, D., A. Schirmer, and H. G. Schlegel.** 1996. Biodegradation of polyhydroxyalkanoic acids. Appl. Microbiol. Biotechnol. **46:**451–463.

19. **Kobayashi, T., M. Shiraki, T. Abe, A. Sugiyama, and T. Saito.** 2003. Purification and properties of an intracellular 3-hydroxybutyrate-oligomer hydrolase (PhaZ2) in *Ralstonia eutropha* H16 and its identification as a novel intracellular poly(3-hydroxybutyrate) depolymerase. J. Bacteriol. **185:**3485–3490.

20. **Kobayashi, T., K. Uchino, T. Abe, Y. Yamazaki, and T. Saito.** 2005. Novel intracellular 3-hydroxybutyrate-oligomer hydrolase in *Wautersia eutropha* H16. J. Bacteriol. **187:**5129–5135.

21. **Lee, S.-Y., and Y. Lee.** June 2003. (*R*)-Hydroxycarboxylic acid producing recombinant microorganisms and process for preparing (*R*)-hydroxycarboxylic acid using the same. WIPO patent WO03/046159.

22. **Lee, S. Y., and N. H. Chang.** 1993. High cell density cultivation of *Escherichia coli* using sucrose as a carbon source. Biotechnol. Lett. **15:**971–974.

23. **Lee, S. Y., and Y. Lee.** 2003. Metabolic engineering of *Escherichia coli* for production of enantiomerically pure (*R*)-(−)-hydroxycarboxylic acids. Appl. Environ. Microbiol. **69:**3421–3426.

24. **Lee, S. Y., Y. Lee, and F. Wang.** 1999. Chiral compounds from bacterial polyesters: sugars to plastics to fine chemicals. Biotechnol. Bioeng. **65:**363–368.

25. **Merrick, J. M., and M. Doudoroff.** 1964. Depolymerization of poly-β-hydroxybutyrate by intracellular enzyme system. J. Bacteriol. **88:**60–71.

26. **Merrick, J. M., D. G. Lundgren, and R. M. Pfister.** 1965. Morphological changes in poly-beta-hydroxybutyrate granules associated with decreased susceptibility to enzymatic hydrolysis. J. Bacteriol. **89:**234–239.

27. **Merrick, J. M., R. Steger, and D. Dombroski.** 1999. Hydrolysis of native poly(hydroxybutyrate) granules (PHB), crystalline PHB, and artificial amorphous PHB granules by intracellular and extracellular depolymerases. Int. J. Biol. Macromol. **25:**129–134.

28. **Park, S. J., S. Y. Lee, and Y. Lee.** 2004. Biosynthesis of R-3-hydroxyalkanoic acids by metabolically engineered *Escherichia coli*. Appl. Biochem. Biotechnol. **113-116:**373–379.

29. **Pohlmann, A., W. F. Fricke, F. Reinecke, B. Kusian, H. Liesegang, R. Cramm, T. Eitinger, C. Ewering, M. Potter, E. Schwartz, A. Strittmatter, I. Voss, G. Gottschalk, A. Steinbuchel, B. Friedrich, and B. Bowien.** 2006. Genome sequence of the bioplastic-producing "Knallgas" bacterium *Ralstonia eutropha* H16. Nat. Biotechnol. **24:**1257–1262.

30. **Ren, Q., A. Grubelnik, M. Hoerler, K. Ruth, R. Hartmann, H. Felber, and M. Zinn.** 2005. Bacterial poly(hydroxyalkanoates) as a source of chiral hydroxyalkanoic acids. Biomacromolecules **6:**2290–2298.

31. **Ruth, K., A. Grubelnik, R. Hartmann, T. Egli, M. Zinn, and Q. Ren.** 2007. Efficient production of (*R*)-3-hydroxycarboxylic acids by biotechnological conversion of polyhydroxyalkanoates and their purification. Biomacromolecules **8:**279–286.

32. **Saegusa, H., M. Shiraki, C. Kanai, and T. Saito.** 2001. Cloning of an intracellular poly[D(−)-3-hydroxybutyrate] depolymerase gene from *Ralstonia eutropha* H16 and characterization of the gene product. J. Bacteriol. **183:**94–100.

33. **Saegusa, H., M. Shiraki, and T. Saito.** 2002. Cloning of an intracellular D(−)-3-hydroxybutyrate-oligomer hydrolase gene from *Ralstonia eutropha* H16 and identification of the active site serine residue by site-directed mutagenesis. J. Biosci. Bioeng. **94:**106–112.

34. **Saito, T., and T. Kobayashi.** 2002. Intracellular degradation of PHAs, p. 23–40. *In* Y. Doi and A. Steinbüchel (ed.), Biopolymers, vol. 3b. Polyesters II. Wiley-VCH, Hoboken, NJ.

35. **Schlegel, H. G., G. Gottschalk, and R. Von Bartha.** 1961. Formation and utilization of poly-beta-hydroxybutyric acid by Knallgas bacteria (*Hydrogenomonas*). Nature **191:**463–465.

36. **Shinohe, T., M. Nojiri, T. Saito, T. Stanislawski, and D. Jendrossek.** 1996. Determination of the active sites serine of the poly(3-hydroxybutyrate) depolymerases of *Pseudomonas lemoignei* (PhaZ5) and of *Alcaligenes faecalis*. FEMS Microbiol. Lett. **141:**103–109.

37. **Shiraki, M., T. Endo, and T. Saito.** 2006. Fermentative production of (*R*)-(−)-3-hydroxybutyrate using 3-hydroxybutyrate dehydrogenase null mutant of *Ralstonia eutropha* and recombinant *Escherichia coli*. J. Biosci. Bioeng. **102:**529–534.

38. **Shuster, C. W., and M. Doudoroff.** 1962. A cold-sensitive D(−)beta-hydroxybutyric acid dehydrogenase from *Rhodospirillum rubrum*. J. Biol. Chem. **237:**603–607.

39. **Spiekermann, P., B. H. Rehm, R. Kalscheuer, D. Baumeister, and A. Steinbüchel.** 1999. A sensitive, viable-colony staining method using Nile red for direct screening of bacteria that accumulate polyhydroxyalkanoic acids and other lipid storage compounds. Arch. Microbiol. **171:**73–80.

40. **Tseng, C. L., H. J. Chen, and G. C. Shaw.** 2006. Identification and characterization of the *Bacillus thuringiensis phaZ* gene, encoding new intracellular poly-3-hydroxybutyrate depolymerase. J. Bacteriol. **188:**7592–7599.

41. **Uchino, K., T. Saito, B. Gebauer, and D. Jendrossek.** 2007. Isolated poly(3-hydroxybutyrate) (PHB) granules are complex bacterial organelles catalyzing formation of PHB from acetyl coenzyme A (CoA) and degradation of PHB to acetyl-CoA. J. Bacteriol. **189:**8250–8256.

42. **Wang, L., W. Armbruster, and D. Jendrossek.** 2007. Production of medium-chain-length hydroxyalkanoic acids from *Pseudomonas putida* in pH stat. Appl. Microbiol. Biotechnol. **74:**1047–1053.

43. **York, G. M., J. Lupberger, J. Tian, A. G. Lawrence, J. Stubbe, and A. J. Sinskey.** 2003. *Ralstonia eutropha* H16 encodes two and possibly three intracellular poly[D-(−)-3-hydroxybutyrate] depolymerase genes. J. Bacteriol. **185:**3788–3794.