Studies on the Influence of Phasins on Accumulation and Degradation of PHB and Nanostructure of PHB Granules in *Ralstonia eutropha* H16

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Received September 24, 2006; Revised Manuscript Received November 6, 2006

Phasins play an important role in the formation of poly(3-hydroxybutyrate) [PHB] granules and affect their size and number in the cells. Recent studies on the PHB granule proteome and analysis of the complete genomic DNA sequence of *Ralstonia eutropha* H16 have identified three homologues of the phasin protein PhaP1. In this study, mutants of *R. eutropha* deficient in the expression of the phasin genes *phaP1*, *phaP2*, *phaP3*, *phaP4*, *phaP12*, *phaP123*, and *phaP1234* were examined by gas chromatography. In addition, the nanostructures of the PHB granules of the wild-type and of the mutants were imaged by atomic force microscopy (AFM), and the molecular masses of the accumulated PHB were analyzed by gel permeation chromatography. For this, cells were cultivated under conditions permissive for accumulation of PHB and were then cultivated under conditions permissive for degradation of PHB. Mutants deficient in the expression of *phaP2*, *phaP3*, or *phaP4* genes mobilized the stored PHB only slowly like the wild-type, whereas degradation occurred much earlier and faster in the *phaP1* single mutant as well as in all multiple mutants defective in the *phaP1* gene plus one or more other phasin genes. This indicated that the presence of the major phasin PhaP1 on the granule surface is important for PHB degradation and that this phasin is therefore of particular relevance for PHB accumulation. It was also shown that the molecular weights of the accumulated PHB were identical in all examined strains; phasins have therefore no influence on the molecular weight of PHB. The AFM images obtained in this study showed that the PHB granules of *R. eutropha* H16 form a single interconnected system inside the wild-type cells.

Introduction

Polyhydroxyalkanoates (PHAs) are found in many prokaryotes as intracellular storage compounds for carbon and energy, and they are synthesized under unbalanced growth conditions (i.e., when a carbon source is available in excess and if another nutrient is depleting).[1] PHAs are then accumulated in the cells and deposited as insoluble inclusions (PHA granules) in the cytoplasm. PHAs exhibit thermoplastic and/or elastomeric properties and are nontoxic and biodegradable; in addition, they can be produced from renewable resources. PHAs are therefore considered for many technical and medical applications.[2,3] Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) copolymers and the homopolymer poly(3-hydroxybutyrate), PHB, have been commercialized under the trade name Biopol.[4]

The facultative chemolithoautotrophic hydrogen-oxidizing bacterium *Ralstonia eutropha*, which serves as a model organism to study various aspects of PHB metabolism and the structure of PHB granules in bacteria, was the first organism from which the PHA biosynthesis genes were cloned and heterologously expressed in *Escherichia coli*.[5−7] The genome of *R. eutropha* contains the PHA operon and comprises three genes encoding a β-ketothiolase (*phaA*), an acetoacetyl-CoA reductase (*phaB*), and a PHA synthase (*phaC*). Recent studies revealed interesting information regarding the PHB granule surface and have shown that four classes of proteins constitute the layer at the surface of the granules. These are, beside PhaC, PHA depolymerases (PhaZ) including the 3HB-oligomer hydrolase (PhaY) for the degradation of PHB, small amphiphilic proteins referred to as phasins (PhaP) that are regarded as the main structural components, and a regulator protein of phasin expression (PhaR).[8] During PHB production, PhaC is covalently linked to the growing polymer chain; the enzyme is thereby attached to the granule surface.[9] The concentration of PhaC in the cells of *Paracoccus denitrificans* influences the molecular weight of the accumulated PHB, with its molecular weight inversely related to the concentration of PhaC.[10]

Phasins contribute up to 5% of the total cell protein of PHB accumulating cells and cover most of the granule surface, thereby exerting a severe influence on the size and number of PHB granules in the cells.[11] PhaP1 is the major phasin of *R. eutropha* and has been studied in the most detail. Beside PhaP1, three homologues were recently detected in *R. eutropha*.[12] Cells of mutants lacking PhaP1 produce only one single granule per cell, and the amounts of PHB accumulated in the cells are significantly lower than in the wild-type.[13] On the other hand, cells of recombinant strains that overexpress PhaP1 contain a large number of very small granules. Remarkably, the amounts of PhaP1 protein produced are exactly regulated by means of the transcriptional regulator PhaR,[14] thereby ensuring that on one hand the surface is entirely covered and that on the other hand no excess PhaP1 is produced by the cells. This regulation also insures that no PhaP1 is produced, if PHB formation is impaired and when conditions are not permissive for PHB accumulation or if *phaC* is deleted.[13,14,15]

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10.1021/bm060912e CCC: $37.00
Published on Web 12/22/2006

Table 1. Bacterial Strains Used in This Study

R. eutropha strain	description	ref
H16	wild-type	25
Re1052 (Δ*phaP1*)	*phaP1*-precise-deletion gene replacement strain (Re1052), derived from *R. eutropha* H16	15
Δ*phaP2*ΩKm (Δ*phaP2*)	*phaP2*-negative (Km^r), derived from *R. eutropha* H16	26
Δ*phaP3* (Δ*phaP3*)	*phaP3*-precise-deletion gene replacement strain, derived from *R. eutropha* H16	26
Δ*phaP4* (Δ*phaP4*)	*phaP4*-precise-deletion gene replacement strain, derived from *R. eutropha* H16	26
Δ*phaP1*Δ*phaP2*ΩKm (Δ*phaP12*)	*phaP1*- and *phaP2*-negative; (Km^r), precise-deletion gene replacement strain, derived from *R. eutropha* Δ*phaP1*	26
Δ*phaP1* Δ*phaP2*ΩKmΔ*phaP3* (Δ*phaP123*)	*phaP1*, *phaP2*-negative; (Km^r) and *phaP3* precise-deletion gene replacement strain, derived from *R. eutropha* Δ*phaP1* Δ*phaP2 Km^r*	26
Δ*phaP1* Δ*phaP2*ΩKmΔ*phaP3* Δ*phaP4* (Δ*phaP1234*)	*phaP1*-, *phaP2*-negative; (Km^r), *phaP3*, and *phaP4* precise-deletion gene replacement strain, derived from *R. eutropha* Δ*phaP1* Δ*phaP2*Ω*WKm^r* Δ*phaP3*	26

The in vivo function of the additional three PhaP1 homologues, which occur only in minor amounts in the cells, and their influence on the nanostructure of PHB granules is unknown as yet. Whereas PhaP3 and PhaP4 are also bound to the surface of the PHB granules in vivo, PhaP2 was detected in the cytoplasm but is capable of binding to PHB granules in vitro.[12] It has been proposed that at least one of these phasins might support the PHA depolymerases to obtain access to their substrate (PHB) across the layer of phospholipids and the abundant PhaP1.[16,17]

PHB accumulation and degradation were studied in the wild-type strain H16 and in various phasin-negative mutants to examine the in vivo function of phasins in *R. eutropha*. The molecular masses of PHB synthesized in the wild-type, the Δ*phaP1* mutant, and the quadruple mutant Δ*phaP1234* of *R. eutropha* H16 were analyzed by gel permeation chromatography. In addition, the surface structures of PHB granules isolated from cells of the wild-type and of the phasin mutants of *R. eutropha* were examined at the nanoscale by means of atomic force microscopy (AFM). So far, only a few AFM studies of PHB granules of the wild-type but not on phasin-negative mutants were published.[18,19]

Experimental Procedures

Bacterial Strains. All strains of *R. eutropha* used in this study are listed in Table 1.

Culture Conditions and Cell Harvest. The wild-type strain H16 and the mutants Δ*phaP1*, Δ*phaP2*ΩKm, Δ*phaP3*, Δ*phaP4*, Δ*phaP1*Δ*phaP2*ΩKm, Δ*phaP1*Δ*phaP2*ΩKmΔ*phaP3*, and Δ*phaP1*-Δ*phaP2*ΩKmΔ*phaP3*Δ*phaP4* were cultivated in 120 mL of mineral salt medium (MSM)[1] containing 0.5 g/L NH_4Cl and 1.5% (w/v) sodium gluconate for 24 h at 30 °C in 1 L Erlenmeyer flasks with baffles to isolate PHB granules for AFM investigation. These cultures were inoculated with 10 mL of pre-cultures grown overnight on NB medium[20] in 100 mL Erlenmeyer flasks without baffles. Cells were harvested by centrifugation for 20 min at 3300*g* and then washed in 50 mL of 0.9% (w/v) NaCl. The cell pellets were frozen at −70 °C for 12 h and subsequently lyophilized.

The wild-type strain H16 and the mutant strains Δ*phaP1* and Δ*phaP1*Δ*phaP2*ΩKmΔ*phaP3*Δ*phaP4* were also cultivated for 72 h and at 30 °C in 500 mL of MSM containing 0.5 g/L NH_4Cl and 1.5% (w/v) sodium gluconate in 2 L Erlenmeyer flasks with baffles to obtain PHB for gel permeation chromatography (GPC) measurements. These cultures were inoculated with 50 mL of pre-cultures grown overnight on MSM containing 0.5 g/L NH_4Cl and 1.5% (w/v) sodium gluconate in 250 mL Erlenmeyer flasks with baffles. Cells were harvested by centrifugation for 20 min using a Sorvall GS3 rotor at 5000 rpm and washed in 250 mL of 0.9% (w/v) NaCl.

For recording their long time PHB storage behavior, cells of the wild-type strain H16 and of the mutant strains Δ*phaP1*, Δ*phaP2*ΩKm, Δ*phaP3*, Δ*phaP4*, Δ*phaP1*Δ*phaP2*ΩKm, Δ*phaP1*Δ*phaP2*ΩKmΔ*phaP3*, or Δ*phaP1*Δ*phaP2*ΩKmΔ*phaP3*Δ*phaP4* were cultivated in 500 mL of MSM containing 1.0 g/L NH_4Cl and 1.5% (w/v) sodium gluconate as a carbon source in 2 L flasks with baffles. Cultures were inoculated with 50 mL of pre-cultures grown overnight in NB medium at 30 °C. In the latter cultures, after 64 h, the original NH_4Cl concentration of 1.0 g/L was restored by adding sterilized NH_4Cl solution to the medium.

PHA Quantification. Samples were subjected to methanolysis in the presence of 15% (w/v) sulfuric acid, and the methyl esters of 3-hydroxybutyric acid were analyzed by gas chromatography (HP 6850 gas chromatograph, Agilent Technologies Inc., Palo Alto, CA).[21]

Gel Permeation Chromatography (GPC). Lyophilized cells were ground, dissolved in chloroform, and stirred constantly with a magnetic stirrer at room temperature for 72 h. This solution was filtered through a paper filter to separate the dissolved PHB from the remaining insoluble cell components. The solution was poured into chilled ethanol (−70 °C), leading to the precipitation of PHB to separate PHB from other compounds dissolved in chloroform. PHB was separated from the solution by filtration through a paper filter and air-dried for 24 h. For determining the molecular weight of PHB, 10 mg of the isolated polymer was dissolved in 2 mL of chloroform and filtered through a Minisart SRP4 filter (Sartorius, Göttingen, Germany) to remove fine insoluble particles. A Waters HPLC apparatus (Milford, MA) consisting of a 717plus auto sampler (Rheodyne 7725i), a 515 HPLC pump, four consecutive columns (Styragel HR3, Styragel HR4, Styragel HR5, and Styragel HR6), a Jetstream 2 column oven, and a RI detector (410 differential refractometer) was used for analyses. A total of 100 μL of the solutions obtained was injected; chloroform was used as the mobile phase at a flow rate of 0.5 mL/min and at a temperature of 35 °C. The data were analyzed using the Millenium Chromatography Manager GPC software (Waters, Milford, MA). The molecular masses were analyzed in relation to polystyrene standards (Polymer Standards Service, Mainz, Germany).

Atomic Force Microscopy (AFM). To obtain protein free PHB granules without destroying their microstructures, lyophilized cells were weighed and ground. One sample of each strain was suspended in 25 μL of 5% (v/v) sodium hypochlorite per milligram of lyophilized cells and stirred on a rotation shaker at room temperature for 48 h. After all non-PHB components of the cells had dissolved in the sodium hypochlorite solution, the granules were harvested by centrifugation for 20 min at 3300*g* and washed 6 times in 50 mL of double-distilled H_2O. The harvested PHB granules were frozen at −70 °C for 12 h, lyophilized afterward, suspended in 5 mL of acetone/diethyl ether (1:2; v/v), and shaken for 1 h to remove all remaining lipids. The PHB granules were harvested by centrifugation for 20 min at 3300*g*, and the acetone/diethyl ether treatment was repeated. After the second harvest, the granules were dried under flowing air. In the final step, the dried granules were suspended in 1 mL of H_2O_{bidest} by two treatments with a MS72 sonic probe (Sonifier 250, Branson Sonic Power

Company, Danbury, CT) for 15 s each. The granule suspensions were cooled on ice during sonication. This suspension was used for AFM measurements. To provide a smooth, hydrophilic, and negatively charged surface as a background, 20 μL of the granule suspension was dripped on a sheet of freshly broken mica and air-dried. The atomic force microscopy measurements were carried out in the tapping mode using an apparatus of Digital Instruments (Santa Barbara, CA) and equipped with a Q-control unit.[22]

Results and Discussion

PHB Storage Behavior. In this study, the PHB storage behavior of several mutants of *R. eutropha* (*phaP1*, *phaP2*, *phaP3*, *phaP4*, *phaP1phaP2*, *phaP1phaP2phaP3*, or *phaP1phaP2phaP3phaP4*) deficient in the expression of phasins was examined by gas chromatographic analyses of the PHB contents of the cells and compared to that of the wild-type. These long time observations of the PHB contents of phasin-negative mutants revealed some very interesting and unexpected findings, and three different types of PHB storage behavior could be distinguished (Figure 1).

A high PHB content was reached relatively quickly by cells of the wild-type H16 and of the single mutants Δ*phaP2*, Δ*phaP3*, and Δ*phaP*4 (group I). Maximal PHB contents of about 45% (w/w) of cell dry weight (CDW) were reached after about 37 h of cultivation (Figure 1A), and a high level was kept over the entire cultivation period under nitrogen limitation (until 64 h). This indicated that the absence of the phasins PhaP2, PhaP3, or PhaP4 had no significant impact on PHB synthesis and accumulation in comparison to the wild-type. Cells of the wild-type and of the single mutants Δ*phaP2*, Δ*phaP3*, and Δ*phaP4* degraded the accumulated PHB relatively slowly at the beginning; however, when ammonium was added after 64 h, degradation of the accumulated PHB was enhanced in all four strains. It was noticed that cells of the mutants Δ*phaP2* and Δ*phaP4* degraded the accumulated PHB faster than cells of the mutant Δ*phaP3* and of the wild-type. Whereas the PHB contents of the cells of mutants Δ*phaP2* and Δ*phaP4* were about only 5−7% of CDW (w/w) at the end of the cultivation after 114 h, PHB amounted still to about 14−16% of CDW (w/w) in cells of the wild-type and of mutant Δ*phaP3* (Figure 1B).

Cells of the three multiple mutants Δ*phaP12*, Δ*phaP123*, and Δ*phaP1234* (group II) accumulated PHB much more slowly than the cells of all other strains and reached maximal PHB contents of about 35−40% of CDW (w/w) after 37 h of cultivation. Afterward, the PHB contents of the cells began to decrease sharply (Figure 1A). This very interesting behavior, which is shared by all multiple mutants defective in at least *phaP1* plus *phaP2* and to some extent also by the Δ*phaP1* single mutant, and is a remarkable difference from the behavior of all other mutants, thus indicating that the presence of PhaP1 or PhaP2 or of both phasins is important for PHB degradation in *R. eutropha*. After only about 70 h of cultivation, cells of the strains belonging to group II had already degraded almost the entire PHB and retained a low but nearly constant PHB content of 3−4% of CDW (w/w) only (Figure 1B). Interestingly, the accumulated PHB was not completely degraded to zero. Thus, the lack of PhaP3 or PhaP4 alone seems not to have a clearly measurable effect on PHB accumulation or degradation. An effect of PhaP2 on PHB metabolism is difficult to understand because PhaP2 was recently shown to not be associated with the PHB granules in vivo,[12,23] although the purified PhaP2 binds to PHB granules in vitro.[12]

Cells of the single mutant Δ*phaP1* exhibited a PHB storage behavior that was between that of groups I and II. They also

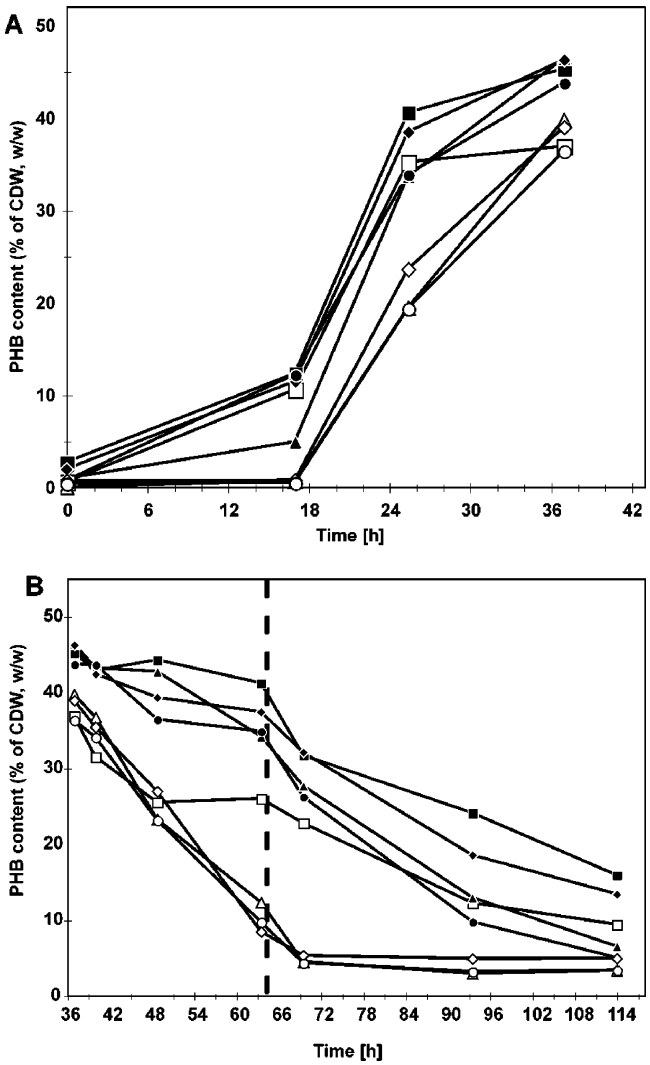

Figure 1. PHB storage behavior of *R. eutropha* wild-type strain H16 and all examined phasin-negative mutants. Cells were cultivated at 30 °C in 120 mL of MSM containing 15 g/L sodium gluconate and 1.0 g/L NH_4Cl in 2 L Erlenmeyer flasks equipped with baffles as described in the Experimental Procedures. After 64 h of cultivation (marked by dashed line), NH_4Cl was added to the cultures to a concentration of 1.0 g/L. Panel A: behavior of cells during the PHB accumulation phase during 37 h incubation. Panel B: behavior of cells after exhaustion of NH_4Cl (37−64 h) and after addition of NH_4Cl until the end of the cultivation period (64−114 h). Symbols: ■, H16; □, Δ*phaP1*; ▲, Δ*phaP2*; ◆, Δ*phaP3*; ●, Δ*phaP4*; ◇, Δ*phaP12*; △, Δ*phaP123*; and ○, Δ*phaP1234*.

accumulated PHB relatively fast to their maximal contents like the cells of group I; however, the maximum PHB content was lower (35% of CDW, w/w), and as soon as the maximum PHB content was reached after about 24 h, the PHB content of the cells began to decrease rapidly (Figure 1A). This behavior is also shared by all double, triple, and quadruple mutants lacking an intact *phaP1* gene. However, cells of the single mutant Δ*phaP1* degraded PHB faster than cells belonging to group II only at the beginning. Later, degradation continued at a lower rate that was comparable to the rates in the wild-type and in mutant Δ*phaP3*; degradation was therefore lower than in the single mutants Δ*phaP2* and Δ*phaP4* or even in the multiple mutants belonging to group II. At the end of the cultivation experiment, the Δ*phaP1* cells contained about 9.5% PHB of CDW (w/w) (Figure 1B).

The *phaP1* mutants as well as the double, triple, and quadruple mutants, which lacked an intact *phaP1* gene in

addition to at least one other phasin, accumulated PHB at a lower rate but degraded PHB at a higher rate. This may be explained by constitutive PHB degradation occurring in parallel to PHB accumulation in the cells.[24] In accordance with this assumption, the maximum PHB accumulation rate or PHB content of the mutants should be lower than the maximum PHB content of the wild-type as it was observed (Figure 1).

Molecular Weights of Accumulated PHB. After the first phasin, PhaP1, of *R. eutropha* H16 was discovered more than 10 years ago,[13] the in vivo function of these amphiphilic proteins, which occur at the surface of PHB granules, was intensively discussed. One of several proposed theories was that phasins might stabilize the growing PHB chain during PHB synthesis, thus preventing breakage of the growing PHB molecule.[25] Consequently, the molecular weight of PHB synthesized in cells possessing a phasin should be higher than in cells lacking a phasin. Phasins may also interact with the catalytically active enzymes (i.e., PHA synthases and PHB depolymerases) at the surface of the granules, thereby influencing the activity of these enzymes and thus the molecular weight of the accumulated PHB. In 1998, Maehara and co-workers showed that an increase of the gene dosage of the PHA synthase PhaC led to a decrease of the molecular weight of the PHA accumulated in *Paracoccus denitrificans*.[10] Therefore, one of the aims of this study was to examine whether or not the presence of phasins impact the molecular weight of PHB.

PHB was isolated from cells of the wild-type H16 and of the two mutants Δ*phaP1* and Δ*phaP1234*, which were cultivated for 72 h in the presence of sodium gluconate as described in the Experimental Procedures. These three strains were chosen because they represented the broadest possible spectrum of variations: all phasins present (H16), the main phasin PhaP1 absent (Δ*phaP1*), and all phasins absent (Δ*phaP1234*). The molecular weights were determined by GPC as described in the Experimental Procedures. The data demonstrated very clearly that the molecular weights of PHB isolated from cells of the mutants Δ*phaP1* and Δ*phaP1234* were almost exactly the same with the molecular weight of PHB isolated from cells of the wild-type. All isolated PHB samples exhibited an average value of M_w 1700 ± 100 kDa with polydispersities (M_w/M_n) of 1.8. Therefore, the molecular weights of PHB isolated from the three strains did not exhibit significant differences, and the phasins do obviously not have an impact on the molecular weights. This makes it very unlikely that phasin proteins influence the degree of polymerization. If phasins have a stabilizing influence on the nascent PHB polymer chain, the molecular weight at least in the quadruple mutant Δ*phaP1234* should be lower than in the wild-type.

AFM Measurements. Although phasins have been shown to have an immense impact on the macro- and microstructures of PHB granules in living *R. eutropha* cells, phasin-negative mutants have not been investigated by AFM until now. We therefore performed tapping mode AFM measurements of PHB granules that were isolated from the wild-type H16 and all currently available phasin-negative mutants.

The most unexpected result was obtained with PHB granules isolated from cells of the wild-type H16 and from the single mutants Δ*phaP2*, Δ*phaP3*, and Δ*phaP4* (group I). These PHB granules stuck together and formed one interconnected structure inside every cell like a skeleton and retained the form of the cells after their disintegration. The connections between the single granules must be caused by PHB itself as all proteins and membranes were removed by hypochlorite. On the macrostructural level, no differences between the granules of these strains belonging to group I, which have PhaP1 expression in common, were observed (Figure 2). This observation was not made in the Δ*phaP1* single mutant or in the multiple mutants Δ*phaP12*, Δ*phaP123*, and Δ*phaP1234* belonging to group II,

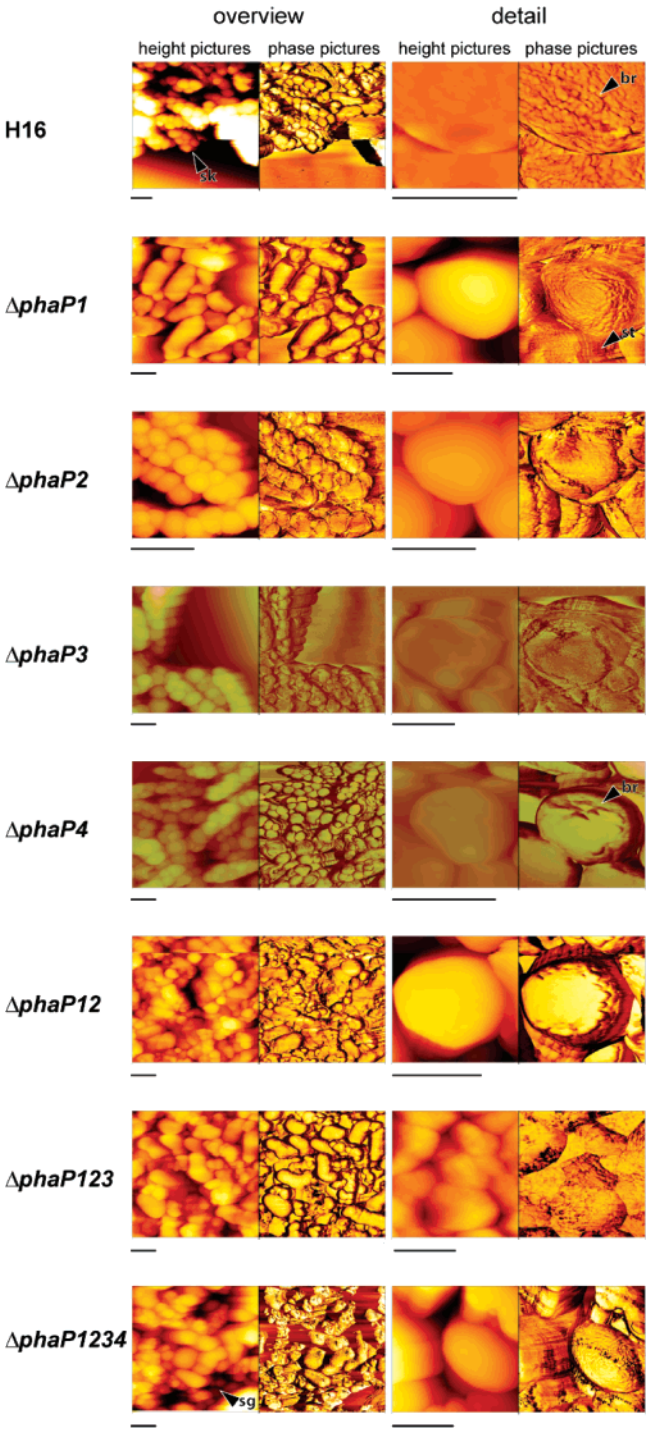

Figure 2. AFM pictures performed in the tapping mode of PHB granules isolated from cells of the wild-type and all examined phasin-negative mutants of *R. eutropha*. Cells were cultivated in 120 mL of MSM containing 0.5 g/L NH_4Cl and 15 g/L sodium gluconate for 24 h at 30 °C in 1 L Erlenmeyer flasks with baffles. PHB granules were isolated with 25 μL of 5% (v/v) sodium hypochlorite per milligram of lyophilized cells. The bars shown on the left side at the overview photographs represent a length of 1 μm, whereas the bars shown on the right side at the detailed photographs represent a length of 0.5 μm. Arrows indicate the linear strands (st) and the brain-like structures (br) as well as the skeleton structures of the granules (sk) and the separated granules (sg).

which have in common a lack of PhaP1. The granules isolated from the Δ*phaP1* mutant were much larger than all others. Their actual size was the same as the size of the whole granule skeletons of the strains belonging to group I, suggesting that all PHB granules coalesced to one single large granule in these cells. In fact, the formation of one large single granule per cell was already previously shown for a Tn*5*-induced *phaP1* insertion mutant by electron microscopic studies.[13] These interconnected PHB granules were not observed in other AFM studies on the PHB granules of the wild-type of *R. eutropha*.[18] Dennis and co-workers[18] observed instead separated PHB granules. This may be due to the very drastic methods employed to isolate PHB granules from the cells by sonication for 5 min using 5 s on and 7 s off cycles. Afterward, the isolated PHB granules were added to a 45 °C SDS solution (0.5%, w/v). This solution was gently mixed and incubated at 45 °C for 5 min.[18] Washing with SDS and also disruption of the cells in a French press or by ultrasonication imposes very harsh conditions on the PHB granules and might cause severe damage of the microstructures of the granules, thereby leading to artifacts. For our investigations, the harvested cells were lyophilized and directly dissolved in hypochlorous acid. This procedure led to the disintegration of all proteins, yielding purified PHB. During the whole isolation process, the PHB granules never encountered any strong mechanical forces. Only when the PHB granules were resuspended were they shortly exposed to two 15 s pulses of mild ultrasonication; this procedure probably prevented the filigree structures from any serious harm. Therefore, our study depicts the original structures of the PHB granules in the wild-type and in the various phasin-negative mutants of *R. eutropha*. These granule skeletons are usually not recognized in electron microscopic examinations since only two-dimensional images of the structures are obtained. One exception is the study of McCool and co-workers on the structure of PHB granules from *Bacillus megaterium*.[27] Transmission electron microscopy images of PHB granules isolated from cells after different periods of cultivation revealed thin bridges between granules. However, the influence of phasins was not investigated and discussed in this study.[27]

A comparison of the AFM images obtained from PHB granules isolated from strains Δ*phaP12*, Δ*phaP123*, and Δ*phaP1234* with images obtained from PHB granules of the single mutants belonging to group I revealed at first glance that the granules of these mutants are very far from having a normal shape (Figure 2). The more the phasins were missing in the mutant strains, the more the regular shape of their granules was lost. Very small and very large granules could be observed side by side in all these samples. The large granules did not exhibit the round and smooth appearance of group I granules. Whereas the average size of these PHB granules was about the same as the average size of the PHB granules of the wild-type, the sizes of individual PHB granules exhibited a greater variance. The majority of these granules was either much bigger or much smaller than the average, and many of them showed an oval shape rather than a round and regular shape like the PHB granules of strains belonging to group I. Apart from this, the most striking difference of granules from all mutants, which were unable to produce one of the other phasins in addition to PhaP1, to the PHB granules of group I was the lack of the granule skeleton phenotype typical for granules isolated from mutant strains belonging to group I (Figure 2). The most probable explanation for this effect is that the newly synthesized small granules in cells of these strains are not sufficiently covered by an envelope or a structure providing system, respectively. The granules obviously merge into irregular granules as soon as two of them establish a closer contact in the cell. In contrast, PHB granules, which did not establish such contacts, did not merge with each other and remained relatively small.

This occurrence of these three groups is of particular interest as it corresponds perfectly with the groupings made due to differences in PHB accumulation and degradation. The most astonishing effect in both cases is that a mutation of *phaP1* and *phaP2* has a completely different effect than a mutation of *phaP1* alone, although the mutation of *phaP2* alone has no measurable effect at all. The most striking nanostructures were the linear strands arranged in parallel arrays, which occurred on almost all PHB granules in any strain (Figure 2). These strands traversed the PHB granule surface in distances of approximately 7 nm and were already discovered previously by Dennis et al.[18] These 7 nm parallel arrays were perfectly confirmed as the original surface structure of the isolated PHB granules. As they were observed on almost all protein-free PHB granules isolated from the wild-type and also from all mutant strains, phasins obviously do not have an influence on these structures, making it very probable that they are related to the PHA synthase protein.

In many cases, the relatively smooth PHB granule surface was interrupted by carves forming a structure resembling the brain of mammals (Figure 2). They resembled the rough surface structures of granules that were described by Dennis and co-workers as being beside smooth granules.[18] These brain-like structures were especially frequent in PHB granules of the wild-type H16 and of the mutant strains Δ*phaP4*, Δ*phaP123*, and Δ*phaP1234*. Other structures, which appeared in almost all types of granules, were imprints or hunches. Sometimes the granules look as if physical force was applied to the them, pulling parts of them out, or if a footprint was pressed into their surface.

Another element, which has been described as typical for the native structure of PHB granules isolated from cells of the wild-type of *R. eutropha*, is globular structures with a central pore that occurs at the surface of PHB granules.[18] If these globular structures really consist of phasins, it is impossible that they are necessary for either PHB accumulation or degradation because the long-term cultivation experiments of the quadruple phasin-negative mutant showed that this mutant is able to accumulate almost as much PHB as the wild-type. Therefore, phasins are not essential for PHB synthesis and accumulation. Our AFM experiments did not identify these globular structures in the wild-type or in the mutant strains of *R. eutropha* H16 because all proteins were removed from the PHB granules by sodium hypochlorite treatment.

Conclusion

Summing up all results of this study, it became obvious that the phasins exert in *R. eutropha* not only a severe influence on the structure of PHB granules but also on PHB accumulation and in particular on PHB degradation. Besides a stabilizing effect on the dispersion of the water-insoluble PHB in the cytoplasm, which is most probably the function of the major phasin PhaP1, phasins affect directly or indirectly PHB degradation in the cells when they utilize PHB as an energy and carbon source in the absence of an external carbon source. Indirect effects might, for example, be caused by altered surface to volume ratios of the PHB granules, whereas a direct effect might be caused by interactions of a phasin with PHB depolymerases. Since PHB depolymerases are constitutively expressed in the

wild-type of *R. eutropha*,[24] the lack of a phasin in a mutant will have an immediate effect on PHB accumulation and degradation rates. The effect caused by a particular phasin could theoretically be positive, for example, by providing access of a PHB depolymerase to the surface of the PHB granules and thereby to the substrate or by stimulating the activity of the PHB depolymerase. Evidence was provided that this could in some mutants also cause constitutive PHB degradation in parallel to PHB synthesis, thereby resulting in a lower PHB accumulation rate under conditions permissive for PHB biosynthesis and in a higher PHB degradation rate under conditions permissive for PHB degradation. It is unlikely that the effects of phasins are indirectly caused by the enormous amount of phasin protein that is synthesized by the ribosomes because phasin synthesis occurs only in the stationary growth phase when growth has ceased and when PHA is synthesized as a storage compound since the cells require much less proteins for other purposes.

This study again indicated that the PHB metabolism is rather complex in the model organism *R. eutropha* and probably also in most if not all PHA accumulating bacteria. Detailed experiments and extensive experiments will be necessary to unravel the interactions of the phasins with the other enzymes involved in PHB metabolism in *R. eutropha* because four different phasins and at least seven different PHB depolymerases have been detected in this bacterium.[28]

Acknowledgment. This study was supported by a grant provided by the Deutsche Forschungsgemeinschaft (Ste 386/6-4).

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BM060912E