The *Ralstonia eutropha* H16 phasin PhaP1 is targeted to intracellular triacylglycerol inclusions in *Rhodococcus opacus* PD630 and *Mycobacterium smegmatis* mc²155, and provides an anchor to target other proteins

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In *Ralstonia eutropha*, the H16 phasin PhaP1 represents the major phasin that binds to the surface of polyhydroxyalkanoate (PHA) inclusions. In this study, C-terminal fusions of PhaP1 with enhanced green fluorescent protein (eGFP) and with *Escherichia coli* β -galactosidase (LacZ) were expressed separately in the triacylglycerol (TAG)-accumulating actinomycetes *Rhodococcus opacus* PD630 and *Mycobacterium smegmatis* mc²155, employing the *M. smegmatis* acetamidase (*ace*) promoter of the *Escherichia*–*Mycobacterium*/*Rhodococcus* shuttle plasmid pJAM2. PhaP1 and the PhaP1 fusion proteins were expressed stably in the recombinant strains. Western blot analysis of cell fractions of *Rh. opacus* revealed that PhaP1 and the PhaP1–eGFP fusion protein were associated with the TAG inclusions, whereas no phasin or phasin fusion protein was detected in the soluble and membrane fractions. Additional electron microscopy/immunocytochemistry studies demonstrated that PhaP1 was mainly located on the surface of intracellular TAG inclusions; in addition, some PhaP1 also occurred at the plasma membrane. Fluorescence microscopic investigations of the subcellular distribution of the PhaP1–eGFP fusion protein *in vivo* and on isolated TAG inclusions revealed that the fusion protein was bound to TAG inclusions at all stages of their formation, and to some extent at the cytoplasmic membrane. The PhaP1–LacZ fusion protein also bound to the TAG inclusions, and could be separated together with the inclusions from *Rh. opacus* crude extracts, thus demonstrating the immobilization of β -galactosidase activity on the inclusions. This is believed to be the first report demonstrating the ability of PhaP1 to bind to lipid inclusions in addition to PHA inclusions. Furthermore, it was demonstrated that this non-specificity of PhaP1 can be utilized to anchor enzymically active fusion proteins to a matrix of bacterial TAG inclusions.

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INTRODUCTION

The accumulation of various types of lipophilic compounds as storage compounds for energy and carbon is widespread among all kinds of organisms. These compounds are deposited as insoluble inclusions in the cytoplasm (Murphy, 2001; Wältermann & Steinbüchel, 2006). In

prokaryotes, polyhydroxyalkanoates (PHAs) are the most common type of such storage compounds (Steinbüchel & Valentin, 1995). As the most prominent example, *Ralstonia eutropha* H16 is able to accumulate poly(3-hydroxybutyrate) (PHB) at up to 90% of its cell dry weight (Steinbüchel, 1991). In contrast, inclusions composed of triacylglycerols (TAGs) or wax esters (WEs) occur less frequently in prokaryotes. The latter seem to be restricted to nocardioform actinomycetes, streptomycetes and some Gram-negative strains (Alvarez & Steinbüchel, 2002; Wältermann & Steinbüchel, 2005).

Until recently, the molecular architecture of hydrophobic inclusions was not well understood. They are generally

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Abbreviations: DIC, differential interference contrast; eGFP, enhanced green fluorescent protein; PH, phase contrast; PHA, polyhydroxyalkanoate; PHB, poly(3-hydroxybutyrate); TAG, triacylglycerol; WE, wax ester; WS/DGAT, WE synthase/acyl-CoA:diacylglycerol acyltransferase.

composed of the respective storage lipid or polyester representing the hydrophobic core of the inclusion, and are surrounded by a shell consisting of a monolayer of phospholipids and proteins attached to the surface. This amphiphilic shell separates the core from the cytoplasm, thereby preventing the inclusions from coalescing, or other proteins from binding non-specifically to the inclusions (Wältermann *et al.*, 2005; Jurassek & Marchessault, 2004). However, besides having this in common, large differences have been revealed regarding the protein composition and formation between PHA inclusions on the one hand, and WE or TAG inclusions on the other. Whereas no specific proteins are known to be abundantly associated with bacterial TAG and WE inclusions, PHA inclusions are coated by phasins, which represent a unique class of proteins (Pötter & Steinbüchel, 2005; Wältermann & Steinbüchel, 2005; Steinbüchel *et al.*, 1995). PhaP1, which represents the major phasin on the surface of PHA inclusions in *Ra. eutropha* H16, plays an important role in the formation and structure of these inclusions, because its presence or absence affects the number and size of the inclusions, and the amount of PHB in the cells (Wieczorek *et al.*, 1995; Pötter *et al.*, 2002, 2004; York *et al.*, 2002). According to the most accepted model, PHA inclusions are formed from soluble PHA synthases that polymerize the 3-hydroxybutyrate (3HB) of 3HB-CoA to PHB, with concomitant release of CoA. Since PHA synthases remain covalently linked to the growing PHB chain, an amphiphilic complex composed of the hydrophilic synthase and the elongating polymer chain is formed (Gerngross *et al.*, 1993). These complexes are thought to aggregate to form micelle-like structures, which enlarge to PHA granules due to subsequent extension of the PHA chains. During granule growth, phasins and phospholipids are thought to migrate into the exposed hydrophobic surface of the polymer core, thereby generating an interphase between the hydrophobic core and the cytoplasm (Stubbe & Tian, 2003). However, how phasin molecules are targeted to the hydrophobic surface of growing PHA granules, and whether this recognition is specific for PHA granules or also occurs with other types of hydrophobic inclusions, is unknown (Pieper-Fürst *et al.*, 1995).

In contrast to the formation of PHA granules, TAG and WE inclusions are formed at the cytoplasmic surface of the plasma membrane by WE synthase/acyl-CoA : diacylglycerol acyltransferase (WS/DGAT). The latter is the key enzyme for biosynthesis of these lipids in bacteria, and is bound to lipid droplets (Kalscheuer & Steinbüchel, 2003; Wältermann *et al.*, 2005). These small droplets coalesce to larger structures which are then released into the cytoplasm, and appear finally as large lipid inclusions (Wältermann *et al.*, 2005).

In this study, we demonstrate the heterologous expression of *Ra. eutropha* H16 *phaP1* in TAG-accumulating cells of *Rhodococcus opacus* and *Mycobacterium smegmatis*, the distribution of the phasin protein in the cells, and its binding to intracellular TAG inclusions. In addition, we

demonstrate the binding of fusion proteins consisting of PhaP1 and the enhanced green fluorescent protein (eGFP) or β -galactosidase to lipid inclusions, and that this binding is mediated by the phasin.

METHODS

Strains, plasmids and culture conditions. Cells of *Escherichia coli* strains XL-1 Blue (Stratagene) and S17-1 (Simon *et al.*, 1983) were routinely cultivated in Luria-Bertani medium (Sambrook *et al.*, 1989). Cells of *Rh. opacus* PD630 (DSM 44193; Alvarez *et al.*, 1996) and *M. smegmatis* mc²155 (ATCC 700084; Snapper *et al.*, 1990) were cultivated in Standard I (Std1) medium (Merck). To promote biosynthesis of TAGs and formation of inclusions, cells were transferred to mineral salt medium (MSM) containing 0.1 g NH₄Cl l⁻¹, and cultivated for 24, 48, 72 and 96 h (Schlegel *et al.*, 1961). After 72 h, cells achieved their maximum TAG content, which was routinely assessed by TLC, according to a previously described method (Wältermann *et al.*, 2000), and by microscopic examination. In addition, *M. smegmatis* was also cultivated in Sauton's medium (SM) (Darzins, 1958). To promote TAG accumulation in SM, the potassium phosphate concentration was reduced to 0.05 g l⁻¹. Carbon was supplied in MSM and SM as sodium gluconate or glucose (10 g l⁻¹) for *Rh. opacus* or *M. smegmatis*, respectively. To maintain plasmid pJAM2 and its derivatives, kanamycin was used at a final concentration of 50 µg ml⁻¹, following Sambrook *et al.* (1989). Induction of the acetamidase (*ace*) promoter of pJAM2 and its derivatives was routinely achieved by addition of 0.5% (w/v) acetamide to the respective cultures, if not otherwise mentioned (Triccas *et al.*, 1998). All liquid cultures were incubated in Erlenmeyer flasks equipped with baffles at 37 °C for *E. coli* or at 30 °C for *Rh. opacus* and *M. smegmatis*. Solid media were prepared by the addition of 18 g agar l⁻¹.

Cloning of *phaP1* downstream of the *ace* promoter of pJAM2. Standard molecular biology protocols were used (Sambrook *et al.*, 1989). All PCR products were first cloned into a TA vector (pGEM-T Easy; Promega). Ligation products were first checked by DNA sequencing, and then released by digestion with appropriate restriction enzymes before they were cloned into the expression vector pJAM2, which represents an *E. coli*-*Mycobacterium/Rhodococcus* shuttle vector containing the 1.5 kb *ace* promoter region (Triccas *et al.*, 1998). For subcloning, restriction enzyme recognition sites (as underlined below) were incorporated into the oligonucleotide sequences. The coding region of PhaP1 was amplified without its native start and stop codons (582 bp) by PCR from *Ra. eutropha* H16 genomic DNA, using the oligonucleotides phaP1-5' (5'-AAAGGATCCATCCTCACCCCGGAACAAGTT-3') and phaP1-3' (5'-AAAGGATCCCGATATGCTTTGCCAACGGAC-3'). Subsequently, the PCR product was cloned downstream of the *ace* promoter into the *Bam*HI site of pJAM2. In this way, a functional in-frame fusion with the first six codons of the *amiE* gene was generated, yielding pJAM2::*phaP1*. The *phaP1* gene in the constructed fusion lacked its own stop codon, but contained a stop codon after the His₆-tag linker sequence of pJAM2. Therefore, the amino acids SRHHHHHH occurred at the C-terminal region of the protein.

Construction of the *phaP1*-*egfp* and *phaP1*-*lacZ* fusion-expressing plasmids. A 720 bp fragment representing the complete eGFP gene was amplified, without the start codon, from pEGFP-N3 (BD Bioscience Clontech) using PCR primers egfp-5' (5'-AAATCTAGAGTGAGCAAGGGCGAGGAGCTG-3') and egfp-3' (5'-AAATCTAGATTACTTGTCACAGCTCGTCCATG-3'), harbouring the native stop codon (bold type). The PCR product was then cloned downstream of the *ace* promoter and *phaP1* into the *Xba*I

site of pJAM2::*phaP1*, yielding pJAM2::*phaP1-egfp*. To investigate the expression and distribution of unfused eGFP in control experiments, the *phaP1* portion of pJAM2::*phaP1-egfp* was released from the expression plasmid by *Bam*HI restriction and religation, yielding pJAM2::*egfp*. For construction of pJAM2::*phaP1-lacZ*, the 3075 bp coding region of *lacZ* was amplified from genomic DNA of *E. coli* S17-1 without its native start codon, using PCR primers lacZ-5' (5'-AAATCTAGAACCATTGATTACGGATTCACTGG-3') and lacZ-3' (5'-AAATCTAGATTATTTTGTACACCAGACCAACTG-3') harbouring the native stop codon (bold type). The PCR product was then cloned downstream of the *ace* promoter and *phaP1* into the *Xba*I site of pJAM2::*phaP1*.

Preparations of the electrocompetent cells and transfer of the constructed plasmids to *M. smegmatis* and *Rh. opacus* were carried out according to Snapper *et al.* (1990) and Kalscheuer *et al.* (1999), respectively, by electroporation in a model 2550 electroporator (Eppendorf–Netheler–Hinz).

Preparation of crude cell extracts, soluble fractions, membrane fractions and TAG inclusions. Cells of *Rh. opacus* and *M. smegmatis* were grown in MSM with reduced ammonium concentration, as described above, harvested by centrifugation (20 min, 6000 g, 4 °C), and resuspended in two volumes of 0.1 M sodium phosphate buffer (pH 7.5). After threefold passage through a French pressure cell (1000 MPa), crude extracts were obtained. To obtain soluble fractions, cell debris was removed from crude extracts by centrifugation for 30 min at 16 000 g at 4 °C, followed by 90 min at 100 000 g at 4 °C in a Sorvall Discovery 90SE ultracentrifuge. Membrane fragments were pelleted by the 100 000 g ultracentrifugation step, and subsequently resuspended in 0.1 mM sodium phosphate buffer (pH 7.5), after washing in the same buffer. TAG inclusions were prepared by loading 1–2 ml crude extracts onto the top of a discontinuous glycerol gradient consisting of 3 ml each of 22, 44 and 88 % (v/v) glycerol in 0.1 M sodium phosphate buffer (pH 7.5). The gradient was centrifuged for 1 h at 170 000 g at 4 °C. The TAG inclusions were withdrawn and subsequently washed twice in 0.1 M sodium phosphate buffer (pH 7.5), and used for further analyses.

SDS-PAGE and immunoblot analysis. Samples from crude cell extracts were analysed for their protein content according to the method of Bradford (1976), using BSA as a standard. Samples of crude extracts representing a quantity of 50 µg protein, and samples of soluble fractions, membrane fragments and TAG inclusions obtained from corresponding amounts of the extracts, were separated on SDS-polyacrylamide gels. Proteins in the gels were visualized using Coomassie brilliant blue R250 according to the method of Weber & Osbourne (1969). For immunological detection of eGFP, and of PhaP1 and its fusions, the proteins in the gels were transferred onto a PVDF membrane, according to the method of Towbin *et al.* (1979). Proteins on the membrane were stained with Ponceau S and analysed immunologically employing 0.5 µg polyclonal rabbit anti-PhaP1 IgGs ml⁻¹ (Wieczorek *et al.*, 1995) or 0.5 µg polyclonal rabbit anti-eGFP IgGs ml⁻¹ (BD Bioscience). IgGs were visualized on immunoblots using goat anti-rabbit IgG–alkaline phosphatase conjugates (diluted 1:10 000 in 10 mM Tris/HCl, pH 7.5), converting 5-bromo-4-chloro-3-indolyl-phosphate dipotassium/nitrotetrazolium blue chloride (Sigma) into an insoluble dark product.

Preparation of light microscopy samples. Nile Red-labelled cells and isolated TAG inclusions were prepared by incubating samples for 30 min at 4 °C in 0.1 M sodium phosphate buffer (pH 7.5) containing 0.5 µg Nile Red ml⁻¹ (stock solution 0.5 mg ml⁻¹ in DMSO). After labelling, cells and inclusions were sedimented by centrifugation at 16 000 g at 4 °C, and resuspended in 0.1 M sodium phosphate buffer (pH 7.5). Cells and TAG inclusions were attached to glass slides via electrostatic interaction, employing slides that were

positively charged through adsorption of poly(α-L-lysine) (PL) hydrobromide. In order to coat a glass surface with PL hydrobromide, cleaned glass slides were rinsed thoroughly with tap water, dipped in methanol, and rinsed again with demineralized water. Afterwards, a drop of 0.01 % (w/v) PL hydrobromide solution was added. After air-drying, slides were rinsed with demineralized water, and a drop of a cell suspension or TAG inclusions was added. After 15 min, the coated slides were rinsed with demineralized water to remove loosely attached bacteria or TAG inclusions, and analysed by fluorescence microscopy.

Microscopy and image processing. Slides were examined on a Zeiss Axio Imager M1 upright wide field fluorescence microscope fitted with a ×100/1.4 NA oil-immersion Plan-Apochromat objective lens, and ×4 or ×2.5 auxiliary tube lenses in phase contrast (PH) or differential interference contrast (DIC) mode. Images were collected by using a Peltier-cooled AxioCam MRm 16 bit digital monochrome charge-coupled device (CCD) camera. The two-thirds of an inch-sized CCD chip consisted of 1388 (horizontal) × 1040 (vertical) pixels, each 6.45 × 6.45 nm in size. Nile Red and eGFP fluorescence were excited using a Zeiss HBO 103 W/2 high-pressure mercury arc lamp. Recording of single and multichannel fluorescence images was performed by using emission bandpass filters at EX/EM 470 ± 40/525 ± 50 nm for eGFP and EX/EM 550 ± 25/605 ± 70 nm for Nile Red. Image stacks consisting of 45–96 planes of optical sections covering the entire z axis were generated by collecting images at focal positions differing in increments of 0.275 µm, by employing a high-precision motorized xyz stage. Depending on samples and fluorescence channels, the exposure times varied between 50 and 1000 ms to obtain sufficiently saturated images suitable for deconvolution. To reduce photobleaching, illumination was controlled by a Zeiss high-speed shutter device. Care was taken to avoid exposing the field to be recorded to the fluorescence light source until recording had begun and the camera had been adjusted to provide the optimum image. Images were stored in zvi data format for subsequent image data processing. All images were acquired using the Zeiss Axiovision 4.5 software. Where indicated, constrained iterative deconvolution of acquired images was performed using the Zeiss AxioVision 3D deconvolution module. All image processing was performed on a Siemens 2.8 GHz Line Celsius R630 workstation.

Cryosectioning and immunogold labelling. For cryosectioning, cell suspensions of *Rh. opacus* were prefixed for 5 min by adding an equal volume of 4 % (w/v) paraformaldehyde in PBS (pH 7.4). Cells were washed briefly in the same buffer, and fixed further in 4 % (w/v) paraformaldehyde for 1 h, followed by 1 h incubation in 4 % (w/v) paraformaldehyde with 0.9 M sucrose and 90 % (w/v) polyvinylpyrrolidone 25 buffered with 50 mM sodium carbonate (pH 7.0) as a cryoprotectant. The cells were concentrated by centrifugation at 16 000 g for 20 min at 4 °C, placed on pins in a small volume of cryoprotectant, and frozen in liquid nitrogen. Ultrathin sections were performed as described by Tokuyasu (1980). For immunogold labelling of cryosections, 5 µg polyclonal rabbit anti-PhaP1 IgGs ml⁻¹ (Wieczorek *et al.*, 1995) was used, followed by gold-conjugated (12 nm gold) goat anti-rabbit IgGs (Jackson ImmunoResearch), diluted 1:20 from the commercially obtained stock solution with 10 mM Tris/HCl (pH 7.5). Each set of three samples of the replicas and control specimens, which were prepared without the first antibody, were analysed by electron microscopy.

Determination of β-galactosidase activity on isolated TAG inclusions. TAG inclusions isolated from cells of *Rh. opacus* grown for 72 h in ammonium-reduced MSM harbouring pJAM2::*phaP1-lacZ* or pJAM2::*phaP1* as a control were prepared as described above. Inclusions (10 mg wet weight) were suspended in 100 µl 0.1 M sodium phosphate buffer (pH 7.5), followed by addition of 650 µl reaction solution consisting of 17 ml 0.1 M

sodium phosphate buffer (pH 7.5), 3 ml ONPG solution (8%, w/v), 1 mM magnesium chloride, 45 mM β -mercaptoethanol and 4 μ l SDS solution (20%, w/v). The assay mixture was incubated for 30 min at 37 °C. To stop the reaction, 400 μ l 1 M disodium carbonate were added. Subsequently, TAG inclusions were eliminated from the assay by filtration through a 0.22 μ m syringe filter, and the absorbance of the filtrate was examined at 405 nm to analyse the amount of cleaved ONPG. For calculation of enzyme activities an ϵ_{405} of 4.6 mM⁻¹ cm⁻¹ was used for the product *o*-nitrophenol (ONP). Measured β -galactosidase activity was essentially associated with TAG inclusions, since further cleavage of ONPG did not occur in assays after the inclusions were removed. β -Galactosidase activity assays were done in triplicate in three separate experiments.

RESULTS

Expression of *egfp*, *phaP1* and *phaP1-egfp* in recombinant strains of *M. smegmatis* and *Rh. opacus*, and distribution of the translation products in subcellular fractions

To determine heterologous expression of *egfp*, *phaP1* and *phaP1-egfp* in the recombinant actinomycetes, crude extracts and fractions of cells grown for 72 h under ammonium-reduced conditions were analysed by SDS-PAGE and Western blotting, as described in Methods. Electropherograms of cells of *M. smegmatis* harbouring pJAM2::*phaP1* exhibited an additional protein with an apparent molecular mass of 25 kDa when induced with 0.5% (w/v) acetamide. This molecular mass corresponded to that calculated for the His₆-tagged PhaP1. The His₆-tagged PhaP1 was easily recognized on corresponding Western blots by applying anti-PhaP1 IgGs. However, synthesis of His₆-tagged PhaP1 was significantly lower compared to that in the strains synthesizing the 52 kDa PhaP1-eGFP fusion protein and the unfused 27 kDa eGFP, which were also demonstrated on Western blots using the anti-PhaP1 and anti-eGFP IgGs. No IgGs recognized any proteins in crude cell extracts of the non-induced cultures, indicating that in *M. smegmatis*, the synthesis of the recombinant proteins was strictly regulated by the addition of acetamide. As no products of lower molecular mass were detected in the electropherograms and Western blots of cells harbouring pJAM2::*phaP1* and pJAM2::*egfp*, these proteins seemed to be stable against proteolysis in the cytoplasm. However, applying the anti-PhaP1 and anti-eGFP IgGs to crude extracts of *M. smegmatis* pJAM2::*phaP1-egfp* revealed that slight cleavage of the fusion protein occurred. In addition, all SDS-PAGE electropherograms of crude extracts of *M. smegmatis* cells exhibited an additional protein of 44 kDa, which most likely represented the chromosomally encoded acetamidase when cells were induced with 0.5% (w/v) acetamide (Fig. 1A). The intracellular stability of PhaP1 in recombinant *M. smegmatis* was also demonstrated by extending the expression time to 96 h (Fig. 1B).

We tried to determine the distribution of PhaP1 in subcellular fractions of recombinant cells of *M. smegmatis*.

Unfortunately, induction of the cells with acetamide resulted in a severe decrease of TAG accumulation and the number of TAG inclusions, even when the concentration of acetamide was reduced to 0.05% (Fig. 1C). This might have been due to cleavage of the inducer by the chromosomally encoded acetamidase, thus providing the cells with sufficient ammonium for growth. Attempts to achieve a sufficient accumulation of TAG inclusions under phosphate limitation in SM, as described in Methods, failed due to poor growth and little lipid accumulation (data not shown).

To circumvent this obstacle, all constructed plasmids were subsequently introduced into *Rh. opacus*. In contrast to *M. smegmatis*, SDS-PAGE electropherograms of crude extracts of the corresponding recombinant strains of *Rh. opacus* revealed no additional visible protein bands, in comparison to those obtained from the wild-type, when grown for 72 h in ammonium-reduced MSM, even when the cells were induced with 0.5% (w/v) acetamide, indicating that expression of genes controlled by the *M. smegmatis* *ace* promoter was significantly lower in *Rh. opacus*. However, according to the results obtained in recombinant *M. smegmatis*, the anti-PhaP1 IgGs recognized a 25 kDa protein in Western blots obtained from crude extracts of cells harbouring pJAM2::*phaP1*, although immunological recognition of the phasin was significantly weaker than that in recombinant *M. smegmatis*. As in *M. smegmatis*, no degradation products of the phasin were detected in *Rh. opacus*. Similarly, eGFP and the PhaP1-eGFP fusion protein were easily recognized on Western blots of crude cell extracts harbouring pJAM2::*egfp* and pJAM2::*phaP1-egfp*, respectively, as was demonstrated by employing the anti-eGFP IgGs (Fig. 2). In contrast to *M. smegmatis*, addition of 0.5% (w/v) acetamide to the cultures did not affect TAG accumulation in *Rh. opacus* (not shown). To investigate the cellular distribution of PhaP1, eGFP and the PhaP1-eGFP fusion protein in *Rh. opacus*, crude extracts of induced cells were fractionated into soluble fractions, membrane fractions and fractions representing the TAG inclusions. On Western blots of the respective fractions of *Rh. opacus* pJAM2::*phaP1*, the phasin was recognized by the anti-PhaP1 IgGs in the fraction representing the TAG inclusions, whereas no signal occurred in the soluble fraction. This indicates that PhaP1 was associated with the TAG inclusions in recombinant *Rh. opacus*. The result obtained for the distribution of PhaP1 in cell fractions of *Rh. opacus* was also confirmed by the localization of the PhaP1-eGFP fusion protein by employing anti-eGFP IgGs on Western blots of the strain harbouring pJAM2::*phaP1-egfp*. In this recombinant strain, the fusion protein also occurred only in the fraction representing the TAG inclusions. We also tried to localize PhaP1 and its eGFP fusion in electropherograms of total membrane fractions of the recombinant strains, but failed (data not shown). As expected, the unfused eGFP was only localized in the soluble fraction of the control strain harbouring pJAM2::*egfp* (Fig. 2).

Fluorescence microscopic localization of PhaP1-eGFP fusion protein in recombinant *Rh. opacus*

To verify the association of PhaP1-eGFP with the TAG inclusions in *Rh. opacus*, the distribution of the fusion protein was investigated by fluorescence microscopy in cells grown in Std1 medium, and also for 24, 48 and 72 h in ammonium-reduced MSM under conditions permissive for TAG accumulation when formation of large intracellular TAG inclusions occurs in the cytoplasm. The fluorescence of the fusion protein was predominantly associated with TAG inclusions at all stages of their formation. In cells grown in Std1 medium, fluorescence was associated with nascent TAG inclusions at the plasma membrane, whereas, in cells grown in ammonium-reduced MSM for 24, 48 and 72 h, it was

predominantly associated with matured TAG inclusions in the cytoplasm (Fig. 3A–D). As revealed by constrained iterative deconvolution of images obtained from Std1-grown cells, fluorescence also occurred to some extent in areas of the cell wall and plasma membrane. However, fluorescence at these sites was much weaker than that of intracellular TAG inclusions (see deconvolved image in Fig. 3A). After 72 h in ammonium-reduced MSM, cells were fully packed with brightly fluorescent TAG inclusions. In fact, after deconvolution, large TAG inclusions in these cells often exhibited a ring of fluorescence, indicating a localization of the fusion protein at the surface of the inclusions (Fig. 3D). Fluorescence of the fusion protein was thoroughly distinguishable from Nile Red fluorescence at all stages of TAG accumulation, which, in addition to the TAG inclusions, also clearly labelled the cellular envelope (Fig. 3A–D).

Fig. 1. (A) Effect of acetamide induction on synthesis of PhaP1, eGFP and the C-terminal PhaP1-eGFP fusion protein in *M. smegmatis* harbouring the constructed expression plasmids, as shown by SDS-PAGE (left) and immunological detection of the respective recombinant proteins by Western blot analysis (right). Antibodies used for the detection of the respective proteins are indicated. Std, molecular mass standard; lane 1, *M. smegmatis* pJAM2::phaP1 in the absence of acetamide; lane 2, *M. smegmatis* pJAM2::phaP1 induced with 0.5% (w/v) acetamide; lane 3, *M. smegmatis* pJAM2::egfp in the absence of acetamide; lane 4, *M. smegmatis* pJAM2::egfp induced with 0.5% (w/v) acetamide; lane 5, *M. smegmatis* pJAM2::phaP1-egfp in the absence of acetamide; lane 6, pJAM2::phaP1-egfp induced with 0.5% (w/v) acetamide. (B) Time-course analysis of recombinant PhaP1 synthesis and stability in *M. smegmatis* harbouring pJAM2::phaP1. Electropherograms (left) of crude cell extracts, and immunological detection of PhaP1 by employing anti-PhaP1 IgGs on a Western blot corresponding to the SDS-PAGE (right) after 24 h (lane 1), 48 h (lane 2), 72 h (lane 3) and 96 h (lane 4) growth in ammonium-reduced MSM supplemented with 0.5% (w/v) acetamide. Proteins in the SDS-PAGE gels presented in (A) and (B) were visualized by Coomassie brilliant blue R250. (C) Effect of different concentrations of acetamide on intracellular TAG accumulation in *M. smegmatis* after 72 h growth in ammonium-reduced MSM, as revealed by TLC. Std, triolein standard; lane 1, 0.5% (w/v); lane 2, 0.3% (w/v); lane 3, 0.1% (w/v); lane 4, 0.05% (w/v); lane 5, 0.01% (w/v); lane 6, 0.005% (w/v), lane 7, 0.001% (w/v) acetamide.

Fig. 2. Immunological detection of PhaP1, eGFP and the PhaP1–eGFP fusion protein in crude cell extracts and subcellular fractions obtained from *Rh. opacus* wild-type cells and respective recombinant strains harbouring pJAM2::*phaP1*, pJAM2::*egfp* or pJAM2::*phaP1-egfp*. Left image, SDS-PAGE electropherograms of the crude extracts and cellular fractions; centre and right images, immunological assays employing anti-PhaP1 and anti-eGFP IgGs, respectively, on Western blots corresponding to the SDS-PAGE. Proteins in the gel were stained with Coomassie brilliant blue R250. Std, molecular mass standard; lane 1, crude extract of wild-type cells; lane 2, soluble fraction of wild-type cells; lane 3, TAG inclusions isolated from wild-type cells; lane 4, crude extract of cells harbouring pJAM2::*phaP1*; lane 5, soluble fraction obtained from cells harbouring pJAM2::*phaP1*; lane 6, TAG inclusions isolated from cells harbouring pJAM2::*phaP1*; lane 7, crude extract of cells harbouring pJAM2::*egfp*; lane 8, soluble fraction of cells harbouring pJAM2::*egfp*; lane 9, TAG inclusions of cells harbouring pJAM2::*egfp*; lane 10, crude cell extract of cells harbouring pJAM2::*phaP1-egfp*; lane 11, soluble fraction of cells harbouring pJAM2::*phaP1-egfp*; lane 12, TAG inclusions isolated from cells harbouring pJAM2::*phaP1-egfp*. Cells were grown for 72 h in ammonium-reduced MSM supplemented with 0.5% (w/v) acetamide.

After disruption of the cells, fluorescence of PhaP1–eGFP was observed in association with isolated TAG inclusions, indicating that the fusion protein was stably associated with the inclusions. Similar to the observation in whole cells, isolated inclusions showed a ring of green fluorescence at their periphery in deconvolved images (Fig. 3E). In contrast to this, TAG inclusions from cells expressing unfused eGFP exhibited no fluorescence when observed without Nile Red labelling (not shown). Cells expressing unfused *egfp*, which served as a negative control, exhibited a diffuse green fluorescence throughout the cytoplasm, whereas intracellular TAG inclusions were easily detectable by their Nile Red fluorescence (Fig. 3F).

Immunogold labelling of cryosections

To investigate whether PhaP1 is targeted exclusively to the surface of TAG inclusions in *Rh. opacus*, or also to other components of the cells, the fusion protein was localized on cryosections by postembedding immunogold labelling. Ultrathin cryosections were prepared from recombinant cells harbouring pJAM2::*phaP1-egfp* grown under ammonium-reduced conditions for 72 h. For immunogold labelling of cryosections, rabbit anti-PhaP1 IgGs were used in combination with goat anti-rabbit IgG gold conjugates. In cryosectioned *Rh. opacus* cells, TAG inclusions appeared as nearly spherical, electron-translucent areas with little internal structure. Strong labelling of PhaP1

was found at the surface of the inclusions, whereas almost no label was observed in the cytoplasm. Label was also detected at the plasma membrane, thus confirming the fluorescence-microscopic localization of the PhaP1–eGFP fusion protein at this site in the cells. However, although immunogold labelling of sections is not a suitable method for the quantification of proteins, the concentration of PhaP1 label at the periphery of the cells was lower than that at the surface of the TAG inclusions (Fig. 4A). Control specimens, prepared without the primary antibody, were essentially free from gold particles (Fig. 4B).

Immobilization of *E. coli* LacZ on TAG inclusions in *Rh. opacus*

Once the binding of the native PhaP1 and of the PhaP1–eGFP fusion protein to the TAG inclusions was demonstrated, we investigated whether PhaP1 could be used as an anchor for immobilization of active enzymes on the surface of TAG inclusions. For this purpose, a fusion of *E. coli lacZ* as a reporter gene to the 3'-terminal region of phaP1 was constructed in pJAM2. The resulting plasmid pJAM2::*phaP1-lacZ* was transferred to *Rh. opacus*, and the cells were cultivated for 72 h under ammonium-reduced conditions. Subsequently, the TAG inclusions were isolated and used for enzymic conversion of ONPG. It has previously been shown that various cytosolic proteins bind non-specifically to the TAG inclusions, and are then co-purified

Fig. 3. Fluorescence microscopic localization of Nile Red (NR) and the PhaP1–eGFP fusion protein in recombinant cells of *Rh. opacus* grown in (A) Std1 medium and for 24 h (B), 48 h (C) or 72 h (D) in ammonium-reduced MSM. Images at the top of each panel show PH, DIC and three-channel fluorescence microscopic overlay images merged from PH, NR and eGFP fluorescent images. Images at the bottom of each panel show single-channel eGFP and NR images, and a two-channel fluorescence microscopic overlay image merged from NR and eGFP fluorescence. In addition, panel (A) shows a deconvolved image of *Rh. opacus* grown in Std1, revealing slight PhaP1–eGFP fluorescence at the cytoplasmic membrane (arrow), whereas the additional deconvolved image in panel (D) demonstrates PhaP1–eGFP fluorescence at the surface of intracellular TAG inclusions in a cell grown for 72 h in ammonium-reduced MSM. (E) A PH and deconvolved two-channel eGFP/NR fluorescent image of a TAG inclusion isolated from a *phaP1-egfp*-expressing *Rh. opacus* cell grown for 72 h under storage conditions, showing distribution of the fusion protein at the surface, and NR labelling of the lipids in the core of the inclusion. (F) PH and fluorescence images of cells of *Rh. opacus* transformed with pJAM2::egfp grown for 48 h under storage conditions, showing a diffuse cytoplasmic fluorescence of unfused eGFP (upper panel), whereas intracellular TAG inclusions are clearly labelled by NR in a two-channel eGFP/NR fluorescent image (lower panel). All images were obtained from cells cultivated in the presence of 0.5% (w/v) acetamide. Bars, 1 µm, if not otherwise stated.

Fig. 4. (A) PhaP1 is associated with intracellular TAG inclusions and the plasma membrane in recombinant *Rh. opacus* PD630. Immunocytochemistry was done on a cryosection applying rabbit anti-PhaP1 IgGs followed by 18 nm gold-conjugated goat anti-rabbit pig IgGs (black dots). Cells were transformed with pJAM2::phaP1-egfp and grown for 72 h under storage conditions before sections were prepared as described in Methods. (B) Control specimens, prepared without rabbit anti-PhaP1 IgGs, were essentially free of gold particles. Abbreviations: CM, cytoplasmic membrane; CW, cell wall; CY, cytoplasm; TAG, TAG inclusion. Bars, (A), 200 nm; (B), 300 nm.

with the inclusions (Kalscheuer et al., 2001; Wältermann & Steinbüchel, 2005). The specific β -galactosidase activity [9.5 ± 0.5 U (mg wet weight) $^{-1}$] was significantly higher in samples containing TAG inclusions which were isolated from *Rh. opacus* PD630 harbouring pJAM2::phaP1-lacZ. When TAG inclusions were removed from the assay, specific β -galactosidase activity was only 0.3 ± 0.3 U (mg wet weight) $^{-1}$. For control experiments, cells harbouring pJAM2::phaP1 were analysed in the same way. Since *Rh. opacus* also expresses a chromosomally encoded β -galactosidase, low enzyme activity was also expected to occur in the control samples. In the controls, i.e. in samples containing TAG inclusions, which were isolated from *Rh. opacus* harbouring pJAM2::phaP1, specific β -galactosidase activity was only 1.8 ± 0.8 or 0.3 ± 0.3 U (mg wet weight) $^{-1}$ before and after removal of the TAG inclusions, respectively. The data represent the mean $\pm$ SD of three independent experiments. These data demonstrate a stable immobilization of LacZ to bacterial TAG inclusions mediated by PhaP1 as an anchor.

DISCUSSION

In this study, it was shown that cells of recombinant strains of *Rh. opacus* and *M. smegmatis* transformed with the *Ra. eutropha* H16 phaP1 gene synthesized the phasin PhaP1. The key findings of our study are that the phasin remains stable in the cells, and that PhaP1 and PhaP1 fusion proteins are also targeted to TAG inclusions. This is believed to be the first report on the binding of a phasin protein to TAG inclusions. In *Ra. eutropha* H16, PhaP1 is strictly associated with the PHB granule fraction, and its expression is highly

associated with PHB synthesis due to the regulation exerted by the transcriptional repressor PhaR (Pötter et al., 2002). The motif in PhaP1 which targets the phasin to PHB granules in *Ra. eutropha* H16 has not yet been identified. However, PHB granules, as well as TAG inclusions, possess a hydrophobic core of the polyester or lipid, respectively, which is thought to be surrounded by a monolayer of phospholipids (de Koning & Maxwell, 1993; Hocking & Marchessault, 1994; Mayer & Hoppert, 1997; Wältermann et al., 2005). This common structure allows the targeting of PhaP1 to PHB granules and also TAG inclusions, as demonstrated in this study. Therefore, our data indicate that targeting of PhaP1 to PHB granules in *Ra. eutropha* H16 is most probably not mediated by a direct mutual recognition of the phasin and the polymer in the granules. Our results indicate that PhaP1 has the ability to bind to any type of hydrophobic inclusion, irrespective of whether a PHA or a different hydrophobic compound is present in the core of the inclusions. Furthermore, it is also unlikely that an additional as-yet-unidentified component involved in PHA metabolism mediates targeting of PhaP1 to the inclusions, since such components would have been absent in the strains used in our study. Most probably, binding of PhaP1 to the inclusions is mediated only by the presence of the amphiphilic interphase consisting of the monolayer membrane between the inclusions and the surrounding cytoplasm, by the hydrophobic surface of the core, or by a combination of both.

Combined electron microscopy and postembedding immunocytochemistry revealed that PhaP1 is distributed mostly on the amphiphilic surface of the TAG inclusions. However,

in contrast to its exclusive distribution on the surface of PHB granules in *Ra. eutropha* H16, it was demonstrated that some PhaP1 was also present at the plasma membrane and cell wall regions in *Rh. opacus*. This distribution has also been reported by Pieper-Fürst *et al.* (1994) while investigating the cellular distribution of the 14 kDa phasin in *Rhodococcus ruber*, which is able to synthesize equal amounts of TAGs and the copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [poly(3HB-co-3HV)]. Although, in *R. ruber*, it is not known whether TAGs and poly(3HB-co-3HV) occur separately or simultaneously in the inclusions, it has been demonstrated that the phasin occurs on the surface of any inclusion in the cells, and also at the cytoplasmic surface of the plasma membrane. According to our recently proposed model, TAGs are synthesized by WS/DGAT localized at the cytoplasmic surface of the plasma membrane as small lipid droplets, which coalesce to form larger inclusions by prolonged TAG synthesis, and are then released into the cytoplasm (Wältermann *et al.*, 2005). Thus, a binding of phasins to nascent TAG inclusions at their site of synthesis is the most probable explanation for this distribution, rather than a direct interaction of the phasin with the cytoplasmic membrane. This was also confirmed by our unsuccessful experiments, which aimed at localizing the phasin in the total membrane fractions of *Rh. opacus*. Most probably, this was caused by the disintegration of the plasma membrane–lipid droplet association during cell disruption.

In *Ra. eutropha* H16, the amount of PHB and the number of granules is directly influenced by the amount of phasin molecules in the cells (Wieczorek *et al.*, 1995; Pötter *et al.*, 2002). The presence of the phasin neither altered the amount of TAGs in *Rh. opacus*, nor influenced the size or number of the TAG inclusions. As revealed by our expression analysis, the total amount of PhaP1 in *Rh. opacus* was very low, since expression of the protein was limited by the *ace* promoter of pJAM2. Whether the presence of a high amount of phasins can influence TAG metabolism in the cells remains to be elucidated. This may be clarified by inactivation of the chromosomally encoded acetamidase in *M. smegmatis*, through the use of an alternative gene expression system that does not influence cellular TAG metabolism. However, a more suitable expression system or an acetamidase-negative *M. smegmatis* strain are as yet unavailable.

We demonstrated that TAG inclusions tagged with a PhaP1–LacZ fusion protein exhibited β -galactosidase activity *in vitro*. The immobilization of enzymes and other surface proteins or defined particles offers interesting applications. One example could be the synthesis of functionalized nanoparticles, e.g. to carry antibodies for analytic purposes, or hormones and other therapeutic agents. Such nanoparticles can be purified easily from crude cell extracts. Moldes *et al.* (2004) have created a system for the synthesis and purification of enzymes using PHA granules as a matrix, and the N terminus of the phasin PhaF from *Pseudomonas putida* as a linker. Furthermore, PHB granules in recombinant *E.*

coli have been successfully demonstrated as a matrix for the purification of target proteins by fusions with phasins and self-cleaving affinity tags based on protein-splicing elements known as inteins (Banki *et al.*, 2005). Also, TAG inclusions have been utilized as a matrix for purification of enzymes (Moloney, 1998, 2002). The latter author created an analogous system based on plant cells, by attaching target enzymes to oil bodies via oleosins. Both purification systems described above are patented and commercially available (Prieto *et al.*, 2004; Moloney, 1997; Moloney *et al.*, 2005). In addition, anchoring of enzymes and other proteins to TAG inclusions by a PhaP1 tag offers an interesting possibility to establish alternative, bioengineered pathways on the monolayer surface of intracellular TAG inclusions.

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