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A phasin with extra talents: a polyhydroxyalkanoate granule-associated protein has chaperone activity

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Summary

Phasins are proteins associated to intracellular polyhydroxyalkanoate granules that affect polymer accumulation and the number and size of the granules. Previous work demonstrated that a phasin from *Azotobacter* sp FA-8 ($PhaP_{Az}$) had an unexpected growth-promoting and stress-protecting effect in *Escherichia coli*, suggesting it could have chaperone-like activities. In this work, *in vitro* and *in vivo* experiments were performed in order to investigate this possibility. $PhaP_{Az}$ was shown to prevent *in vitro* thermal aggregation of the model protein citrate synthase and to facilitate the refolding process of this enzyme after chemical denaturation. Microscopy techniques were used to analyse the subcellular localization of $PhaP_{Az}$ in *E. coli* strains and to study the role of $PhaP_{Az}$ in *in vivo* protein folding and aggregation. $PhaP_{Az}$ was shown to colocalize with inclusion bodies of PD, a protein that aggregates when overexpressed. A reduction in the number of inclusion bodies of PD was observed when it was coexpressed with $PhaP_{Az}$ or with the known chaperone GroELS. These results demonstrate that $PhaP_{Az}$ has chaperone-like functions both *in vitro* and *in vivo* in *E. coli* recombinants, and suggests that phasins could have a general protective role in natural polyhydroxyalkanoate producers.

Introduction

Polyhydroxyalkanoates (PHAs) are biodegradable polymers accumulated in intracellular granules by many bacteria as a reserve material. These polymers have been extensively studied due to their thermoplastic properties that make them attractive candidates for the replacement of traditional plastics (Reddy *et al*., 2003; Keshavarz and Roy, 2010; Wang *et al*., 2014). PHA granules are dynamic intracellular structures that can respond to different physiological stresses by synthesis or degradation (Pötter *et al*., 2004). The capability to accumulate PHAs has been demonstrated to provide a selective advantage to bacteria, as it allows cells to survive in nutrient poor ecosystems (López *et al*., 1995; Kadouri *et al*., 2005) and to resist biotic and abiotic stresses such as heat (Ruiz *et al*., 2001), cold (Ayub *et al*., 2009), UV irradiation, osmotic pressure and desiccation (Tal and Okon, 1985).

Natural PHA producers have several proteins involved in the regulation of polymer synthesis, degradation and/or granule formation and distribution. Phasins are proteins found on the surface of PHA granules, and are thought to generate an interphase between the cytoplasm and the hydrophobic core of the granules, preventing them from coalescence (Wieczorek *et al*., 1995). The capability of phasins to bind to PHA granules in recombinant *E. coli* has been used to develop purification procedures in which other proteins fused to phasins can be recovered by co-purification with polymer granules (Banki *et al*., 2005). Phasin fusions have also been developed using the N-terminal of PhaF, a bimodular phasin of *Pseudomonas putida*, to purify recombinant proteins expressed in this organism (Moldes *et al*., 2004).

Apart from their structural role as part of the PHA granule cover, different functions have been associated to many phasins. Some of them were observed to possess regulatory activity, such as PhaF of *P. putida*, that regulates PHA synthesis (Galán *et al*., 2011), ApdA of *Rhodospirillum rubrum*, that activates PHB depolymerization (Handrick *et al*., 2004), and PhaP of *Ralstonia eutropha* (currently denominated *Cupriavidus necator*) and *Aeromonas hydrophila*, that have been observed to affect PHA polymerase at different levels (Qi *et al*., 2000; Tian *et al*., 2005). Experiments involving phasin mutants have revealed that these proteins affect the number and size of PHA granules (Kuchta *et al*., 2007) and several studies have reported that phasins promote PHA accumulation in natural PHA producers (Wieczorek *et al*., 1995),

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and increase both growth and polymer synthesis in recombinant *E. coli* (York *et al*., 2001; de Almeida *et al*., 2007). The effect of PhaP on PHA accumulation has been attributed to several possible causes, ranging from the activation of genes and/or enzymes involved in PHA synthesis, to acting as a barrier between the polymer and other cellular components, thus avoiding deleterious effects of PHA production (Wieczorek *et al*., 1995).

$PhaP_{Az}$ is the most abundant PHB granule-associated protein observed in *Azotobacter* sp. FA-8 (Pettinari *et al*., 2003). Previous studies showed that this phasin not only enhanced growth and polyhydroxybutyrate (PHB) accumulation in recombinant *E. coli*, but it also had an unexpected protective effect in non-PHB synthesizing *E. coli*, under both normal and stress conditions, resulting in increased growth and higher resistance to both heat shock and superoxide stress by paraquat (de Almeida *et al*., 2011). The protective effect of $PhaP_{Az}$ is similar to that observed in *E. coli* overexpressing the small heat shock proteins *ibpA* and *ibpB* (Kitagawa *et al*., 2000). Small heat shock proteins are chaperones involved in protein folding and cell survival under thermal stress, along with DnaK, DnaJ and GroELS, the main drivers of *E. coli* protein folding (Laskowskaa *et al*., 1996; Thomas and Baneyx, 1998; Kitagawa *et al*., 2000). Chaperones are also involved in the construction/deconstruction of inclusion bodies (IBs), dynamic structures composed of proteins aggregated as a result of incorrect protein folding or protein unfolding (Carrió and Villaverde, 2003).

Synthesis of chaperones is induced by several stresses, and responds to the levels of unfolded proteins present in the cells (Guisbert *et al*., 2008). Furthermore, stress levels are very often estimated through the induction of heat shock proteins. $PhaP_{Az}$ expression was shown to reduce induction of heat shock-related genes in *E. coli*, and to result in lower levels of RpoH, the main heat shock regulator, both during normal growth at 37°C and in response to heat shock, providing further evidence for the stress alleviating effect of $PhaP_{Az}$ (de Almeida *et al*., 2011).

The increased resistance to different kinds of stress observed in cells expressing $PhaP_{Az}$, together with a decrease in the expression of heat shock proteins, suggested that $PhaP_{Az}$ could affect protein folding in a chaperone-like manner. This work investigates this hypothesis both *in vivo* and *in vitro*.

Results

Subcellular localization of $PhaP_{Az}$ in recombinant E. coli

The localization of PhaP from *Azotobacter* sp. FA8 in *E. coli* was investigated by fluorescence microscopy using pACP-GFP, a plasmid that carries a fusion of $PhaP_{Az}$ to GFP in the C-terminus. SDS-PAGE showed that a protein of the expected size was produced in *E. coli* strains (data not shown). Localization of $PhaP_{Az}$ and its relationship with PHB granules was studied in *E. coli* PHB-L21 that contains a copy of the *pha* cluster of *R. eutropha* under the control of the Ptrc promoter integrated into the chromosome by transposition, transformed with pACP-GFP. The capability of the fusion protein to bind to PHB granules was verified by microscopic observation of the purified granules and analysis of granule-associated proteins. An intense green fluorescence was observed in the purified granules, and SDS-PAGE analysis revealed a strong band corresponding to the fusion protein (Fig. 1).

The intracellular localization of $PhaP_{Az}$ in PHB accumulating *E. coli* was then analysed to investigate if PhaP-GFP was able to interact with polymer granules *in vivo*, and if the intracellular localization of the protein in the recombinants is similar to what was described in natural polymer producers. PhaP-GFP was identified through its green fluorescence, while the PHB granules were stained with Nile Red. Strong green fluorescence indicating the presence of $PhaP_{Az}$ was observed in all cells in 24 h cultures. However, red fluorescence due to PHB granules stained with Nile Red varied, resulting in low intensities for some cell populations. In cells in which PHB granules were more visible, $PhaP_{Az}$ colocalized with the polymer (Fig. 2). These results indicate that the fusion to GFP does not alter the ability of the phasin to interact with PHB granules *in vivo*.

Subcellular localization of $PhaP_{Az}$ in cells devoid of PHB was analysed by fluorescence microscopy in *E. coli* BL21 (DE3) transformed with pACP-GFP. Uniform fluorescence distribution was observed in the cells during the first 3 h

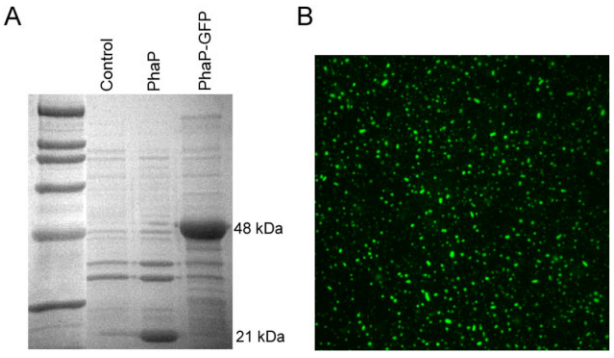

Fig. 1. Binding of PhaP-GFP to purified PHB granules.
A. SDS-PAGE analysis of proteins associated to the PHB fraction after purification through a glycerol gradient. Lane 1: MW Marker (Broad Range Prestained SDS-PAGE standard from BioRad), lane 2: PHB-L21/pACYC (empty vector), lane 3: PHB-L21/pACP (expressing *phaP*), lane 4: PHB-L21/pACP-GFP (expressing *phaP-gfp*).
B. Confocal Fluorescence microscopy image of PhaP-GFP bound to purified PHB granules.

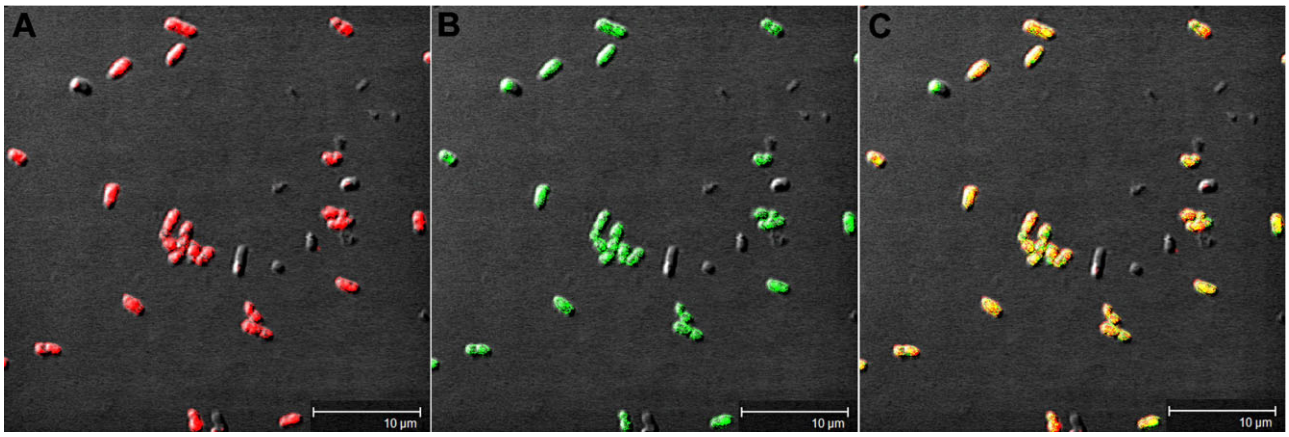

Fig. 2. Localization of $PhaP_{Az}$ in PHB accumulating *E. coli*. Confocal Fluorescence microscopy image of PHB-L21/pACP-GFP expressing *phaCAB* and *phaP-gfp*.
A. PHB granules stained with Nile Red.
B. PhaP-GFP.
C. Merge.

after induction with IPTG, indicating soluble localization of PhaP-GFP (Fig. 3A). After 4 h of induction, PhaP-GFP was observed to migrate to the cell poles and to the septum of the cells in division. This localization was maintained after 24 h (Fig. 3B).

In vitro *chaperone activity*

Previous results suggested that $PhaP_{Az}$ could have chaperone-like properties. To test the chaperone activity of $PhaP_{Az}$ *in vitro*, thermal and chemical denaturation

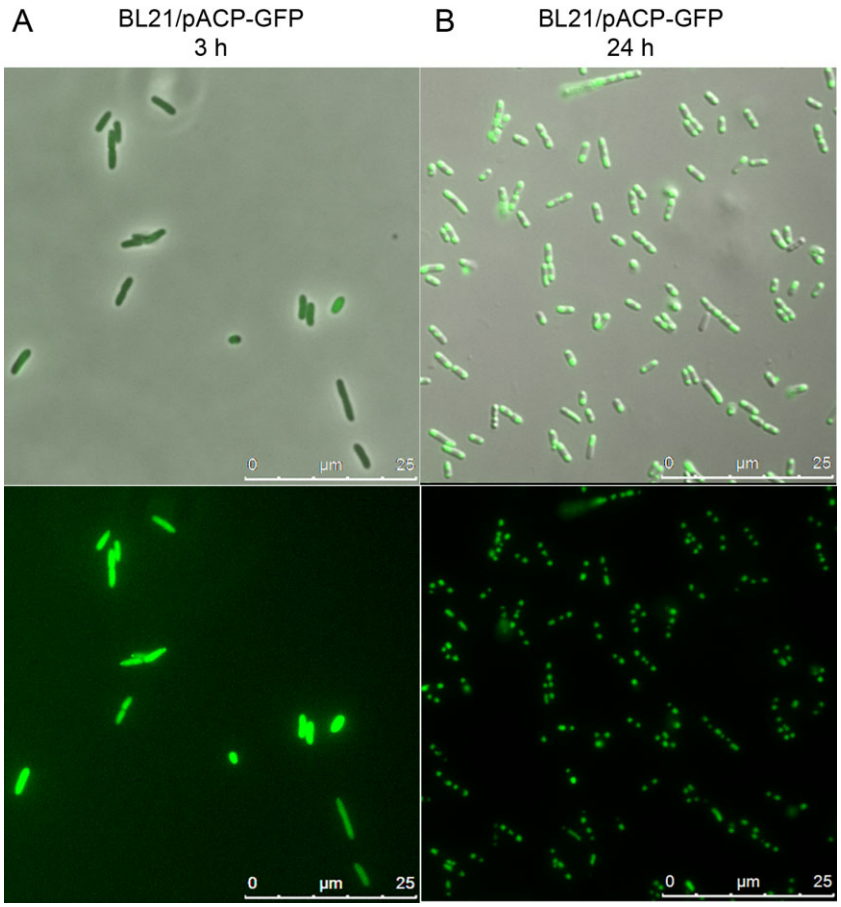

Fig. 3. Localization of $PhaP_{Az}$ in non-PHB-producing *E. coli*. Confocal Fluorescence microscopy images of strain BL21/pACP-GFP expressing *phaP-gfp*. Upper panels: transmission image superimposed to fluorescence image. Bottom panels: fluorescence image.
A. Cells after 3 h of induction with IPTG.
B. Cells after 24 h of induction with IPTG.

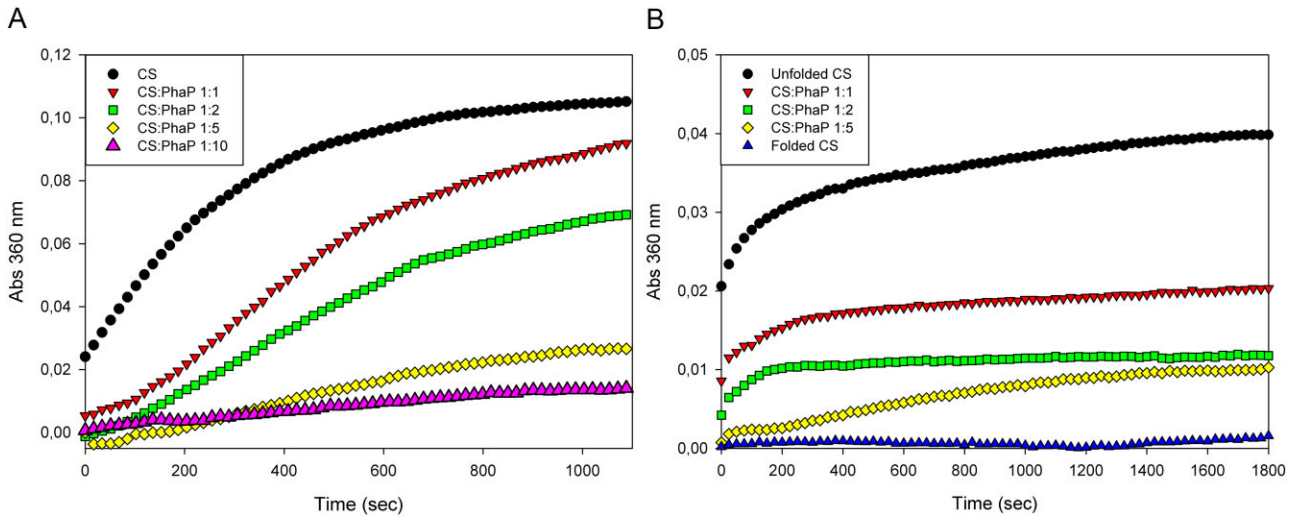

Fig. 4. $PhaP_{Az}$ chaperone activity assays.
A. Influence on CS thermal aggregation kinetics with increasing CS:PhaP molar ratios followed by light scattering at 360 nm during 20 min.
B. Chaperone activity of $PhaP_{Az}$ upon chemically unfolded CS. Chemically unfolded CS was diluted in refolding buffer (see Materials and Methods) in the absence and in the presence of increasing CS:PhaP molar ratios. Formation of aggregates was followed by light scattering.

experiments were carried out using the dimeric citrate synthase (CS) as a model protein in the presence of $PhaP_{Az}$. Incubation of CS at 45°C leads to slow aggregation in the absence of additives that can be monitored through light scattering measurements (Buchner *et al.*, 1998). The addition of $PhaP_{Az}$ to the sample containing CS prior to heat treatment not only slowed the aggregation process but also decreased the amount of aggregates formed (Fig. 4A). This effect is more evident when the $PhaP_{Az}$ to CS ratio increases, and the amount of aggregates formed tends to be negligible at higher ratios. As previously reported in the literature (Alonso *et al.*, 2006), the addition of BSA as a control protein instead of $PhaP_{Az}$ did not prevent CS aggregation (data not shown). A control experiment containing only $PhaP_{Az}$ revealed that the protein by itself did not aggregate (data not shown).

The folding state of $PhaP_{Az}$ in the conditions used in the *in vitro* chaperone activity assays was further analysed by circular dichroism (CD), revealing that between 50% and 65% of the protein retains its secondary structure after incubation at 45°C during 30 min. After cooling, the protein recovered 90% of its structure (data not shown). The results obtained in the light scattering and CD experiments indicated that $PhaP_{Az}$ alone does not aggregate in the conditions used.

Another way to test the chaperone activity of a protein *in vitro* is by measuring its capacity to avoid the spontaneous aggregation that takes place upon dilution of chemically unfolded CS into a folding condition (Buchner *et al.*, 1998). When CS unfolded by incubation with 6M guanidine chloride was diluted hundredfold into a buffer without denaturant in the absence of $PhaP_{Az}$, the protein aggregated slowly. If the dilution was performed in the presence of $PhaP_{Az}$, the aggregation was observed to decrease both in speed and final amount (Fig. 4B).

To test if CS remains in its native form after treatment with $PhaP_{Az}$, fluorescence spectroscopy experiments were performed. CS contains tryptophan residues that can be used to study protein-folding processes because their intrinsic fluorescence properties are sensitive to changes in the environment that take place as the protein folds/unfolds. On the other hand, $PhaP_{Az}$ does not contain any tryptophans in its sequence. This characteristic allowed us to study the folding state of CS in the absence and presence of $PhaP_{Az}$ by analysing the CS fluorescence spectra at 25°C and at 45°C. The fluorescence spectrum of CS observed at 45°C in the presence of $PhaP_{Az}$ corresponds to the spectrum of the native form of the protein, indicating that CS is correctly folded after treatment at 45°C in the presence of $PhaP_{Az}$ (Fig. 5).

From these results, it can be concluded that $PhaP_{Az}$ has chaperone activity *in vitro*, because it prevents formation of CS thermal aggregates and it allows the correct refolding of CS after chemical denaturation.

In vivo *chaperone activity*

As chaperones are known to prevent protein misfolding and formation of protein aggregates or IBs, the effects of $PhaP_{Az}$ on IBs were analysed using PD, an insoluble domain of TolR from *Azoarcus* sp. CIB (Eduardo Díaz Collection) that was used in this work as a model protein. When overproduced in *E. coli*, PD forms large IBs that

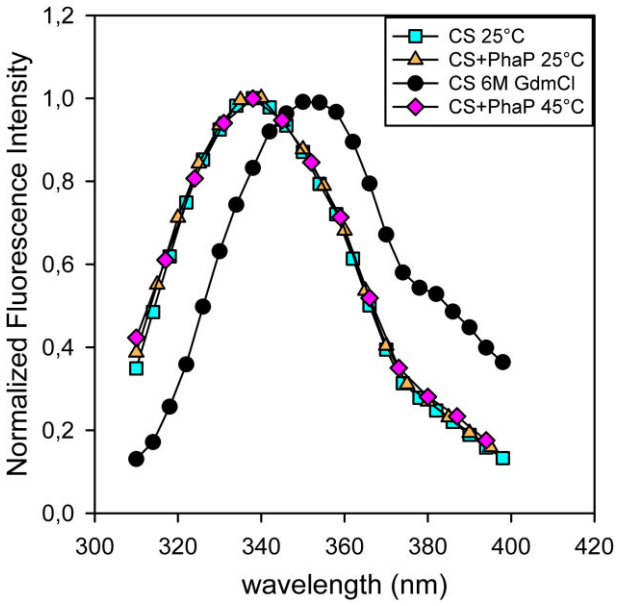

Fig. 5. Fluorescence spectra of folded and unfolded CS. The folding state of CS in the presence of $PhaP_{Az}$ was detected by fluorescence spectroscopy. Squares: CS spectrum at 25°C; triangles: CS spectrum in the presence of $PhaP_{Az}$ after incubation at 25°C; diamonds: CS spectrum in the presence of $PhaP_{Az}$ after incubation at 45°C; circles: chemically unfolded CS spectrum.

can be easily detected by transmission electron microscopy (TEM) (Fig. 6A). This system enabled the study of $PhaP_{Az}$ chaperone activity *in vivo* in *E. coli* BL21 (DE3) carrying pACP and pPD that coproduces $PhaP_{Az}$ and PD. As a positive control of chaperone activity, cells producing PD and GroELS, a well-known chaperone, were used.

PD IBs were observed by phase contrast microscopy and also by TEM, appearing as large and dense intracellular bodies (Fig. 6A). Cells that presented IBs were large, had an irregular morphology and contained between one and three IBs. When PD was coproduced with $PhaP_{Az}$, the total number of cells with IBs (Fig. 6D) and the number of IBs per cell (Fig. 6E) decreased. In addition, the morphology of the cells changed, becoming more regular (Fig. 6B). These effects were similar to those observed in cells producing GroELS (Fig. 6C–E). Furthermore, the decrease in IB formation was even more pronounced in cells expressing *phaP*. These results indicate that $PhaP_{Az}$ prevents formation of IBs in *E. coli*, evidencing the chaperone activity of $PhaP_{Az}$ *in vivo.*

Interaction of $PhaP_{Az}$ with PD IBs

To further study the interaction between $PhaP_{Az}$ and IBs, fluorescence microscopy experiments were performed using BL21 (DE3) transformed with pACP-GFP – carrying a PhaP-GFP fusion – and pPD. Proteins in IBs are not correctly folded, causing hydrophobic residues to be exposed. As PHA granules can be stained with Nile Red because of their hydrophobicity, we tested if PD IBs could be visualized by this technique. Large PD IBs were detected using a 10-fold higher concentration of this dye (see *Experimental procedures* for details). These experiments showed that 2 h after induction, PhaP-GFP colocalizes with PD IBs, indicating that $PhaP_{Az}$ effectively binds to IBs *in vivo*. The M1/M2 colocalization coefficients were $M1 = 0.939 \pm 0.037$ and $M2 = 0.692 \pm 0.098$, being M1 the fraction of green overlapping red and M2 the fraction of red overlapping green (Fig. 7).

Discussion

In natural PHA producers, phasins interact with the PHA granules and also between themselves (Pfeiffer *et al.*, 2011), forming a complex network that involves many different proteins related to polymer synthesis (Dennis *et al.*, 2008). In the present work, no PHA-related proteins are expected to be present in *E. coli* other than the three PHB biosynthetic enzymes and $PhaP_{Az}$, indicating that $PhaP_{Az}$ is able to bind to the granules both *in vitro* and *in vivo* in the absence of other phasins. Experiments using the PhaP-GFP fusion protein showed that addition of GFP does not affect the granule binding ability of the phasin. These results are in agreement with previous studies, in which the capacity of PhaP1 of *R. eutropha* to bind to PHB granules in recombinant *E. coli* has been used to develop purification procedures in which other proteins fused to phasin can be recovered by copurification with PHB granules (Banki *et al.*, 2005).

In the absence of the polymer, PhaP-GFP was first observed to be homogenously distributed in the cytoplasm, but it concentrated around the cell poles several hours after induction, forming bright *foci*. This phenomenon has also been reported for phasins of *R. eutropha* (Pfeiffer and Jendrossek, 2012), and proposed to be due to the formation of phasin oligomers. However, this explanation does not seem to be applicable to $PhaP_{Az}$. This protein is tetrameric in solution, but results obtained both in previous work (Mezzina *et al.*, 2014) and in this study indicate that it is very soluble and does not form detectable aggregates or IBs. On the other hand, it has been observed that as cells age, misfolded proteins tend to accumulate around the cell poles, and chaperones such as IbpA bind to them (Lindner *et al.*, 2008). Experiments performed in the present study have shown that $PhaP_{Az}$ binds to IBs, so it could be hypothesized that localization of $PhaP_{Az}$ (or other phasins) in the poles of older cells could be related to this phenomenon.

In our previous work, PHB producing *E. coli* strains overexpressing *phaP* were observed to grow more, and

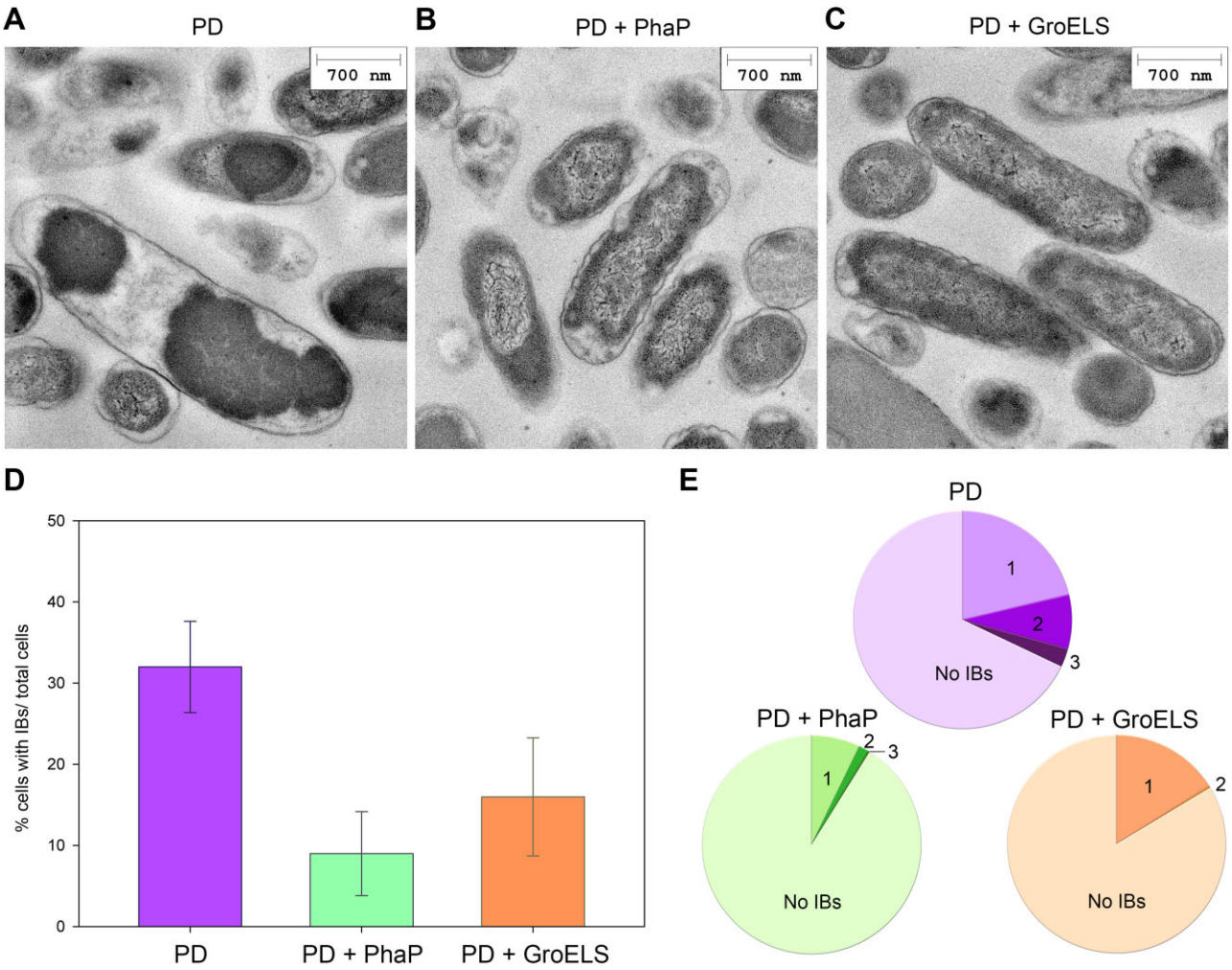

Fig. 6. *In vivo* chaperone activity: effect of $PhaP_{Az}$ on inclusion body construction/deconstruction. TEM images of BL21 (DE3) *E. coli* cells producing (A) PD, (B) PD and $PhaP_{Az}$, (C) PD and GroELS. (D) Percentage of cells containing IBs after 24 h of induction. Error bars correspond to one SD. Statistics: non-parametric test ($P < 0.0001$). (E) Percentage of cells containing no IBs, 1, 2 or 3 IBs after 24 h of induction. Cells producing PD contained 21.3; 8.0 and 2.7% cells with 1, 2 or 3 IBs respectively. Cells coproducing PD and PhaP contained 7.2; 1.5 and 0.2% cells with 1, 2 or 3 IBs respectively. Cells coproducing PD and GroELS contained 16.1; 0.3 and 0% cells with 1, 2 or 3 IBs respectively.

to accumulate more polymer (de Almeida *et al.*, 2007). These results suggested that $PhaP_{Az}$ exerts a growth-promoting effect in the recombinants. Proteome analyses of PHB-producing recombinant *E. coli* strains have shown an increase in heat shock proteins, when compared with non-producing strains, which indicates that the accumulation of the polymer causes a stress to the cells (Han *et al.*, 2001). This stress can be compared with the one observed during the production of heterologous proteins in *E. coli*, which generates an increase in the transcription (Gill *et al.*, 2000; Jürgen *et al.*, 2000) and concentration (Hoffmann and Rinas, 2004) of several chaperones, such as GroEL, GroES, DnaK, IbpA and IbpB. It is likely that PHB production induces the heat shock response by titrating available cytoplasmic chaperones onto the surfaces of the PHB granules (de Almeida *et al.*, 2011). Analysis of proteins associated to PHB granules have shown that several heat shock proteins bind to the granules in the absence of $PhaP_{Az}$, but are excluded from the granules in the presence of $PhaP_{Az}$ (Tessmer *et al.*, 2007). In view of this, it was not surprising to observe that in PHB-producing strains expressing $PhaP_{Az}$, expression levels of heat shock proteins were lower than in strains without $PhaP_{Az}$. However, the finding that these levels were also lower than in the control strains without PHB was totally unexpected. The lack of induction of heat shock proteins in cells producing $PhaP_{Az}$, together with a protective effect observed in cells exposed to heat and oxidative stress, led to the hypothesis that $PhaP_{Az}$ could have chaperone-like activities (de Almeida *et al.*, 2011).

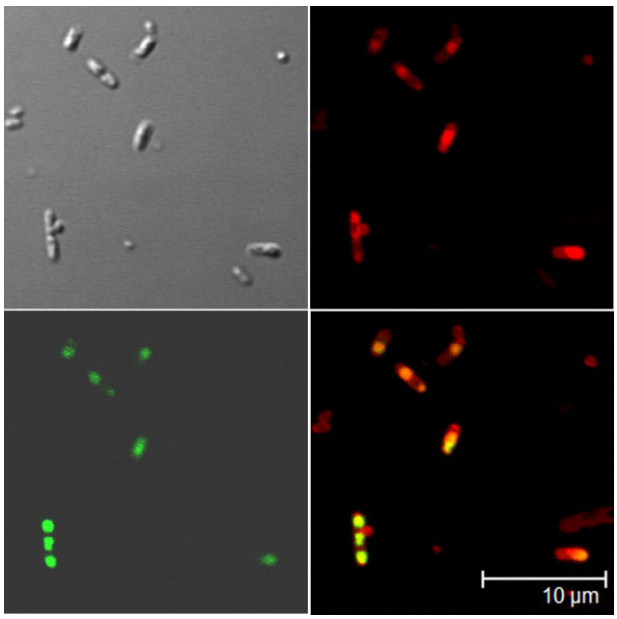

Fig. 7. Interaction of $PhaP_{Az}$ with PD inclusion bodies. Cells (top left) were examined by confocal fluorescence microscopy: IBs after staining with Nile Red (top right), PhaP-GFP (bottom left), merge (bottom right). The M1/M2 colocalization coefficients were M1 = 0.939 ± 0.037 and M2 = 0.692 ± 0.098, being M1 the fraction of green overlapping red and M2 the fraction of red overlapping green.

This hypothesis was verified in the present work by both *in vivo* and *in vitro* results. *In vitro* chaperone activity was monitored using CS, one of the most widely used proteins for chaperone activity assays. These experiments showed that $PhaP_{Az}$ is able to protect CS from thermal unfolding followed by aggregation. Additionally, incubating chemically denatured CS in the presence of $PhaP_{Az}$ demonstrated that the phasin enhances both the rate and the level of CS refolding, helping the protein achieve its native state. These results demonstrated that $PhaP_{Az}$ has chaperone activity *in vitro* (Fig. 8).

The capability of $PhaP_{Az}$ to protect cells from thermal and oxidative stress suggested that it has chaperone activity *in vivo* (de Almeida *et al.*, 2011). To further analyse this hypothesis, its effect on the construction/deconstruction of IBs formed by proteins that aggregate when overexpressed in *E. coli* was analysed by microscopy experiments. When the recombinant protein PD was coexpressed with GroELS or $PhaP_{Az}$, we observed that the number of IBs after 24 h decreased. Furthermore, the effect of $PhaP_{Az}$ on the number of cells containing IBs was even greater that the effect of the known chaperone GroELS. These results indicate that $PhaP_{Az}$ plays a role in IBs construction/deconstruction processes as observed for other chaperones such as DnaK, that has been observed to decrease aggregation and help the solubilization of small aggregates, GroEL, that is involved in the organization of small protein aggregates in IBs and the removal of proteins from IBs, and the small heat-shock proteins IbpA and IbpB, that are known to bind to heat-denatured proteins protecting them from irreversible aggregation (Carrió and Villaverde, 2003). However, it is not possible to distinguish if $PhaP_{Az}$ affects formation of IBs, IBs dissolution due to protein removal or both. $PhaP_{Az}$ was shown to colocalize with IBs *in vivo*, demonstrating that this protein has an effective interaction with the misfolded proteins. This characteristic has been observed in several chaperones, such as DnaK and GroEL, the main drivers of *E. coli* protein folding (together with their cochaperones DnaJ-GrpE and GroES, respectively) that have also been identified as components of *E. coli* IBs (Carrió and Villaverde, 2005).

Phasins are known to be amphiphilic proteins that contain hydrophobic residues (Maestro *et al.*, 2013; Mezzina *et al.*, 2014) that allow them to bind to hydrophobic substrates such as PHAs, and to have surfactant-like properties (Wei *et al.*, 2011). $PhaP_{Az}$ contains three amphipatic helices that expose hydrophobic surfaces (Mezzina *et al.*, 2014) that could interact with exposed hydrophobic regions of unfolded proteins. However, this unspecific interaction alone cannot explain the *in vitro* and *in vivo* chaperone properties of $PhaP_{Az}$. In addition, $PhaP_{Az}$ has a remarkable capacity to change according to its environment, probably due to its unstructured regions, and this structural flexibility suggests that it could interact with different targets, including misfolded proteins (Mezzina *et al.*, 2014). Some well-known molecular chaperones, such as DnaK, have also been observed to include disordered regions that are essential for their activity (Smock *et al.*, 2011). The results presented in this work support the hypothesis that the suppression of heat shock protein induction *in vivo* observed in our previous study is due to the contribution of $PhaP_{Az}$ to prevent the accumulation of misfolded proteins, probably by protecting them from unfolding and/or by helping them refold, thus acting as a chaperone (Fig. 8).

PHAs function as carbon and energy storage compounds in many bacteria, and are known to play an important role in stress protection and survival. However, the mechanism by which PHAs exert this protective effect are not yet fully understood, and have been traditionally attributed exclusively to the capacity of PHAs to provide energy and reducing power for stress response processes. (Kadouri *et al.*, 2005). In natural PHA producers, any possible effects that could be due to phasins would be masked by the polymer or polymer-associated cellular components, as it is well known that phasins affect the synthesis (Wieczorek *et al.*, 1995) and degradation (Handrick *et al.*, 2004) of PHAs. Analysing the effect of $PhaP_{Az}$ in a non-PHA-producing microorganism like

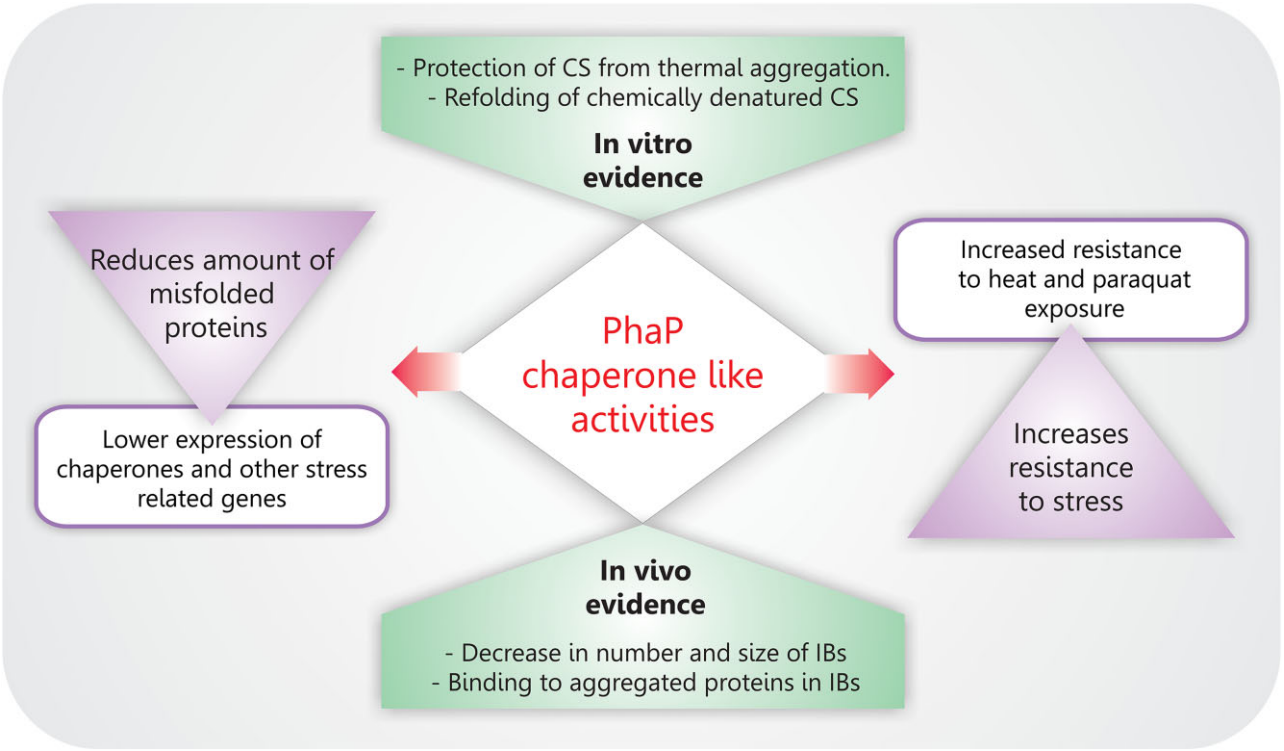

Fig. 8. Chaperone like activities of PhaP from *Azotobacter* sp FA8. *In vivo* and *in vitro* experimental evidence of $PhaP_{Az}$ chaperone activity. *In vitro* (top): $PhaP_{Az}$ was observed to prevent the spontaneous thermal aggregation of citrate synthase (CS) and facilitate its refolding after chemical denaturation. *In vivo* (bottom): $PhaP_{Az}$ was observed to bind to inclusion bodies (IBs) formed by misfolded proteins, and to reduce the number and size of IBs. Effects of $PhaP_{Az}$ on heat shock gene expression and stress resistance (observed in a previous study de Almeida *et al*., 2011) can be explained by its chaperone activity. In the presence of $PhaP_{Az}$ the amount of misfolded proteins, that trigger the heat shock response, is reduced (left triangle). As a result, the heat shock response, that includes the expression of chaperones and other stress-related genes, is not induced. $PhaP_{Az}$ exerts a stress protective effect (right triangle), probably by reducing stress-related protein misfolding, that is reflected by an increased survival to heat shock and paraquat exposure.

E. coli has enabled the analysis of the effects of this protein independently of polymer metabolism, revealing that it has remarkable properties. If the protective effects demonstrated for $PhaP_{Az}$ are a general characteristic of phasins, these proteins could play an important role in protein homeostasis, complementing the resources provided by polymer degradation with specific chaperone activities that could further help cells to cope with different kinds of stresses.

Although the enhancement of polymer synthesis and cell growth of PHA synthesizing recombinant *E. coli* in the presence of phasins has been known for many years (York *et al*., 2001; de Almeida *et al*., 2007), the mechanisms underlying this effect have remained unknown, and were generally attributed to the role of $PhaP_{Az}$ as part of the PHA granule cover that prevents interaction of the polymer with other cellular components, such as proteins (Wieczorek *et al*., 1995). The results obtained in this work suggest that the protective effect of phasins is not only passive, but could involve an active role in protein folding and/or unfolding prevention, expanding the already broad range of different activities associated to phasins.

Experimental procedures

Bacterial strains, plasmids and growth conditions

All *E. coli* strains and plasmids used in this work are listed in Table 1. For strain M15/pREP4/pQP2, Lysogeny broth (LB) medium supplemented with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was used. Cultures were grown at 37°C and shaken at 250 rpm. For strains BL21 (DE3), LB medium supplemented with 0.1 mM IPTG was used. Cultures were grown at 30°C and shaken at 200 rpm. For PHB-L21 strain, LB medium supplemented with 1% glucose and 0.1 mM IPTG was used. For plasmid maintenance, 50 μg ml^{-1} kanamycin, 20 μg ml^{-1} chloramphenicol and 100 μg ml^{-1} ampicillin were added when needed.

Protein expression and purification

$PhaP_{Az}$ was expressed, purified and quantified as previously described (Mezzina *et al*., 2014).

Construction of $PhaP_{Az}$ expression vectors for in vivo *experiments*

Primers phaP FB (5' CATGGGATCCCGTAATGGCTTTTT TTGATC 3') and phaP RX (5' TATCTCGAGGCCGTCAG

Table 1. *E. coli* strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Reference or source
E. coli strains		
BL21 (DE3)	F^- *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3)	Novagen
M15/pREP4	Nal^s, Str^s , Rif^s , Thi^- ,Lac^-, Ara^+, Gal^+, Mtl^-, F^-, $RecA^-$, Uvr^+, Lon^+, carrying pREP4 plasmid containing *lacI*	Qiagen
PHB-L21	Same as BL21, carrying *phaBAC* from *R. eutropha* under the control of the Ptrc promoter, integrated in the chromosome by using pMAB26 minitransposon (Pais *et al*., 2014), Km^r	Ma. Auxiliadora Prieto strain collection
Plasmids		
pRX23	pBlueScript carrying a 5 kb XhoI genomic fragment from *Azotobacter* sp. strain FA8 containing ISr', *phaF*, *phaP* and *phaR*; Ap^r	(de Almeida *et al*., 2007)
pQP2	pQE31 carrying *phaP* from *Azotobacter* sp. FA8, Ap^r	(Mezzina *et al*., 2014)
pACP	pACYC-Duet1 carrying *phaP* from *Azotobacter* sp. FA8, Cm^r	This work
pACP-GFP	pACYC-Duet1 carrying a fusion of $PhaP_{Az}$ to GFP in the C-terminus, Cm^r	This work
pPD	pET-29 carrying *pd,* that encodes an insoluble domain of TolR from *Azoarcus* sp. CIB (Accesion number: DQ988527.1 from position 781 to 1149), Km^r	Eduardo Díaz strain collection
pGroELS	pET-29, carrying *groELS* from *E. coli,* Cm^r	(Yasukawa *et al*., 1995)

GCAGTCTT 3') were used to obtain a 591 bp amplification from pRX23 plasmid. The resulting amplification fragment was cut with BamHI and XhoI and ligated into vector pACYCDuet-1 (Novagen). The resulting plasmid, pACP, confers resistance to chloramphenicol and expresses $phaP_{Az}$ from a promoter operator element consisting of the phage T7 promoter and a *lac* operator sequence.

For fluorescence microscopy experiments, a PhaP-GFP fusion in the C-terminus was synthetically synthesized (Appendix S1) and cloned in a pACYCDuet-1 vector (Novagen).

In vitro *chaperone activity*

Pig heart CS was purchased from Sigma-Aldrich, and dissolved to 20 μM in 20 mM Tris HCl (pH 8.0).

Pig heart CS (150 nM final concentration) was added to a 45°C preheated solution containing varying amounts of $PhaP_{Az}$ or BSA (protein control) in 20 mM sodium phosphate buffer (pH 7.5) and 2.0 mM DTT. A control experiment containing only PhaP (0.15 or 1.5 μM final concentration) was carried out. Aggregation was followed by monitoring light scattering at 360 nm in a JASCO V-650 spectrophotometer with a thermostated cuvette holder.

Chemically denatured CS was prepared as described previously (Buchner *et al*., 1998). Briefly, an aliquot of CS (12 μM final concentration) was dissolved in 20 mM Tris buffer (pH 8.0), 5.0 M GdmCl and 3.0 mM DTT, and incubated for 4 h. For light scattering measurements, chemically denatured CS was diluted 100 times in 25 mM sodium phosphate buffer (pH 7.5) and 2.0 mM DTT containing varying amounts of $PhaP_{Az}$ or BSA at 25°C.

All data are representative of replicate experiments.

CD spectroscopy

CD experiments were carried out in a Jasco J-810 spectropolarimeter equipped with a Peltier PTC-423S system. $PhaP_{Az}$ in a concentration range between 0.3 and 1.5 μM was incubated during 30 min at 45°C in a buffer containing 20 mM sodium phosphate, 50 mM NaCl pH = 7.3, 1 mM DTT. Measurements were performed immediately after incubation at 45°C and after cooling to 20°C. Wavelength spectra were acquired at a scan speed of 100 nm min^{-1} with a response time of 4 s and averaged over at least six scans at 25°C.

Fluorescence spectroscopy

Tryptophan emission spectra were recorded on an Aminco-Bowman spectrofluorometer with an excitation wavelength of 290 nm at 25°C.

CS (0.15 μM) samples were either untreated, chemically denatured (by treatment with 6 M GdmCl) or incubated at 25°C or 45°C in the presence of a 10-fold molar excess of $PhaP_{Az}$ (considering monomeric concentration) for 20 min.

Treated and untreated samples were centrifuged for 20 min at 13 000 rpm and fluorescence spectroscopy measurements were performed at 25°C for 25 min, in 20 mM sodium phosphate buffer (pH 7.4).

All data are representative of replicate experiments.

In vivo *localization of PhaP-GFP by fluorescence microscopy*

Escherichia coli strains were cultivated overnight in LB medium. Then, cells were inoculated at 0.1 OD_{600} in LB and incubated at 30°C. Cultures were induced with 0.1 mM IPTG in the exponential phase of growth to induce the production of the PhaP-GFP fusion protein. PD IBs were visualized after staining with 30 μg ml^{-1} Nile Red for 15 min in the dark. PHB granules were stained with 3 μg ml^{-1} Nile Red for 15 min in the dark. Cells were then fixed with 4% paraformaldehyde at room temperature for 1 h, washed tree times with phosphate-buffered saline (PBS), 10 mM, pH 7.2 and visualized by confocal microscopy laser confocal espectral Leica TCS SP2-AOBS or Zeiss Axioplan Universal epifluorescence microscope operated for incident light fluorescence and contrasting techniques of bright field and phase contrast.

Images were analysed using ImageJ (NIH). Colocalization coefficients M1/M2 were calculated using JACoP plug in Bolte and Cordelières (2006).

All data are representative of replicate experiments.

TEM

Cells were harvested, washed twice in PBS and fixed in 5% (w/v) glutaraldehyde in the same solution. Afterwards, cells were suspended in 2.5% (w/v) OsO4 for 1 h, gradually dehydrated in ethanol [30%, 50%, 70%, 90% and 100% (v/v); 30 min each] and propylene oxide (1 h) and embedded in Epon 812 resin. Ultrathin sections (thickness 70 nm) were cut with a microtome using a Diatome diamond knife. The sections were picked up with 400 mesh copper grids coated with a layer of carbon and subsequently observed in a Jeol-1230 electron microscope (Jeol, Akishima, Japan). The minimum number of total cells counted for each strain analysed was 750. The number of IBs per cell was determined only from cells which were fully visible in the electron micrographs, considering cells in which the width/length relationship was approximately 1:2, indicating a longitudinal section.

Images were analysed using ImageJ (NIH). Cells and IBs were counted using Cell Counter plug in.

Statistic analysis was performed using Graph Pad. All data are representative of replicate experiments.

PHB granule extraction

PHB was isolated from PHB-L21-grown in LB medium with glucose (1% wt/vol) as the carbon source, disrupted by passage through a French press. Granule isolation was done by ultracentrifugation through 2 gradients: 50 and 85 w/v % glycerol for the first one and 40, 60, 80 and 85 w/v% glycerol for the second one. SDS-PAGE of the PHB fraction, obtained in the 60% fraction of the second gradient, was performed in order to analyse the proteins associated to the granules.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Sequence of *phaP-gfp* (1313 bp).