

Phasins, Multifaceted Polyhydroxyalkanoate Granule-Associated Proteins

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Phasins are the major polyhydroxyalkanoate (PHA) granule-associated proteins. They promote bacterial growth and PHA synthesis and affect the number, size, and distribution of the granules. These proteins can be classified in 4 families with distinctive characteristics. Low-resolution structural studies and *in silico* predictions were performed in order to elucidate the structure of different phasins. Most of these proteins share some common structural features, such as a preponderant α -helix composition, the presence of disordered regions that provide flexibility to the protein, and coiled-coil interacting regions that form oligomerization domains. Due to their amphiphilic nature, these proteins play an important structural function, forming an interphase between the hydrophobic content of PHA granules and the hydrophilic cytoplasm content. Phasins have been observed to affect both PHA accumulation and utilization. Apart from their role as granule structural proteins, phasins have a remarkable variety of additional functions. Different phasins have been determined to (i) activate PHA depolymerization, (ii) increase the expression and activity of PHA synthases, (iii) participate in PHA granule segregation, and (iv) have both *in vivo* and *in vitro* chaperone activities. These properties suggest that phasins might play an active role in PHA-related stress protection and fitness enhancement. Due to their granule binding capacity and structural flexibility, several biotechnological applications have been developed using different phasins, increasing the interest in the study of these remarkable proteins.

Polyhydroxyalkanoic acids (PHAs) are polymers synthesized by many bacteria that function as intracellular carbon and energy storage compounds. According to the length of the monomer, these polymers are classified as short (C_3 to C_5)- or medium (C_6 to C_{16})-chain-length PHAs (1, 2). The best known and most common PHA is poly(3-hydroxybutyrate) (PHB), composed of C_4 monomers. PHAs are accumulated as intracellular insoluble granules that are surrounded by an organized protein layer composed of several granule-associated proteins (PGAPs). Among the proteins associated with PHA granules are PHA synthases, PHA depolymerases, and a group of low-molecular-weight proteins known as phasins. The presence of structural, biosynthetic, catabolic, and regulatory proteins in the granule surface indicates that they are organized and complex subcellular structures that were designated carbonosomes (3).

The first phasin was identified in 1994 by Pieper-Fürst and Steinbüchel when they found a low-molecular-weight protein (GA14) associated with PHA granules in *Rhodococcus ruber*. GA14 was the predominant protein present in the granules, forming a layer at their surface. Due to these properties, the name phasin (PhaP) was proposed, analogous to the oleosins at the surface of triacylglycerol inclusions in plants (4).

Since then, phasins have been found in the surfaces of the granules of all natural PHA-producing microorganisms studied (5), constituting the most abundant and widespread granule-associated proteins. Several phasins were identified in many PHA-producing bacteria, such as *Ralstonia eutropha* (also known as *Cupriavidus necator*) (5–7), *Bacillus megaterium* (8), *R. ruber* (4), *Paracoccus denitrificans* (9), *Pseudomonas putida* (10), *Azotobacter vinelandii* (11), and *Synechocystis* sp. strain PCC 6803 (12), among many others. Phasins have also been identified in *Archaea* such as *Haloferax mediterranei* (13).

This review summarizes the main characteristics of phasins, the multiple roles associated with these proteins, and their biotechnological applications.

PHASIN PROTEIN FAMILIES

Although phasins do not constitute a highly conserved group of proteins, and early reports indicated that the degree of conservation among them was very low, several protein motifs have been defined using the great number of phasins that have already been described, and several types of phasin families have been distinguished based on sequence similarity.

Considering the Pfam database (<http://pfam.xfam.org/>), there are four phasin-related families, each containing a characteristic domain (Table 1). The first family (PF09361) is the largest one and includes sequences found in bacteria belonging to *Alpha*-, *Beta*-, and *Gammaproteobacteria*, such as the most studied phasin, PhaP1 from *R. eutropha* (PhaP_{Re}). The second (PF09602) corresponds to phasins found in *Bacillus* species, and the third (PF09650) contains a diverse group of mostly uncharacterized proteins belonging to different *Proteobacteria*. The last family (PF05597) contains proteins from different *Proteobacteria* and includes all characterized phasins belonging to *Pseudomonas* that accumulate medium-chain-length PHAs (PHAmcl), such as PhaF and PhaI from *P. putida*.

Although most phasins described to date belong to one of these four families, there are a few that show very little similarity to the rest and contain no recognizable phasin-related domains. Among them is the first phasin described, GA14 from *R. ruber*, PhaP from *Synechocystis* sp. PCC 6803, and archaeal phasins. Analysis of

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TABLE 1 Phasin families according to the Pfam database

Pfam database family name	Domain	Representative taxon/taxa	Representative protein(s)
PF09361	Phasin_2	<i>Ralstonia</i> , <i>Azotobacter</i>	PhaP from <i>Ralstonia eutropha</i>
PF09602	PhaP_Bmeg	<i>Bacillus</i>	PhaP from <i>Bacillus megaterium</i>
PF09650	PHA_gran_rgn	Diverse <i>Proteobacteria</i>	Uncharacterized group of phasins
PF05597	Phasin	<i>Pseudomonas</i>	PhaF from <i>Pseudomonas putida</i>

PhaP from *Synechocystis* revealed that it is similar to thylakoid-associated proteins from different algal species and to a protein from *Arthrospira platensis* that contains a Phasin_2 motif (12). Many phasins have been identified in *Archaea*, but to date, only that of *H. mediterranei* has been experimentally studied. This phasin has been reported to be relatively similar to other putative archaeal phasins but not to those from bacteria (13).

The different similarity groups in which phasins can be classified seem to reflect both the phylogenetic origin of the phasins and the kind of PHAs to which they bind. Most phasins characterized to date belong to *Proteobacteria*. Among them, PF09650 and PF09361 group phasins are related to short-chain PHAs, while phasins related to PHAmcl, mostly belonging to *Pseudomonas* species, constitute a separate group (PF05597 family). Although most *Pseudomonas* spp. accumulate only PHAmcl, several strains, such as *Pseudomonas* sp. 61-3 (14), *P. extremaustralis* (15), and *P. pseudoalcaligenes* (16), have been observed to accumulate both PHAmcl and PHB. These bacteria contain separate biosynthesis gene clusters for each polymer and different phasins that belong to different phasin families. A recent study that characterized granules containing different polymer compositions in *Pseudomonas* sp. 61-3 determined that phasins PhaF and PhaI, which have the Phasin domain (Table 1), were found on the surface of PHAmcl granules while PhbP, which belongs to the largest phasin family (PF09361) containing the Phasin_2 domain, was bound to PHB granules in this organism (14), suggesting that phasins have a certain degree of specificity. The ability of *Pseudomonas* strains to accumulate PHB could have been acquired by horizontal gene transfer (15, 16), so it is possible that genes encoding the PHB-related phasins in these bacteria could have also been acquired in this manner.

STRUCTURAL ASPECTS OF PHASINS

The structure of phasins has been analyzed in relatively few studies. Low-resolution structural studies and *in silico* predictions were performed in order to elucidate the structure of different phasins, such as PhaP from *R. eutropha* (PhaP_{Re}) (17), PhaP from *Azotobacter* sp. strain FA8 (PhaP_{Az}) (18), PhaF from *P. putida* (PhaF_{Pp}) (19), PhaP from *Aeromonas hydrophila* (PhaP_{Ah}) (20), and PhaP from *Synechocystis* sp. PCC 6803 (PhaP_{Sp}) (12).

Secondary-structure *in silico* predictions for phasins PhaP1 to PhaP4 from *R. eutropha*, PhaP1 from *Ralstonia solanacearum*, PhaP from *Ralstonia metallidurans* (17), PhaP_{Az} (18), and PhaP_{Sp} (12) revealed that these phasins have a high percentage (close to 90%) of amino acids in an α -helix conformation. This was found

to be a general characteristic of phasins (18–20). However, the proportion of residues in an α -helix conformation was observed to change according to the environment in experiments performed with PhaP_{Az} and the N-terminal domain of PhaF_{Pp}, as this proportion was shown to increase in the presence of structure-stabilizing solvents, like 2,2,2-trifluoroethanol, or with the addition of sodium oleate, which creates a hydrophobic environment that mimics PHB (18, 19).

A structural feature that has been recently recognized in phasins is the presence of disordered regions, which are predicted in many of these proteins (18, 19). Intrinsically disordered proteins or disordered regions are interconverting dynamic ensembles of structures that do not fold in a single structure and are important for the regulatory functions of the proteins because of their highly flexible nature (21). Maestro et al. proposed that the multifunctional phasin PhaF_{Pp} belonged to a new family of intrinsically disordered proteins due to the unstructured regions predicted in its N-terminal domain, which was also predicted to contain a long α -helix that is partially disordered in the absence of PHA (19). Unstructured regions were also found in PhaP_{Az}. It was estimated that around 40 to 45% of this phasin could be disordered when it is not binding any target, and this value would decrease to 23 to 30% when interacting with other molecules. The capacity of PhaP_{Az} to change according to the environment could be attributed to the presence of disordered regions that provide flexibility to the protein (18). Disordered regions might contribute to the functional diversity of these proteins.

All phasins form oligomers, and most studies have indicated that these proteins are normally tetramers in solution (12, 18–20). However, Neumann et al. proposed that PhaP_{Re} occurs as a trimer (17), and some phasins have been reported to form other kinds of oligomers, such as dimers and dodecamers (corresponding to PhaP5 and PhaM from *R. eutropha*, respectively) (22). A common structural characteristic of phasins is the presence of coiled-coil interacting regions. Bioinformatics predictions have revealed that many of them have a high coiled-coil probability (>75%), such as phasins from *R. eutropha* and from different species of *Pseudomonas* and *Bacillus* (19). On the other hand, other phasins have an intermediate coiled-coil probability (25% to 75%), such as PhaP_{Az} (18) and a phasin from *Azotobacter vinelandii* (19). These regions have been proposed to be involved in oligomerization and/or interaction with other proteins (18, 19). Although coiled-coil regions have been found in almost all phasins described to date, the location and extension of these regions differ greatly, even among phasins belonging to the same family (18). This suggests that the interaction between monomers and with PHA granules, other phasins, and other proteins might differ for different phasins. PhaP_{Sp} is the only phasin devoid of coiled-coil regions described to date, suggesting that the mode of oligomerization of this protein, which was observed to form tetramers in solution, is different from that described for other phasins (12).

INTERACTION WITH PHA GRANULES

As phasins are proteins that bind to hydrophobic PHA granules, the presence of hydrophobic domains that could mediate interactions with the polymer has been analyzed in many of them. GA14 from *R. ruber* contains two hydrophobic domains in the C terminus that were proposed to be interaction domains with PHA based on experimental results (4). A later study that analyzed PhaP1 to PhaP4 from *R. eutropha* revealed that these phasins do not have

clear hydrophobic domains in common, although they share homologous hydrophobic regions. Experimental results obtained with PhaP_{Re} mutants revealed that the entire protein is involved in the interaction with polymer granules and that there is no particular region responsible for this interaction (17). The absence of hydrophobic domains appears to be a common characteristic of most phasins, despite their lipid granule binding function. Analysis of the primary structure of other phasins, like PhaP_{Az}, PhaP_{Pp}, and PhaP_{Sp}, revealed that they lack clear hydrophobic domains, and their ability to bind to PHA granules was associated with the amphiphilic nature of at least one of their helices (12, 18, 23). Furthermore, PhaP from *B. megaterium* (PhaP_{Bm}) was observed to be an extremely hydrophilic phasin that can effectively bind to the granules in spite of the lack of hydrophobic domains (8). The hydrophobic nature of GA14, which has no characteristic phasin domains, seems to be uncommon among phasins (18). This protein has been observed to interact with granules containing both PHA and triacylglycerols in *R. ruber* (24), so its characteristics might be associated with the particular composition of the lipid granules accumulated by this oleaginous bacterium. However, phasins that lack clear hydrophobic domains, such as PhaP_{Re}, have been observed to bind to triacylglycerol inclusions when expressed in *R. opacus* and *Mycobacterium smegmatis* (25). Amphipathic helices in phasins might be important not only for the interactions of these proteins with the polymer but also for interactions with other granule-associated proteins and with hydrophobic regions of misfolded proteins and inclusion bodies (IBs) (23). Pfeiffer and Jendrossek studied the interactions between known PHB granule-associated proteins from *R. eutropha* *in vitro*. According to their results, phasins not only are able to form homo-oligomers but can also associate with each other to form hetero-oligomers, and they are also able to interact with other proteins, such as the regulator PhaR and the PHB depolymerase PhaZ1 (26).

ROLE OF PHASINS IN PHA METABOLISM

Studies of phasin PhaP_{Re} showed that this protein plays an important role in PHB synthesis. The first genetic analysis of the role of PhaP_{Re} in PHB metabolism was performed by Wieczorek et al. (5), who studied the effect of *phaP* mutations on PHB synthesis in *R. eutropha*. Their study revealed that this phasin promotes PHA synthesis and affects the number and size of the granules (5), an effect that has also been observed in other bacterial producers (9, 12, 27, 28).

It has been reported that the occurrence of PhaP_{Re} in the cells is strictly dependent on PHA biosynthesis and that cells cultivated under conditions not permissive for PHA synthesis or mutants defective in the PHA synthase structural gene do not synthesize detectable levels of PhaP_{Re} (5, 29, 30). Intracellular levels of this phasin were observed to increase concomitantly with PHB accumulation in cells producing either low, intermediate, or high levels of the polymer (31), suggesting that in natural PHA producers the synthesis of phasins is tightly coupled with PHA synthesis. Analysis of the genetic regulation of phasin PhaP_{Re} revealed that it is regulated by PhaR, a repressor that binds upstream of *phaP* and was also found to associate with PHA granules (32). Pötter et al. (33) and Pötter and Steinbüchel (34) proposed that during active PHA biosynthesis in *R. eutropha*, PhaR binds to PHA granules, allowing the transcription of *phaP*, so at the later stages of PHA accumulation, when PhaR is no longer bound to the granules, the

transcription of *phaP* is again repressed (33, 34). In *B. megaterium*, PhaQ was proposed to act in a manner similar to that of PhaR from *R. eutropha*, as it binds to PHB granules and suffers transcriptional autoregulation (35). This mechanism is analogous to the one proposed by Prieto et al. for the regulation of the PHA biosynthesis genes in *Pseudomonas oleovorans* (currently known as *P. putida*) by phasin PhaF_{Pp} (10). PhaR was also found in *H. mediterranei*, but the regulation pattern of *phaP* by PhaR in this archaeon is slightly different from that of its bacterial counterparts, as it was shown to repress the expression of both its own gene and *phaP*, which are transcribed from the same operon (*phaRP*) (36).

Apart from its structural role as part of the PHA granule cover, many other functions related to PHA accumulation and degradation have been described for different phasins. For example, ApdA from *Rhodospirillum rubrum* was observed to activate PHB granules isolated from different species, including recombinant *Escherichia coli*, so that the granules could be hydrolyzed by soluble *R. rubrum* PHB depolymerase *in vitro* (28). This effect was demonstrated to be due to the capability of this phasin to activate the PHB depolymerase (37). In *R. eutropha*, the presence of PhaP_{Re} on the granule surface was also reported to be important for PHB degradation. This study suggested that the effect of this phasin on PHB degradation might be direct, through interaction with the PHB depolymerase, or indirect, by providing access of a PHB depolymerase to the surface of the PHB granules (38).

Mms16 from *Magnetospirillum gryphiswaldense* was first described as a protein associated with magnetosomes, but this was later observed to be an artifact attributable to unspecific adsorption during preparation of the magnetosomes. This protein was observed to bind to PHB granules *in vivo* and to activate the PHB depolymerase of *R. rubrum* (PhaZ1) *in vitro*. Accordingly, the authors suggested renaming the Mms16 protein of *Magnetospirillum* species ApdA, the name used for the phasin from *R. rubrum* that activates depolymerization (39).

PhaP_{Re} was shown to increase the activity of class II PHA synthases PhaC1 and PhaC2 from *P. aeruginosa* *in vitro*, activating PHA synthesis (40). Other phasins were also observed to affect PHA synthesis. For example, a mutant strain of *Synechocystis* sp. strain PCC 6803 that does not produce phasin PhaP_{Sp} showed reduced activity of the PHB synthase (12), and phasin PhaM from *R. eutropha* was described as the physiological activator of the PHB synthase in this microorganism (41).

The role of phasins from *Aeromonas caviae* (PhaP_{Ac}) and *R. eutropha* (PhaP_{Re}) in the modulation of the activity of PHA synthase (PhaC) from different microorganisms was studied by *in vitro* polymerization assays. These experiments revealed that both phasins increased the activity of PhaC from *A. caviae* (PhaC_{Ac}), but they inhibited the activity of the PHB synthases from *R. eutropha* (PhaC_{Re}) and *Delftia acidovorans* (PhaC_{Da}) *in vitro*, despite the fact that the presence of PhaP_{Ac} increased *in vivo* polymer production in recombinant *E. coli* strains that express either *phaC_{Ac}* or *phaC_{Re}*. These results led the authors to propose that there is PhaP-mediated PhaC_{Ac} activation at the molecular level (42). Another study performed using recombinant *E. coli* containing the PHA biosynthesis genes from *R. eutropha* revealed that the replacement of PhaP_{Re} by PhaP_{Ac} affected the composition of the polymer accumulated from soybean oil, increasing the amount of 3-hydroxyhexanoate incorporated. These results suggested that

TABLE 2 Additional functions of phasins

Phasin	Microorganism	Additional function(s) ^a	Reference(s)
PhaP _{Re}	<i>Ralstonia eutropha</i>	Increases specific activity of PhaC _{Re} and the activity of PhaC1 and PhaC2 from <i>Pseudomonas aeruginosa</i> <i>in vitro</i>	40
		Activates PHB degradation	38
PhaP _{Ah}	<i>Aeromonas hydrophila</i>	Activates transcription of <i>phaC_{Ah}</i>	45
PhaP _{Sp}	<i>Synechocystis</i> sp. PCC 6803	Increases activity of PHB synthase <i>in vivo</i>	12
PhaM	<i>Ralstonia eutropha</i>	Physiological activator of PhaC _{Re}	41
ApdA	<i>Rhodospirillum rubrum</i>	Stimulates <i>in vitro</i> depolymerization by PHB depolymerase of <i>R. rubrum</i>	28
Mms16	<i>Magnetospirillum gryphiswaldense</i>	Activates PHB depolymerase from <i>R. rubrum</i> <i>in vitro</i>	39
PhbP	<i>Azotobacter vinelandii</i>	Increases alginate production	11, 56, 57
PhaP _{Ac}	<i>Aeromonas caviae</i>	Increases activity of PhaC _{Ac} but inhibits activity of PhaC from <i>R. eutropha</i> and <i>Delftia acidovorans</i> <i>in vitro</i> ; increases the solubility of PhaC _{Re} <i>in vitro</i> (chaperone-like activity)	42
PhaF _{Pp}	<i>Pseudomonas putida</i>	Binds to DNA through its histone-like domain in a nonspecific manner	44
		Is involved in PHA granule segregation during cell division	19
PhaP _{Az}	<i>Azotobacter</i> sp. FA8	Protects <i>E. coli</i> from stress caused by PHB accumulation, heat, and paraquat; has <i>in vitro</i> and <i>in vivo</i> chaperone activity	18

^a The abilities to bind to the PHA granule surface and to affect the number and size of the granules are considered general properties of all phasins and are not indicated in the table.

the phasins affected both PHA synthase activity and its affinity for different monomers (43).

On the other hand, PhaF_{Pp} acts as a negative transcriptional regulator of PhaC in *P. putida*, as the disruption of *phaF* was observed to lead to higher expression levels of PhaC (10). Furthermore, this phasin binds to DNA nonspecifically through a histone-like domain and plays a key role in intracellular localization and equal distribution of PHA granules to daughter cells during cell division (19, 44). Last, PhaP from *A. hydrophila* (PhaP_{Ah}) was also observed to act as a positive regulator of the gene that encodes the PHA synthase in this organism, as the overexpression of *phaP* increased the expression of *phaC* (45).

All these results indicate that phasins have multiple and important roles in different aspects of PHA metabolism, ranging from their structural functions as components of the granule surface to regulatory roles affecting the expression and activity of different enzymes involved in PHA synthesis and depolymerization (Table 2).

PROTECTIVE EFFECT AND CHAPERONE ACTIVITY OF PHASINS

Phasins constitute the major components of the protein layer that stabilizes PHA granules and enhances polymer synthesis (34). In natural polymer producers, *phaP* mutants contain one or a few large granules at the end of the accumulation phase, instead of the several small granules observed in the wild-type strains. This has been proposed to be due to the fact that naked granules exhibit a hydrophobic surface and are therefore not protected from coalescence (5, 9, 12, 27). The protective layer surrounding PHB granules might also prevent damage to cellular components due to interactions with the polymer (5).

The beneficial effect of phasins on PHB production was also observed in *E. coli* recombinants, as the coexpression of phasins was observed to enhance growth and PHB accumulation (27, 31). PHB production was shown to cause stress in recombinant *E. coli*, evidenced by an increment in expression levels of chaperones, sigma factors, and other stress-related genes (46, 47). An analysis of the effect of PHB accumulation in a recombinant *E. coli* strain revealed that the presence of PhaP_{Az} produces a dramatic reduction in the expression of stress-related genes, such as *ibpA* and *dnaK*, compared to the strain that does not synthesize the phasin

(48). Unexpectedly, PhaP_{Az} also had a protective effect in non-PHB-synthesizing *E. coli* strains under both normal and stress conditions, evidenced by a reduction in heat shock protein levels, increased growth, and higher resistance to both heat shock and superoxide stress by paraquat (48). Given that this strain does not accumulate PHB, the protective effect of the phasin could not be due to its capacity to protect cells from the consequences of polymer accumulation, and it reflected a more general protective role. The reduced heat shock protein transcription observed in strains expressing *phaP*, together with reduced expression of *rpoH* during PHB production and reduced RpoH protein levels during heat shock, suggested that PhaP_{Az} might affect protein folding by exerting chaperone-like activity (48) (Fig. 1). PhaP_{Az} was observed to prevent the spontaneous thermal aggregation of citrate synthase and facilitate its refolding after chemical denaturation, indicating that this phasin has *in vitro* chaperone activity. In addition, PhaP_{Az} was observed to bind to inclusion bodies (IBs) formed by the heterologous protein PD, an insoluble domain of TolR from *Azoarcus* sp. strain CIB that aggregates when overexpressed in *E. coli*, and to play a role in the IB construction/deconstruction process by reducing the number and size of IBs *in vivo* (Fig. 1). Taken together, these results indicate that this phasin has *in vivo* chaperone activity (23).

Evidence supporting the chaperone-like functions of other phasins was found while analyzing the interaction of PhaP_{Ac} and PhaP_{Re} with different PHA polymerases. The presence of PhaP_{Ac} was observed to increase the solubility and activity of the synthase PhaC_{Re} in *in vitro* assays, and this effect was attributed to a chaperone-like role of the phasin (42).

Phasin-associated phenotypes have been traditionally attributed to the capability of these proteins to form a protective layer surrounding PHB granules (5). The chaperone activity of phasins might provide an additional explanation for the positive effect of PhaP on polymer accumulation, as these proteins might help assemble the complex structure of PHB granules and achieve active PHA synthesis. Phasins might also facilitate the folding or prevent the degradation of proteins that are not involved in PHA metabolism, thus producing a general protective effect in PHA-producing cells.

As indicated in the previous section, phasins were shown to be

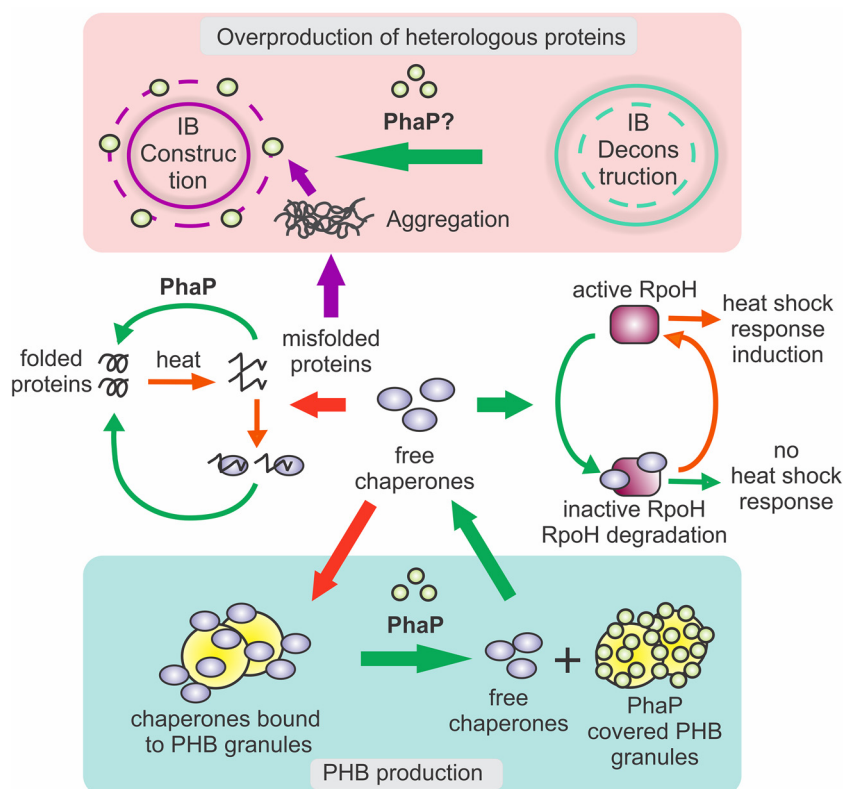


FIG 1 Proposed mechanism for the protective effect of phasins (based on results obtained with PhaP from *Azotobacter* sp. FA8 in *E. coli*). (Center) When misfolded proteins appear in the cell as a result of different kinds of stress, such as heat shock, production of PHB, or overproduction of heterologous proteins, chaperones bind to them, and RpoH freed from chaperones activates the heat shock response. PhaP, through its chaperone activity, exerts a protective effect that produces a reduction in the amount of misfolded proteins, diminishing the recruitment of chaperones, which are available to bind to RpoH, inactivating it. (Top) In cells that overproduce heterologous proteins, the expression of *phaP* reduces the number of IBs. This might occur through different mechanisms: PhaP might facilitate protein folding, preventing the aggregation of proteins in IBs; PhaP might release misfolded proteins from the IBs to the cytoplasm; or both mechanisms might occur. (Bottom) In PHB-producing cells, chaperones bind to PHB granules in the absence of phasins, reducing the pool of free chaperones, which leads to the induction of the heat shock response. On the contrary, when *phaP* is expressed, the presence of the phasin prevents the chaperones from binding to the granules and thus the induction of the heat shock response.

involved in the synthesis (5) and degradation (28) of PHAs. Because of this close association, any possible effects due to the activity of phasins in natural PHA producers could be attributed to the polymer or to polymer-associated cellular components. In contrast, results obtained in a non-PHA-producing microorganism, like *E. coli* expressing *phaP_{FA8}*, have provided evidence that this phasin has a protective role that is independent from polymer metabolism (18).

PHAs are known to act as carbon and energy storage in natural producers, and the effect of PHAs in stress protection and survival has been traditionally attributed solely to the supply of energy and reducing power for stress response processes (49). However, the chaperone-like activities described for some phasins suggest that they might play an important role in stress protection, complementing the resources provided by polymer degradation with specific chaperone activities, thus participating actively in reducing the deleterious effects of different kinds of stress.

BIOTECHNOLOGICAL APPLICATIONS OF PHASINS

As indicated in the previous sections, phasins have been shown to enhance bacterial growth and polymer accumulation (31), and they have also been observed to affect polymer elongation (42)

and even its composition (43). These properties highlight the importance of phasins in PHA production.

The ability of these proteins to bind to PHB granules has been exploited for a number of different applications apart from biopolymer production. For example, PhaP_{Re} has been used to facilitate recombinant protein purification. Fusions of this protein to green fluorescent protein (GFP) and an intein, an autoclavable protein, were constructed. This fusion protein was able to bind to PHB granules. After cell lysis and differential centrifugation, this fusion protein could be obtained bound to purified PHB granules. Then, the addition of thiols activates the intein, resulting in the liberation of GFP. Similar studies have been performed with PHB-producing recombinant *E. coli* strains in order to produce different kinds of recombinant proteins (50, 51).

The N-terminal domain of phasin PhaF_{pp}, denominated BioF, was used as a polypeptide tag to anchor fusion proteins to PHA granules. These granules could be easily purified by a simple centrifugation step and proteins subsequently released by treatment with mild detergents, maintaining their enzymatic activity (52). This method was proposed for the release of proteins to the environment, in particular for the liberation of the Cry1Ab toxin as an insecticide treatment (53).

Phasins were also used to develop a receptor-mediated drug delivery system using PHA nanoparticles. PhaP_{Re} fused with different ligands was attached to PHA nanoparticles containing hydrophobic drugs, allowing the delivery of the drugs to target cells that have receptors recognized by the ligands (51). Phasins bound to PHB beads can also be easily fused to antigens for application in fluorescence-activated cell sorter (FACS) analysis (54).

Phasins were also proposed as biosurfactants because of their amphipathic nature, and PhaP_{Ah} was reported to be able to emulsify petrochemical and vegetable oils and diesel *in vitro* (55).

Last, the chaperone-like activity observed for PhaP_{Az} (23), together with evidence that other phasins, such as PhaP_{Ac} (42), could have similar properties, suggests that phasins could be used to enhance the production of heterologous proteins in *E. coli*. These findings expand the already-wide field of potential applications for phasins.

CONCLUDING REMARKS

Phasins are proteins that were identified as major components of the PHA granule cover and found to promote growth and PHA synthesis and to affect the number, size, and distribution of the granules. Later studies revealed that they have a remarkable functional diversity, as different capabilities were described for different phasins. Apart from their PHA binding capability, phasins have been shown to interact with other proteins. Several phasins act as regulators, activating PHA depolymerization or increasing the expression and activity of PHA synthases. One phasin has been observed to exert a general protective effect in *E. coli*, both in the presence and in the absence of PHA, and has both *in vivo* and *in vitro* chaperone activity. Chaperone-like activities have also been proposed for other phasins in the context of PHA synthesis, and future work should help clarify whether these are common properties among phasins belonging to different groups. In natural producers, PHAs protect the cells against stress and contribute to their adaptability and survival in the environment. Although this effect has been attributed solely to the capacity of the polymer to provide carbon and energy for the different cellular processes, phasins might play an active role in reducing the deleterious effects of different kinds of stress, enhancing fitness and survival.

The amphiphilic nature of phasins, their PHA granule binding capacity, and their structural flexibility make them suitable for diverse biotechnological applications. Among the uses that have already been described are those in protein purification and drug delivery. The increasing evidence of the multiple functions and remarkable properties of these proteins opens the way for new possible applications.

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