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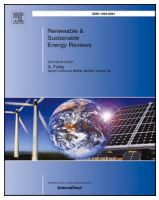

Biotechnical applications of phasins: Small proteins with large potential

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ABSTRACT

Phasins are a particularly fascinating class of small-molecular weight proteins that are the dominant proteins surrounding bioplastic granules produced by bacteria, called polyhydroxyalkanoates (PHAs). PHAs are biopolymers of interest since their thermomechanical properties are comparable to petroleum-based plastics, they are biodegradable, biocompatible, and can be produced from renewable bioresources. As the design and development of sustainable bioproducts from biomass and bioresources is becoming increasingly desirable, efforts to characterize and optimize PHA production have illuminated some exceptional functions of phasins. In addition to their surface performance in PHA granule formation, phasins have been shown to perform chaperone-like activities for bacterial stress mitigation, activate PHA depolymerization, contribute to PHA granule segregation, and boost the expression and activity of PHA synthases. Due to the newfound knowledge of the structures, functions, and strong amphiphilic tendencies of phasins, they have been applied in a wide variety of sustainable applications far beyond bioplastic production. Thus, phasins are emerging as a biotechnology platform for sustainable, next generation bioproduction from biomass. This review provides a synopsis of the biotechnical advances employing phasins, which include optimized bioplastic production, increased tolerance to growth inhibitors in biorefineries, "green" biocatalysis, environmental remediation, and an assortment of sustainable therapeutic bioproducts. Research gaps and suggested applications of phasins are also offered as a potential guide for future direction.

1. Phasins - small proteins with diverse functions

Polyhydroxyalkanoates (PHAs) are biopolymers that have thermomechanical properties similar to petroleum-derived plastics but are also biodegradable and biocompatible. Thus, they have been used in applications ranging from packaging to drug delivery systems. There have been numerous reviews that highlight the applications and recent advancements of PHAs [1–7]. Bacteria produce PHAs during unbalanced growth and typically store them inside their cytoplasm as water-insoluble granules. The size and abundance of granules in the cytoplasm can differ greatly across bacterial species. While it was originally thought that PHAs serve as carbon sinks for bacteria, PHAs have been shown to also enhance bacterial fitness and resilience [8], provide ultra-violet radiation protection [9], and likely be a source of redox balance [10]. PHAs are polyoxoesters of R-hydroxyalkanoic acid monomers and are considered to be the largest group of natural polyesters with over 150 different monomers reported [3]. They are classified based on the numbers of carbon atoms in the monomers, where short-chain-length PHA's (scl-PHAs) monomers have 3–5 carbons, medium-chain-length PHA's (mcl-PHAs) monomers have 6–14 carbons, and long-chain-length PHA's (lcl-PHAs) monomers have more than 14 carbons. Poly-3-hydroxybutyrate (PHB) is arguably the most extensively studied scl-PHA since it is produced by numerous bacteria. The thermomechanical properties of PHAs can be altered and tailored by modifying the monomer composition. For example, the thermomechanical properties of scl-PHAs are comparable to polypropylene,

Abbreviations: Polyhydroxyalkanoates, (PHAs); Short-chain-length polyhydroxyalkanoates, (scl-PHAs); Medium-chain-length polyhydroxyalkanoates, (mcl-PHAs); Long-chain-length polyhydroxyalkanoates, (lcl-PHAs); Polyhydroxybutyrate, (PHB); Granule-associated proteins, (GAPs); Polyhydroxyalkanoate synthases, (PhaC); Polyhydroxyalkanoate phasin regulators, (PhaR); Major phasin from the model bacterium *Pseudomonas putida* KT2440, (PhaF); Phasin affinity tag from *Pseudomonas Putida* KT2440, (BioF).

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whereas mcl-PHAs are more similar to rubber [3]. Unfortunately, the widespread adoption of PHAs is inhibited by high production costs, primarily due to the carbon substrate and extraction methods [11]. As depicted in Fig. 1, there is a general network of PHA granule-associated proteins (GAPs) that contribute to PHA production and composition, which includes PHA polymerases, PHA depolymerases, PHA synthases (PhaC), PHA phasin regulators (PhaR), and phasins. New initiatives in systems and synthetic biology have improved the production and recovery of these bioplastics through exploiting this PHA production network coupled with advances in synthetic biology techniques [12,13]. Engineering bacterial morphology [14], optimizing PHA production pathways [15–18], and improving PHA recovery [19,20] are just a few examples of synthetic biology approaches for enhanced PHA production. Yet, PHAs are still not cost competitive compared to fossil fuel-derived plastics. Phasins are a particularly fascinating class of small-molecular weight proteins that could provide a unique solution to engineering more efficient PHA production as well as other novel sustainable bioproducts from biomass.

Phasins are found in all PHA-producing bacteria and are amphiphilic in nature, creating strong interactions with lipids and hydrophobic structures [21]. Table 1 provides a synopsis of some exceptional functions and characteristics of these small-molecular weight proteins across several species. The functions of phasins can vary considerably between species, and even among multiple phasins employed by a single bacterium. Phasins are the major PHA granule-associated protein surrounding PHA granules, providing an interphase between the granules and the hydrophilic cytoplasm analogous to the oleosins found in plants [22]. Binding strongly to hydrophobic surfaces, phasins manage the surface properties of PHA granules *in vivo* and of other residues *in vitro* [23,24]. As the dominant protein surrounding PHA granules, phasins have a large impact on PHA accumulation and utilization. Some phasins control granule formation in the cell via fostering changes in the size, shape, or abundance of PHA granules [25]. In addition to their surface performance in PHA granule formation, phasins have been shown to have *in vivo* and *in vitro* chaperone activities [27], activate PHA depolymerization [28], contribute to PHA granule segregation [29], and boost the expression and activity of PHA synthases [8,30,31]. Phasins have been revealed to perform chaperone-like activity and stress reduction even in bacteria that do not produce PHAs, such as in *Escherichia coli* [8,32]. Thus, phasins play an active role in stress mitigation and overall fitness protection for bacteria. Several reviews and studies provide detailed synopses of the diverse structures of phasins across bacterial species [21,33]. Based on protein homology, there have been several protein families identified for phasins specifically, allowing bioinformatics analyses to predict new phasins across species based on their sequence and its association with these protein families. For example, *Cupriavidus necator* (formerly *Ralstonia eutropha*) is arguably one of the most characterized model organisms for PHB metabolism and has seven predicted phasins encoded in its genome that perform varying functions [35]. However, we have just begun to scratch the surface of our knowledge of phasins since many PHA-producing strains of bacteria have yet to have phasins experimentally verified or characterized.

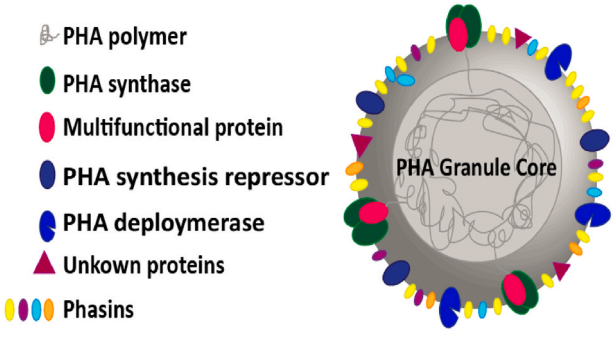

Fig. 1. Representation of the major known proteins associated with bacterial polyhydroxyalkonoate granules.

Table 1
Highlighted extraordinary functions and characteristics of phasins across bacterial species.

Functions and Characteristics	Reference (s)
Strong amphiphilic interactions *in vitro* and *in vivo*	[21,23,24]
PHA granule segregation	[34,36]
PHA depolymerization	[37]
Reduce stress and boost fitness for the bacterium (e.g. chaperone activities)	[32,38,39]
Enhanced PHA synthase expression and activity	[39–41]
Control PHA granule size	[42]
Providing an interphase between the PHA granule and the cytoplasm	[43–45]
Localization of PHA granules	[46]
Foster compositional changes in PHAs	[47]

As the design and development of sustainable bioproducts from biomass and bioresources is becoming increasingly desirable, these remarkable proteins have been employed in a variety of innovative applications. Hence, phasins have become a platform for next-generation industrial biotechnology from biomass feedstocks (e.g. agricultural residues and bacterial biomass) over the past decade. In addition to enhanced bioplastic production, phasins have been engineered for a wide variety of innovative applications ranging from agricultural production to therapeutics (Fig. 2). These advances with phasins include optimized bioplastic production, enhanced biorefinery synthesis, "green" biocatalysis, environmental remediation, and an assortment of sustainable therapeutic bioproducts. However, there has yet to be a holistic review on the vast diversity of sustainable

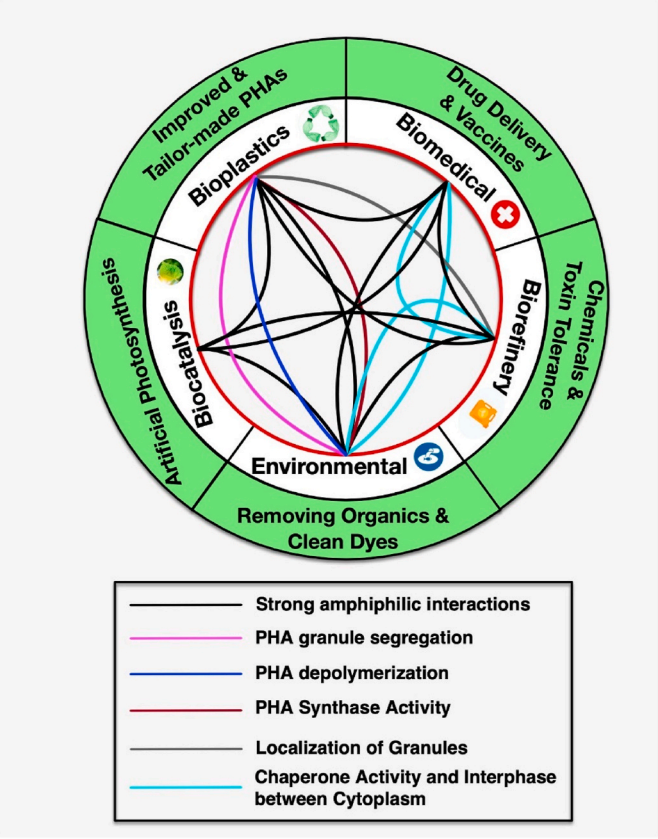

Fig. 2. Overview of the functions and characteristics of phasins discovered thus far that have yielded innovative biotechnical applications across many sectors, as well as suggested future applications.

bioprocessing applications involving phasins. Thus, the scope and motive of this review is to highlight the diverse biotechnical applications of phasins (Table 2) and offer recommendations for future research (Table 3).

2. Engineering phasins for enhanced bioplastic production

As the dominant protein surrounding PHA granules, phasins offer a unique opportunity to test and engineer PHA metabolism for enhanced and tailor-made bioplastic production processes. Phasins have been employed to optimize and alter PHA production in multiple aspects, from altering PHA composition to improving downstream processing.

One of the desirable aspects of PHAs is the ability to create custom PHA compositions to generate anticipated bioplastic thermomechanical properties for various applications. Phasins have been employed to create PHAs with altered composition through synthetic biology approaches. Kawashima et al. (2015) explored the *phaP1* locus for Phasin 1 in *C. necator* as a site of chromosomal modification to generate compositional changes in the PHA produced [47]. Although PHB is the most abundant PHA in nature, its brittleness, high crystallinity, and high melting temperatures limit its potential for application. Some bacteria are able to produce PHAs with more desirable properties, such as *Aeromonas caviae* that can produce poly (3-hydroxybutyrate-co-3-hydroxyhexanoate) [P(3HB-co-3HHx)] from oils and fatty acids. Compared to the PHB homopolymer, P (3HB-co-3HHx) is better suited for practical applications since it is softer and more flexible. There have been many successful studies that have generated recombinant strains of *C. necator* that contain the P (3HB-co-3HHx) production genes from *A. caviae* and produce adequately high fractions of 3HHx from vegetable oils [47]. Of particular interest, Kawashima et al. (2015) utilized the *phaP1* locus in *C. necator* as a site for chromosomal modification to insert the P (3HB-co-3HHx) production proteins from *A. caviae* and to utilize phasins to increase the 3HHx composition [47]. Rather than inserting the encoding (R)-specific enoyl-CoA hydratase from A. caviae (phaJAc) into the *pha* operon as previous studies did, it was inserted into the *phaP1* locus on Chromosome 1 in *C. necator* due to the high expression level of this phasin. Insertion into the *phaP1* locus resulted in efficient production of P(3HB-co-3HHx) on soybean oil with higher 3HHx composition compared to insertion in the *pha* operon. Replacing the PhaP1 phasin from *C. necator* with the major phasin from A. caviae significantly increased 3HHx compositions from 10.5 mol% to 17.2 mol %. Indeed, the *phaP1* locus in *C. necator* was an ideal site for the integration of these genes, and results showed that the recombinant synthase was impacted by the kind of phasins co-existing on the surface of the P(3HB-co-3HHx) granules. Ultimately, phasin replacement enhanced the composition of the P(3HB-co-3HHx), indicating that phasin replacement can be a novel strategy for delivering tailor-made and enhanced compositions of PHA copolyesters.

Although PHB is perhaps the most widely studied scl-PHA, downstream processing significantly increases production costs. Since PHAs are produced intracellularly by the bacteria, they are generally extracted to render access to the biopolymer. This is typically performed by chemical lysis (e.g. methanolysis) or cell disruption (e.g. sonication), which reduces efficiency through non-continuous PHA production. PHA extraction is also generally time consuming and often involves toxic chemicals. Therefore, Rahman et al. (2013) aimed to decrease the downstream processing costs of PHB production by creating a secretion mechanism in *E. coli* that eliminates the need for cell lysis or disruption [48]. Type I secretion was chosen as the mechanism to engineer PHB secretion out of *E. coli* since it is relatively simple and involves translocating proteins from the cytoplasm directly to the extracellular medium. Since *E. coli* does not have a natural PHA production chassis, the PHA production proteins from the model bacterium *C. necator* were synthetically incorporated into *E. coli*. Although PHB is non-proteinaceous and cannot be targeted for secretion, phasins have a

Table 2
Highlighted examples of the diverse applications of phasins.

Topic	Application	Brief Description	Reference (s)
PHA Production	Modified PHA copolymer composition	Increased the 3HHx composition of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) [P (3HB-co-3HHx)] through phasin replacement	[47]
	Production from renewable carbon sources and efficient bioprocessing	Tagged phasins that are bound to PHB with a secretion signal to ultimately excrete PHB out of *E. coli* to generate a continuous process	[48,49]
	Reduce downstream processing and production costs	Increased the size of PHA granules via reducing phasin expression to make it easier for separation in bacterial broth	[50]
Biorefinery Synthesis	Production of industrial chemicals	Immobilized CadA from *E. coli* onto PHB granules via the fusion of phasins to create a cadaverine production system	[51]
	Enhanced bacterial tolerance to biorefinery environments	Expressed a heterologous phasin in *E. coli* that boosted tolerance to butanol and ethanol and enhanced 1,3-propanediol biofuel synthesis	[8]
Biocatalysis	Artificial photosynthesis	Immobilized Cytochrome P_{450} onto PHB to generate solar-to-chemical conversion	[52]
Environmental Remediation	Cleaning wastewater dyes	Utilized phasins as a protein tag to clean wastewaters contaminated with synthetic dyes	[53]
	Ecogenomic sensors for monitoring marine pollution	Discovered that phasins can be biomarkers for hydrocarbon pollution	[54]
	Removing organic pollutants	Created a phasin-mediated *in vivo* immobilization process of an organophosphorus degrading enzyme on PHA granules	[55]
Biomedical	Tissue engineering	Increased chondrogenic differentiation of human bone marrow mesenchymal stem cells via protein-coated PHB copolymer scaffolds	[56]
		Improved fibroblast growth from PHA films	[57]
		Enhanced proliferation and differentiation of neural stem cells grown on PHB copolymers	[58]
		Enhanced proliferation and chondrogenic differentiation of human umbilical cord mesenchymal stem cells	[59]
		Characterized the biocompatibility between PHBHHx films modified by the PhaP-RGD fusion protein and human nasal septum chondrocytes	[60]
	Drug Delivery	Produced a tumor targeting system using phasin-mediated adsorption to present	[61]

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Table 2 (*continued*)

Topic	Application	Brief Description	Reference (s)
		epidermal growth factor receptor (EGFR)-targeting peptide (ETP) onto PHA beads	
		Targeted prostate tumors using PHA beads containing an artificial tumor-homing peptide (iRGD) fused with a phasin	[62]
	Protein Purification	Created improved versions of BioF, a widely used phasin affinity tag from *Pseudomonas putida* KT2440	[63,64]
	Diagnosis	Innovated a novel PCR exclusion assay to detect spotted fever group rickettsiae in the lone star tick (*Amblyomma americanum*) using phasin expression as a primary means of determining prevalence	[65]
	Biomaterials	Efficiently coated implants with the antimicrobial peptide tachyplesin I via phasin immobilization	[66]
	Biosurfactants	Employed *Halomonas* strain TD as an economical host for phasin production for industrial biosurfactants	[67]
		Revealed the interfacial activity of a phasin from *Pseudomonas putida* KT2440 at hydrophobic-hydrophilic interfaces	[68]
		Illuminated the adsorption of a phasin from *Pseudomonas putida* KT2440 onto copolymers and yielded an ideal coating strategy for exposure to hydrophobic residues	[69]

high affinity for PHB and can indeed be targeted. Furthermore, the PhaP1 phasin from *C. necator* has been specifically shown to reduce granule size, and thus translocation of PHB granule was made possible by overexpressing this phasin to decrease the size of the granule and enable translocation across the membrane. The overexpressed phasins were tagged with the Type I secretion target peptide in *E. coli*, called HlyA. This enabled the granules to not only have a reduced size optimal to pass through translocation channels, but also to target the phasins for secretion. Since the targeted phasins are bound to PHB granules in large numbers, PHB is also thereby secreted out of the cell. Through the use of phasins to decrease the size of the granules and to tag them for export with HlyA target peptides, 36% of the total PHB production was secreted after two days. Chen et al. (2018) built upon this system and developed a process called Astroplastic™, which delivers start-to-finish PHB production from solid human waste that can be used by astronauts for 3D printing in space [70].

Alternatively, Shen et al. (2019) applied a synthetic biology approach to increase the size of PHA granules to make it easier for separation in bacterial broth [50]. Since PHAs are typically relatively small (100–500 nm) they are difficult to separate in bacterial broth, and thus Shen et al. (2019) created new *Halomonas bluephagenesis* TD01 strains that were defective in phasin activity to subsequently increase

Table 3
Proposed applications for phasins.

Topic	Application	Brief Description	References
Bioplastics	PHA production for tailor-made polymers	Modify regulatory circuits involved in the control of PHA metabolism to boost production	[71]
	PHA downstream processing	Engineer more efficient secretion of PHAs out of the cell or other methods for extraction for further valorization	[48,70]
	Taking a deeper dive into the mechanisms of phasins	Determine how phasins interact with other proteins (e.g. how phasins contribute to links between PHAs and other types of metabolism)	[72]
	Creating tailor-made PHAs	Control the size and crystallinity of PHAs	[3]
	Creating tailor-made PHAs	Use metabolic engineering for heterogenous phasin production, which could control the composition or even alter the type of PHA produced (e.g. short chain-length to medium chain-length)	[13]
Biorefinery	Coproduction of PHAs	Coproduce PHAs with high-valued chemicals to boost the valorization of PHA production for the biorefinery	[119]
Biocatalysts	Creating new and improved biocatalysts	Employ phasins with industrial biocatalysts (e.g. hydrolases) and create novel systems, such as the construction of enzymatic cascade systems	[79]
	Repurposing of P_{450} systems	Produce new and more sophisticated phasins-mediated bioreactors systems for P_{450} as a biocatalyst	[80]
	Artificial photosynthesis	Generate more systems for a variety of artificial photosynthesis systems, such a hybrid photosynthesis	[81]
Biomedical	Nerve regeneration	Develop more sophisticated systems for nerve regeneration	[58]
	Tissue engineering	Foster stem cell differentiation, interact with various growth factors, 3D printing, custom scaffolding, microfluidics, and possibly even Organ-on-a-Chip	[93]
	Tissue engineering	Engineer noncovalent surface modifications, such as for grafting onto electrospun silica nanofibers	[94]
	Protein production	Create phasin intein systems and affinity tags for largescale protein production	[120]
	Biomaterials	Apply phasins for improved and novel hydrogelators, new biopolymers such as	[99–101]

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Table 3 (*continued*)

Topic	Application	Brief Description	References
		organic-inorganic structures, and phasins characteristics with algal plastics	
	Vaccines	Engineer phasins toward particulate vaccines using biopolyesters	[121]
	Drug Delivery	Innovate novel drug delivery systems or coupling phasins with other proteins (e.g. PHA synthases) could promote more sophisticated drug delivery systems	[95,96]
Agricultural and Aquacultural	PHA production in transgenic plants	Incorporate phasins with the bacterial PHA chassis introduced in transgenic plants	[112,113]
	Mitigating salt stress	Improve the molecular mechanisms of salt response in roots	[102]
	Enhancing crop growth and reducing fertilizer use	Control bacterial colonization and plant growth for nitrogen-fixing bacteria	[103–105, 107]
	Aquaculture	Promote stress resistance, probiotics, reduced infection, and alternatives to antibiotics	[122]
Environmental	Removal of heavy metals	Employ phasins for the removal of heavy metals with PHB nanocomposites	[123]
	Bioemulsifiers	Create designer bioemulsifiers with a variety of phasins and other granule-associated proteins	[89,124]
Symbionts	Industrially relevant symbiont relationships	Engineer novel systems for a variety of industrial applications such as bioactive compounds, discovering new enzymes, and solving practical problems with agricultural pests and diseases	[116,117]

granule size [50]. Defective strains of Phasin 1 (PhaP1) from *H. bluephagenesis* produced larger granules, but with undesirable reduced molecular weights. However, strains defective in either the second or third phasin genes (*phaP2* and *phaP3*) resulted in larger granules with desirably increased PHA molecular weights. Despite the increase of PHA granule sizes through these gene deletions, granule size could not surpass the size of the cell. Thus, genes encoding proteins that block formation of cell fission rings were overexpressed in the PhaP deleted strains. This resulted in larger cell sizes with PHA granules up to 10 μM. Another major finding was that PHA granule size is limited by cytoplasmic space, which could be an additional avenue for optimization across species. The combination of larger cell size with phasin overexpression could be an ideal strategy for PHA-overproduction in the future. This study helps illuminate the variations in function for multiple phasins in a single organism and how phasins can be controlled to manipulate properties of PHA granules.

Ultimately, these studies demonstrate how the unique properties and functions of phasins can be engineered to develop more efficient bioprocessing of bioplastics from biomass feedstocks [47,48,70]. However, there is still much room for growth regarding the applications of phasins towards enhanced PHA production for sustainable bioprocessing. Potential advancements with phasins to boost PHA production include experimentally verifying phasins for microbes that merely have phasins predicted by bioinformatics, creating tailor-made polymers [3,47], optimization of regulatory circuits for PHA metabolism [71], more efficient secretion of PHAs out of the cell, determining how phasins interact with other proteins on and off of PHA granules, if and how phasins contribute to a link between PHA and polyphosphate metabolism [72], and metabolic engineering for heterogenous phasin production [13].

3. Phasins for optimized biorefinery synthesis of sustainable bioproducts

Biorefineries are specific types of refineries that convert biomass to bio-based products such as biofuels, chemicals, and biomaterials. As a more sustainable alternative to conventional refineries, biorefineries can take biomass and turn it into a spectrum of value-added products from a single source. Phasins have been engineered as a possible solution for biorefinery optimization and to improve the economic viability of biorefinery systems.

One of the major limitations in biorefinery production is that the production of biofuels and other valuable bioproducts is limited due to the inherent toxicity of the microbe to these compounds. However, *Azotobacter* sp. strain FA8 is a PHA-producing soil bacterium with a phasin (PhaP) that has been shown to shield *E. coli* from several types of stress and even enhance growth that could provide the solution to increasing resilience during biorefinery production [32]. In an effort to boost bacterial tolerance to common biochemicals used in biorefineries, Mezzina et al. (2017) created a heterologous expression of this phasin (*PhaP*) into *E. coli* [8]. Not only did the phasins significantly enhance *E. coli* tolerance to butanol and ethanol, but ethanol and 1,3-propanediol biofuel synthesis was also boosted. Strains that overexpressed *PhaP* had increased growth in the form of final biomass and product titer produced. For example, when grown in the presence of 5% (vol/vol) ethanol, the *PhaP* strain yielded a 30% increase in biomass after 24 h compared to the control, and the overall percentage of ethanol tolerance for the *PhaP* strain was 1.4 times higher than the control. Hence, phasins can be used to improve bacterial tolerance to toxic biochemical solvents and optimize synthesis of high-valued bioproducts.

Cadaverine (1,5-Diaminopentane) is an industrial chemical used to replace conventional polyamides from petrochemical routes that can be produced by bacterial production via lysine decarboxylase at molar concentration levels [51,73]. If the environment is too acidic *E. coli* naturally produces cadaverine to increase the extracellular pH for survival, and there have been several metabolically engineered strains for the overproduction of cadaverine for industrial uses [74–78]. Seo et al. (2016) used the *phaP1* gene from *C. necator* for *in situ* immobilization of lysine decarboxylase onto PHB granules as a method for conducting enzymatic cadaverine production in *E. coli* [51]. The major phasin employed by *C. necator* (PhaP1) was fused to CadA from *E. coli*, and this chimeric protein was subsequently immobilized onto PHB granules. This strategy resulted in enhanced thermal stability of CadA. By utilizing phasins to fuse lysine carboxylase onto PHB, an enzymatic and reusable process for cadaverine production was created. Therefore, phasin fusion can be a feasible method for producing valuable and sustainable industrial chemicals in biorefineries. This system can be enhanced with more research regarding the optimum phasin that would be even more ideal for extended temperatures and pH changes used in industrial cadaverine production.

More research is desirable to decipher how various phasins can aid in biorefinery optimization, particularly for integrated biorefineries that produce multiple products with assorted processing methods. Significant investigation of specific chaperone activities phasins perform is necessary for more efficient engineering strategies toward biorefinery optimization. As the knowledge of the stress-mitigation roles phasins play across bacteria grows, the potential for phasins to foster improved efficiency (e.g. minimizing product variability), increased productivity,

and lower production costs for biorefineries increases.

4. Phasins as a platform for sustainable biocatalysts

Biocatalysis is the use of living materials to speed up chemical reactions. Biocatalysts are advantageous since they often perform transformations that are too difficult or even impossible with synthetic organic catalysts. Due to the strong amphiphilic tendencies of phasins, they have been applied to generate novel biocatalysis systems.

Cytochromes P_{450} (P_{450}) have received vast attention across many industries due to their unique ability to catabolize a wide variety of oxidative transformations. Although Cytochromes P_{450} can facilitate the synthesis of fine chemicals, they have yet to be produced widely on an industrial scale due to expensive cofactor requirements. Thus, Lee at al. (2016) created a solar-driven system for cofactor regeneration that utilized phasins to immobilize P_{450} on PHB in recombinant *E. coli* [52]. PHB was produced in *E. coli* harboring some of the PHB production chassis from *C. necator*, and P_{450} monooxygenase was immobilized onto the PHB granules by fusion with PhaP. The P_{450}-PHB complex was removed from *E. coli* by cell disruption and purified for further application. This purified complex was then subjected to artificial photosynthesis using cost-effective regeneration of NADPH and Eosin Y as a molecular photosensitizer for light harvesting. The maximum concentration of the product (7-hydroxycoumarin) was four times higher with the P_{450}-PHB complex compared to free P_{450}. The P_{450}-PHB system was subjected to solar tracking under natural sunlight for five consecutive days as a proof of concept for scale-up, and ultimately demonstrated that this P_{450}-PHB design operates as an efficient biocatalyst for artificial photosynthesis. Hence, the application of phasins can potentially foster sustainable platforms for P_{450} use on an industrial scale.

In addition to Cytochromes P_{450}, phasins could be engineered to create new and improved biocatalysts, function with current industrial biocatalysts (e.g. hydrolases), construct enzymatic cascades [79], produce more sophisticated phasin-mediated bioreactors systems with P_{450} via synthetic biology [80], and form advanced hybrid photosynthesis systems [81].

5. Phasins for environmental remediation

Phasins have been applied in innovative environmental remediation applications from cleaning waste waters to identifying anthropogenic pollutants [53–55]. The highlighted studies below showcase how phasins, as sustainable bioproducts from biomass, can be engineered to pioneer solutions to major environmental issues.

PHA granules have been used as natural supports for protein immobilization, in which phasins provide an ideal fusion mechanism. A phasin (PhaF) from the model bacterium *Pseudomonas putida* KT2440 has been widely used an affinity tag, which is a common and standardized method for purifying a fused recombinant protein [63,64,68,82,83]. This affinity tag, dubbed BioF, has been applied extensively for *in vitro* functionalization of several proteins that generate strong and stable interactions [53,63]. Bello-Gil et al. (2018) applied BioF to create an enzymatic system for cleaning wastewater dyes, which is one of the major anthropogenic pollutants worldwide [53]. A laccase-like multicopper oxidase enzyme involved in the copper homeostasis in *E. coli* (CueO) is of particular interest for cleaning wastewater dyes [84]. In this novel system CueO was immobilized onto PHB granules using BioF as the phasin-mediated affinity tag, and the capacity of minibioreactors employing this PHB-CueO fusion systems was evaluated on a various industrial dye. Catalytic activity of CueO increased by up to 40-fold for a variety of dyes, which resulted in decolorization efficiencies between 45 and 90%. Ultimately, this study utilized phasins as a protein tag to clean wastewaters contaminated with synthetic dyes, and offers a promising treatment mechanism for industrial wastewaters using oxidase bioreactors.

The Environmental Sample Processor (ESP), a fully robotized ecogenomic sensor that performs as an autonomous *in situ* liquid handling laboratory, can be equipped with a variety of environmental sensors and is considered one of the most versatile ecogenomic sensors [85]. Knapik et al. (2020) used an ESP to discover functional bacterial gene markers for monitoring anthropogenic hydrocarbon pollution in the marine environment [54]. Metagenomics analyses discovered that phasins were dominantly expressed in the polluted waters, which was hypothesized due to the stress mitigation functions phasins perform for bacteria in these contaminated environments. These data suggest that phasins can serve as ideal biomarkers to report excess carbon pollution and stress in the marine environment.

Organophosphorus compounds are organic pollutants widely used in flame retardants, pesticides, or plasticizers that can cause unintended poisoning to organisms [86]. Li et al. (2019) developed a cost-effective and simple one-step *in vivo* immobilization process of an organophosphorus degrading enzyme in recombinant *E. coli* by displaying it on PHA granules via phasins [55]. Phasins were used to attach the enzyme to the surface of the PHA granule via non-covalent interactions. Anchoring via phasin proteins resulted in greater abundance of the associated enzymes on PHA granules compared to PHA synthase. This phasin-mediated system increased the display density of the organophosphorus degrading enzyme to 6.8% of the total protein. In the end, his study generated a one-step *in vivo* immobilization of self-assembled organophosphorus hydrolase on PHA nano-granules with enhanced catalytic efficiency, high stability, and improved reusability. This is a prime example of how phasins can be used as an anchor protein to functionalize PHA nano-granules containing proteins of interest, and how a sustainable system can be easily prepared by fermentation for novel environmental remediation strategies from biomass feedstocks.

From cleaning wastewaters to identifying anthropogenic pollutants, phasins have been applied to aid in mitigating some of the world's major environmental issues. However, there is still extensive potential for phasins to be applied more in some of the world's leading environmental crises, such as increased carbon footprinting or even soil pollution. For example, phasins would be used as biomarkers to identify soil pollution through expression in the bacteria that naturally thrive in these environments, and subsequently used as protein tags to generate soil cleanup systems similar to the wastewater systems described here previously [53,54].

6. Biomedical applications of phasins

Due to their unique functions and amphiphilic nature, phasins have been engineered in a vast range of therapeutic applications that showcase how these biomass-derived proteins can be engineered for sustainable therapeutic products. In general, PHAs provide an enormous design platform for synthesizing a large variety of polyesters due to their diversity in side chains, variable molecular weights, arrangement of monomers, and chemical modifications [87]. The flexibility, biocompatibility, and biodegradation offered by PHAs has fostered a wave of biomedical applications Click or tap here to enter text. PHA granules have also been engineered as functional PHA-protein assemblies for biomedical applications, which are now commonly known as PHA beads [87,88]. Phasins have been engineered both with and without these PHA beads for an extensive range of applications as discussed further below. Maestro and Sanz (2017) [33] also provide a brief overview of some innovative biomedical applications of phasins that include the use of phasins as biosurfactants [89], affinity tags [90], and protein fusions [57,91,92].

6.1. Phasins fostering tissue engineering

Earlier applications of phasins specific to tissue engineering include increased chondrogenic differentiation of human bone marrow mesenchymal stem cells via protein-coated PHB copolymer scaffolds [56], improved fibroblast growth from PHA films [57], enhanced

proliferation and differentiation of neural stem cells grown on PHB copolymers [58], and the proliferation and chondrogenic differentiation of human umbilical cord mesenchymal stem cells [59].

Building upon the knowledge of phasin-mediated methods in these previous studies, Wang et al. (2016) assessed the biocompatibility of human nasal septum chondrocytes with a peptide called tripeptide Arg-Gly-Asp (RGD) that was attached to PHBHHx films by fusion with phasins [60]. The RGD peptide promotes adhesion between cells and biomaterials, and previous research revealed that differentiation of human mesenchymal stem cells into chondrocytes was enhanced by the PhaP-RGD fusion protein [60]. Thus, it was hypothesized that the PhaP-RGD fusion protein would also foster biocompatibility and growth of human nasal septum chondrocytes on PHBHHx films. Nasal septum chondrocytes can act as a foundation for many forms of cartilage repair, and it is therefore ideal to assess RGD adhesion between cells and biomaterials. Nasal septum chondrocytes from *in vitro* cultures were inoculated to PHBHHx films containing the PhaP-RGD fusion and assessed after three and seven days of cultivation. The water contact angle of the films decreased, the hydrophobicity increased, and there was a relatively high proliferation rate of the chondrocytes with strong viability. Thus, this phasin-mediated method boosts biocompatibility with nasal septum chondrocytes, and therefore serves as an ideal surface biomodification method for tissue engineering. This innovative application of phasins could spawn more developments in cartilage research.

Tissue engineering with phasins can be expanded with new innovations in this field including more insight and engineering into how diverse phasins foster stem cell differentiation, interaction with various growth factors, 3D printing, custom scaffolding, microfluidics, Organ-on-a-Chip [91], and using phasins to create noncovalent surface modifications (e.g. grafting onto electrospun silica nanofibers) [92].

6.2. Engineering phasins for drug delivery systems

Phasins have been widely used to engineer active targeting drug delivery systems, primarily for combating cancer [93,94]. Earlier applications of phasins for active drug delivery include targeted delivery for anticancer treatments [90], and targeted delivery to specific tissues [97].

More recently, Fan et al. (2018) created a tumor targeting system using phasin-mediated adsorption to present the epidermal growth factor receptor (EGFR)-targeting peptide (ETP) onto the surface of PHB copolymer beads [61]. ETP was chosen as the tumor-targeting molecule since EGFR is a transmembrane receptor that is overexpressed in many tumors, making it an ideal choice for anti-cancer drug delivery systems. The fusion protein had strong and efficient adsorption on the surface of the beads, so *in vivo* analysis using subcutaneous human colon cancer cells in mice was conducted. Results unveiled that this phasin-mediated system offers a promising tumor targeting system with specific EGFR targeting capabilities for targeting a wide array of tumors.

Fan et al. (2018) also developed a drug delivery system to target prostate tumors using PHB copolymer beads containing an artificial tumor-homing peptide (iRGD) fused onto the beads with a phasin [62]. This phasin-mediated modification strategy exhibited drastically improved uptake in a human prostate cancer cell line, and tumor target accumulation and retention in prostate tumors was also enhanced.

Thus, phasin-mediated drug delivery systems offer a novel approach to sustainable therapeutic drug delivery systems derived from biomass feedstocks. Applying newly discovered or optimized phasins for novel drug delivery systems or coupling phasins with other proteins (e.g. PHA synthases) could promote more sophisticated drug delivery systems in the future [95,96].

6.3. Phasins for protein purification

Protein purification is often vital for many medical applications, and phasins have been applied to create systems for effective *in vitro* and *in vivo* protein assemblies that can be easily purified for therapeutic applications. Click or tap here to enter text. Moldes et al. (2004) created the first method for selectively immobilizing recombinant proteins onto mcl-PHAs simultaneously with their biosynthesis inside the cell [63]. Another added benefit of this system is that a highly purified soluble protein can be released with only a mild detergent. Ultimately, this method utilized BioF, the phasin affinity tag involving the PhaF phasin from *P. putida* KT2440 previously mentioned, to construct fusion proteins that can be created concurrently with the biosynthesis of their supports (i.e. PHA granules) as well as relatively easily purified.

To further improve this system, Mato et al. (2020) recently conducted a study to gain a more fundamental understanding of the binding domain of the PhaF phasin from *P. putida* KT2440 with the end goal of creating improved shortened versions of BioF that could foster greater biotechnical potential [64]. A minimized BioF tag for PHA functionalization, called MinP, was generated and is of greater interest due to its *in vivo* performance and reduced size compared to BioF. As a proof of concept, the functionality and stability of MinP was further analyzed with fusions of β-galactosidase and the multicopper oxidase (CueO) from *E. coli*. Ultimately, this study generated a versatile platform for protein purification using biocompatible and biodegradable PHAs by easily swapping out affinity tags via improved phasin fusion. This phasin-mediated approach has widespread biomedical applications, from high affinity bioseparation to cell targeting.

Since there are still many gaps in our fundamental understanding of phasins, more discovery and innovation are necessary to further employ phasins for protein purification. Particularly, investigation of phasins involving size exclusion chromatography, separation based on charge or hydrophobicity, and affinity chromatography is of interest.

6.4. Diagnostics with phasins

The development of diagnostic methods capable of detecting miniscule levels of specific molecules is ideal for early diagnosis and more effective treatment for the patient.

A more current initiative innovated a novel polymerase chain reaction (PCR) exclusion assay to detect spotted fever group rickettsiae bacteria in the lone star tick (*Amblyomma americanum*) using phasins as a primary means of determining the prevalence of various categories of spotted fever group rickettsiae [65]. The lone star tick is the most common tick found in the southeastern United States that bites humans, but until this study the roles of transmitting and maintaining pathogenic and non-pathogenic spotted fever group rickettsiae (SFGR) was unclear. Although molecular assays are widely applied for the detection of rickettsiae, low abundance rickettsiae may not be detected. Lydy et al. (2020) created a novel assay to provide more accurate estimates of the prevalence of less common SFGR. This team developed a rapid screen for SFGR that would allow focused detection of other rickettsial agents besides *R. amblyommatis*. There is a deletion in the region upstream of phasin genes in *R. amblyommatis* isolates that is not found in other SFGR. By utilizing this specific region upstream of the phasin, a hemi-nested PCR exclusion assay (RamEX) was generated that detects most SFGR, but not *R. amblyommatis*. Large numbers of tick samples can now be analyzed without the need for quantitative PCR assays. As a result of this targeted application that makes use of phasins, the role of this tick in transmitting and maintaining other important SFGR is more wholly understood.

Yet, more robust baseline analyses of phasin expressions across organisms is necessary to effectively apply diagnostic strategies with phasins. Since phasins play a crucial role in stress mitigation and survival for bacteria, they could be applied as a more pivotal strategy to foster earlier diagnosis for a variety of infections.

6.5. Engineered phasins towards sustainable biomaterials

With the development of alternative and novel biomaterials from

renewable resources becoming ever-more desirable for replacing petroleum-derived materials, biomass feedstocks for biomaterials development are thereby also of increasing interest. Phasins have been utilized for original designs and solutions to sustainable biomaterials, from coating implants with antimicrobial peptides to absorption onto copolyester interfaces.

Coating implants with antimicrobial peptides can prevent infection and help reduce the risk of antibiotic resistance, but the rigidity of the attached peptides often constrains their activities. To rectify this issue, Xue et al. (2018) used a phasin to immobilize tachyplesin I onto a PHB copolymer [66]. This system inhibited the growth of gram-negative bacteria, increased surface hydrophobicity, improved fibroblast proliferation *in vitro*, and was found to shorten wound healing *in vivo*. Hence, phasin-mediated immobilization can foster more flexible display of antimicrobial peptides on the biomaterial surface to promote wound healing.

Phasins have been applied as biosurfactants due to their strong amphiphilic nature. They have also been shown to bind to other substrates besides PHA granules and have been revealed to be strong surfactants while still maintaining their structure [89]. Lan et al. (2016) proposed that *Halomonas* strain TD is an economical host for phasin production as an industrial biosurfactant [67]. Mato et al. (2018) revealed the interfacial activity of PhaF from *P. putida* KT2440 at hydrophobic-hydrophilic interfaces, which paves the way to further utilize PhaF as a biosurfactant [68]. Tarazona et al. (2019) illuminated the adsorption of PhaF onto copolymers of PHB and to poly (lactic-co-glycolic acid (PLGA), and concluded that the phasin provides an ideal platform on copolyester interfaces for exposure to hydrophobic residues [69]. Thus, the adsorption of phasins on biopolymers is an ideal coating strategy for functionalized polymers.

Engineering phasins for improved and novel hydrogelators [99], for new biopolymers such as organic-inorganic structures [100], and to understand phasins characteristics with algal plastics [101] are all desirable achievements in the field of biomaterials research. However, since many bacteria have yet to have their phasins experimentally explored, there is significant advancement needed in our understanding of the particular properties and functions of phasins as they relate to biomaterials research.

7. Concluding remarks and future perspectives

Phasins are a fascinating class of small molecular weight proteins employed by all PHA-producing bacteria that possess extraordinary functions and properties across species. Phasins are the dominant protein surrounding these bioplastic granules and contribute immensely to PHA metabolism and production. Due to the newfound knowledge of phasins and their unique functions, they have now been applied in a vast diversity of applications from enhanced bioplastic production to combating cancer. Thus, over the past decade phasins have emerged as a sustainable biotechnology platform from biomass feedstocks for very diverse applications. However, there are still sectors in which phasins have great potential for engineering and innovation. Table 3 provides an overview of the highlighted potential applications of phasins.

Perhaps the largest untapped potential for phasins thus far is in the agricultural sector. Efficient and sustainable agricultural production is becoming increasingly necessary as the world's population continues to skyrocket, and phasins have been employed in studies that provide a more solid foundation for engineering solutions to some of the world's leading agricultural issues. Phasins have been incorporated in agricultural studies that involved developing a deeper understanding of the molecular mechanisms of salt response in roots to mitigate salt stress [102], employing riboregulation to understand and possibly control the nitrogen fixing symbiont relationship of *Sinorhizobium meliloti* 2011 in alfalfa root nodules [103,104], exploring the role PHA production plays for the plant-bacterium interactions that could reduce chemical fertilizer usage [105,106], and shedding more light on the environments experienced by nitrogen-fixing bean and pea bacteroids [107]. Further investigation and engineering to apply phasins for enhanced endophytic processes for agricultural crops is necessary [108,109]. Employing endophytic bacteria for more eco-friendly and economically viable agricultural production is becoming more widespread and pressing. Endophytic bacteria have shown to enhance crop productivity by alleviating several abiotic and biotic stressors for the plant, promoting plant growth, and maintaining soil health [110]. PHA production provides a competitive advantage for colonization, and phasins could thus provide leverage for improvements in salt and drought tolerance [111]. However, arguably the largest unexploited application for phasins in agriculture is for PHA production in transgenic plants. Transgenic plants have the potential to produce significantly larger volumes of PHAs at drastically lower production costs compared to bacteria [112]. Several plants have been engineered with a variety of bacterial PHA chassis, with a relevant example being the entire *phaCAB* operon of *C. necator* that was driven by a novel Tomato-yellow-leaf-curl-virus (TYLCV)-derived universal vector (SE100) to drive the operon to the plastids and produce PHB in tomatoes [113,114]. However, there are numerous factors still impeding PHA production, including growth defects and low relative yields [114]. Plant cells are more compartmentalized than bacteria, and thus the genes introduced must be efficient at targeting locations of high acetyl-CoA. Phasins have yet to be introduced into plants, and could potentially aid with boosting PHA production, mitigating stress, and fostering more efficient targeting of acetyl-CoA pools in the compartments of plants due to their localization functions.

Phasins have also played a crucial role in illuminating our fundamental understanding of symbionts, which can foster novel or more efficient industrial consortium systems to produce sustainable bioproducts from biomass. A symbiont relationship is a type of long-term interaction between biological organisms. Understanding symbiotic relationships is essential to illuminating evolution, habitual patterns, survival needs, health of the ecosystem, and optimizing industrial bioprocessing with symbionts [115]. Exposing the molecular mechanism of an insect-symbiont system between Burkholderia gut symbionts and their host insect *Riptortus pedestris* [116] and describing a very unique relationship between a species of Paracatenula marine flatworms with its symbiont bacteria *Candidatus Riegeria santandreae* [117] are two highlighted studies in which phasins played crucial roles in characterizing symbiont relationships. Having just skimmed the surface of the impact phasins have on symbiotic relationships, more research is necessary to decipher how phasins contribute to these complex relationships across different environments (e.g. whether phasin activity changes with different stress conditions) and between species. Exploiting symbiosis has great biotechnical potential for engineering bioactive compounds, discovering new enzymes, and solving practical problems with agricultural pests and diseases [118], and phasins could be utilized to engineering novel systems employing industrially relevant symbionts.

However, we have only just begun to scratch the surface on the diversity of phasins. Many species of bacteria that produce PHAs have phasins predicted via bioinformatics that have yet to be experimentally verified or explored. Since the functions and properties of phasins can differ dramatically across species and even between multiple phasins in a single organism, further investigation is necessary to expand our fundamental understanding of these unique proteins. For example, more in-depth analyses on the specific chaperone activities and stress mitigation functions phasins perform is essential. Ultimately, the versatility of phasins and new discoveries of their functions will pave the way for more wide-spread applications and further development of phasins as a sustainable biotechnical platform from biomass feedstocks.

Outstanding questions

What is the benefit to the bacterium of having multiple phasins?
What specific chaperone-like activities do phasins conduct?
What are the genetic indicators that show variations in the functions

of phasins (e.g. is one phasin more active in particular stress environments than another)?

Do phasins impact polyphosphate metabolism?

How does the absorption behavior of phasins differ across proteins and species?

Does phasin activity vary with carbon substrate, and if so, how?

What impact do different phasins have on PHA composition?

Credit author contribution statement

Brandi Brown: Conceptualization, Investigation, Writing – original draft. Cheryl Immethun: Supervision, Writing – review & editing. Mark Wilkins: Resources, Funding acquisition, Supervision, Writing – review & editing. Rajib Saha: Resources, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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