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Review: Regulated polyhydroxyalkanoate granule biology and polymer phenotype in mangrove-associated bacteria

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Abstract. *Darmawan MT, Alviansyah MH, Aditya RD, Fadhila NH, Safika NJ, Salma O, Nugraheni R, Hidayati RK, Nadhira S, Setyawan AD. 2025. Review: Regulated polyhydroxyalkanoate granule biology and polymer phenotype in mangrove-associated bacteria. Cell Biol Dev 9: 129-157.* Polyhydroxyalkanoates (PHAs) are biodegradable biopolymers whose molecular weight, composition, and dispersity strongly determine material performance and industrial applicability. Despite extensive efforts to optimize culture conditions and genetic constructs, reproducible control of PHA polymer phenotype remains challenging. This review reinterprets PHA accumulation from a cell-biological perspective by positioning PHA granules as regulated intracellular organelles rather than passive repositories of excess carbon. Synthesizing evidence from mechanistic studies, culture-based experiments, and gene-centric analyses, the review demonstrates that polymer phenotype-including molecular-weight distribution, structural heterogeneity, and batch-to-batch reproducibility-emerges as a physiological output shaped by regulatory gating and granule state. PHA accumulation is shown to be an active and reversible storage strategy embedded within global stress-response networks, rather than a simple consequence of metabolic overflow. Granule-associated proteins, spatial organization, and turnover dynamics act as critical intermediaries translating regulatory state into material properties. The review further highlights that widespread reliance on production-oriented metrics, such as polymer yield or percentage of cell dry weight, obscures intracellular processes that govern polymer history and quality, contributing to inconsistencies across studies. Mangrove-associated bacteria are used as a biologically informative stress-context model to illustrate how conserved intracellular storage mechanisms operate under recurring physiological constraints, without invoking environmental determinism. Building on these insights, a regulatory-granule-phenotype framework is proposed that reframes reproducibility as a biological property dependent on granule-state stability rather than a purely technical issue. By foregrounding granule-level control, this synthesis provides a coherent foundation for integrating cellular regulation with polymer phenotype and outlines experimental directions for achieving more predictable and interpretable PHA production.

Keywords: Carbonosome, granule-associated proteins, intracellular regulation, polyhydroxyalkanoate, polymer phenotype

INTRODUCTION

Polyhydroxyalkanoates (PHAs) are biodegradable microbial polyesters widely recognized as promising alternatives to petroleum-derived plastics, reflecting broader efforts to develop plastics from renewable biological sources with reduced environmental impact (Chen 2010; Chen and Patel 2012). Early biotechnological studies demonstrated that diverse bacterial and biological systems can synthesize intracellular polyesters, establishing PHAs as a distinct field within microbial biotechnology (Steinbüchel and Füchtenbusch 1998; Verlinden et al. 2007). Despite this progress, PHAs continue to illustrate both the potential and persistent challenges of translating microbial metabolism into reliable and scalable bioprocesses, particularly regarding process stability, reproducibility, and product consistency (Chen 2012).

Beyond their technological relevance, PHAs function as regulated intracellular storage polymers that enable bacteria to manage carbon and energy under fluctuating environmental and physiological conditions. PHA accumulation is not an uncontrolled metabolic overflow but a reversible, stress-responsive process activated by nutrient imbalance, redox perturbations, and cellular stress (Doi 1990; Madison and Huisman 1999). This strategy represents an active physiological decision embedded within global stress-response networks, notably the RpoS-mediated general stress response, which integrates environmental and metabolic signals into coordinated regulatory control (Battesti et al. 2011).

At the cellular level, PHAs are stored within discrete intracellular granules that exhibit conserved organizational features across phylogenetically diverse bacteria. These granules comprise a hydrophobic polyester core surrounded by a dynamic protein layer enriched in phasins and

regulatory proteins, forming structured, organelle-like entities rather than amorphous inclusions (Jendrossek and Pfeiffer 2014; Bresan et al. 2016; Chen et al. 2018). Such non-membranous architectures align with broader recognition of bacterial microcompartments as spatial organizers of metabolic functions (Bobik et al. 2015). Increasing evidence supports interpreting PHA granules as functional intracellular organelles-often termed carbonosomes-that coordinate carbon storage with regulatory processes. Granule-associated proteins contribute not only to structural stabilization but also to regulatory interactions with transcriptional regulators and biosynthetic enzymes, rendering granule architecture and spatial organization integral components of PHA metabolism rather than passive by-products of polymer accumulation (Mitra et al. 2022).

Despite advances in granule biology, much of the PHA literature remains dominated by production-oriented metrics such as polymer yield, percentage of cell dry weight, and substrate conversion efficiency. While valuable for strain screening and process optimization, these metrics provide limited insight into intracellular regulation, granule dynamics, or polymer history. This limitation is critical because PHA performance in downstream applications, including biomedical and tissue engineering uses, depends strongly on polymer phenotype rather than polymer quantity alone (Chen and Wu 2005). Polymer phenotype-encompassing molecular-weight distribution, crystallinity, and batch-to-batch reproducibility-is therefore frequently interpreted independently of the cellular processes that generate it (Rehm 2010; Koller 2017). This separation has contributed to inconsistencies across studies and has obscured mechanistic links between cellular physiology and material properties, leading to reproducibility challenges being framed primarily as technical rather than biological issues.

Mangrove-associated bacteria provide a biologically informative context for examining regulated intracellular storage strategies such as PHA accumulation. Mangrove ecosystems are characterized by pronounced environmental variability, including fluctuating salinity, redox heterogeneity, episodic inundation, and variable water availability, imposing recurrent physiological stress on resident microbial communities (Alongi 2014; Thatoi et al. 2020). Restoration and disturbance dynamics further alter sediment physicochemical properties and bacterial community composition, underscoring the sensitivity of mangrove microbial assemblages to changing stress regimes (Ma et al. 2021). Broader ecological analyses show that water-related stress alone can drive substantial reorganization of microbial metabolism and carbon allocation at the community level (Manzoni et al. 2012). In this review, however, mangrove environments are not treated as deterministic drivers of polymer properties. Instead, mangrove-associated bacteria are viewed as representative of microbial populations repeatedly exposed to stress and nutrient imbalance, conditions under which regulatory flexibility and intracellular storage strategies are especially relevant.

Building on this perspective, polymer phenotype is interpreted here as a physiological output of regulated granule state. Cellular regulatory gating, granule organization, and turnover collectively shape polymer quality, heterogeneity, and reproducibility, independent of polymer quantity or environmental origin alone. Experimental manipulation of intracellular organization has demonstrated that altering granule positioning can directly influence PHA synthesis and distribution, providing causal support for this interpretation (Chen et al. 2015). Recognizing this relationship offers a framework for reconciling discrepancies across studies and reframing reproducibility as a biological property.

The objective of this review is to synthesize current knowledge on the mechanistic links between cellular regulation, granule homeostasis, and polymer phenotype in PHA-accumulating bacteria. By integrating cell-biological, regulatory, and gene-centric evidence, this review advances a regulatory-granule-phenotype framework that emphasizes testable biological mechanisms over descriptive correlations. Mangrove-associated bacteria are used as a stress-context model to illustrate these principles, providing a coherent foundation for interpreting PHA accumulation as an integrated physiological process and for guiding future granule-focused experimental research.

LITERATURE SEARCH AND EVIDENCE FRAMEWORK

This synthesis adopts a structured but non-exhaustive literature approach designed to support conceptual integration of cellular regulation, granule biology, and polymer phenotype, rather than to perform a formal systematic review (Figure 2). The primary objective of this framework is to ensure transparency and consistency in evidence selection while retaining flexibility to integrate mechanistic insights from heterogeneous experimental traditions. Given the diversity of methodological approaches in PHA research, ranging from cell biology to process-oriented microbiology, a scoping-oriented strategy was selected to enable comparison across evidence types without imposing restrictive inclusion criteria.

This review advances the hypothesis that variability and limited reproducibility in Polyhydroxyalkanoate (PHA) polymer phenotype are primarily driven by regulated intracellular granule states rather than by culture conditions or production parameters alone. Specifically, it asks: (i) how cellular regulatory networks and stress responses shape PHA granule organization and turnover; (ii) how granule-associated proteins and spatial dynamics translate regulatory state into polymer molecular characteristics; and (iii) to what extent polymer phenotype can be interpreted as a physiological output reflecting granule-state stability. By addressing these questions, the review reframes reproducibility as a biological property emerging from granule-level regulation.

Search strategy and scope

Literature searches were conducted using major scientific databases, including Scopus, Web of Science, PubMed, and Google Scholar, covering publications from 2015 to 2025. Search terms combined keywords related to polyhydroxyalkanoates with descriptors of cellular regulation, granule-associated proteins, intracellular organization, and microbial ecological contexts, including mangrove-associated systems. Boolean combinations were adjusted iteratively to capture both mechanistic studies and biologically informative observational reports.

The scope of the search was explicitly cell-biological and regulatory. Priority was given to studies that provided interpretable information on regulatory state, granule organization, or polymer phenotype, even when production metrics were not the primary focus. In contrast, reports focusing exclusively on process optimization, yield maximization, or environmental surveys without linkage to intracellular PHA metabolism were not emphasized. This selective scope reflects the objective of synthesizing mechanistic understanding rather than compiling an exhaustive inventory of PHA-producing organisms.

The temporal restriction to the past decade was applied to ensure relevance to contemporary conceptual and methodological advances, particularly in imaging, molecular regulation, and polymer characterization. Earlier foundational studies were included selectively when necessary to establish conceptual continuity. This strategy balances breadth and depth while maintaining coherence with the review's focus on regulated intracellular storage.

Study screening and categorization

Screening was conducted in two sequential stages, beginning with title and abstract evaluation, followed by full-text assessment, following established guidance for scoping and evidence synthesis (Peters et al. 2015; Tricco et al. 2018). Bibliographic management and screening decisions were documented using standardized digital workflows to ensure traceability and reproducibility of study selection (Ouzzani et al. 2016). The overall screening process and study inclusion are summarized in a PRISMA-style flow diagram (Figure 1).

Included studies were categorized into three analytical groups to prevent conflation of evidence types. The first category comprised culture-based isolate studies demonstrating PHA accumulation under defined laboratory conditions. The second included mechanistic cell-biological studies that interrogated regulatory pathways, granule-associated proteins, or intracellular organization through genetic, biochemical, or imaging-based approaches. The third category encompassed metagenomic and gene-centric studies reporting biosynthetic potential without direct phenotypic validation.

This categorization was applied consistently throughout the manuscript to maintain clarity of inference and to avoid extrapolating causal conclusions from descriptive or associative data. By explicitly separating these evidence streams, the synthesis preserves analytical rigor while enabling integrative interpretation across methodological boundaries.

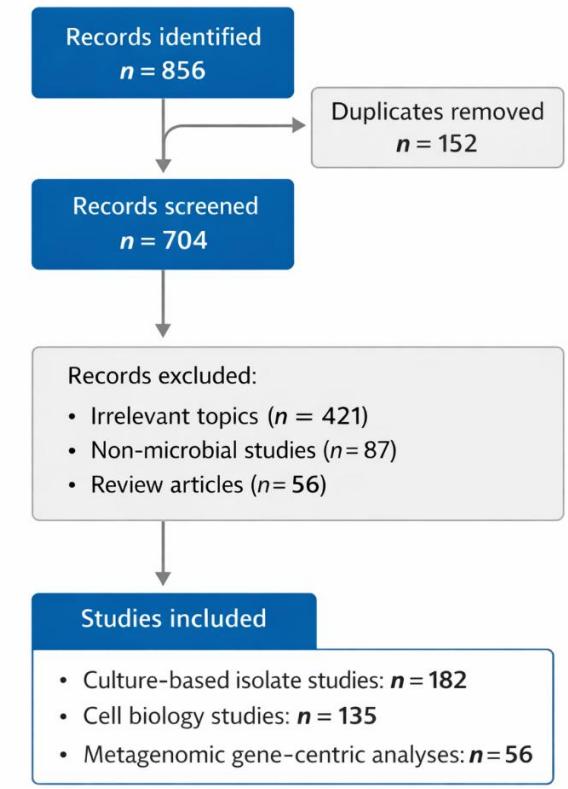

Figure 1. PRISMA-style flow diagram of literature screening, exclusion, and categorization of studies included in this review

Evidence-tier framework (E1-E4)

To further distinguish inference strength across heterogeneous studies, an internal evidence-tier framework (E1-E4) was applied throughout the synthesis (Table 1). Tier E1 includes descriptive or associative observations, such as detection of PHA accumulation or presence of biosynthetic genes, without functional interpretation. Tier E2 comprises correlative studies that combine PHA content measurements with limited polymer or physiological characterization. Tier E3 encompasses perturbation-supported studies in which genetic, regulatory, or physiological manipulation informs mechanistic understanding. Tier E4 represents causal cell-biological evidence demonstrating direct regulatory control of granule homeostasis or polymer phenotype.

This tiered framework functions as an organizational tool rather than as a formal quality appraisal system. Its purpose is to clarify the logical distance between observation, inference, and causation, particularly in a field where production metrics and mechanistic insights are often intermingled. By explicitly indicating the evidence tier, the synthesis avoids overinterpretation of associative findings and maintains alignment between claims and supporting data.

Table 1. Evidence tier definitions for studies linking regulation, granule phenotype, and polymer outputs

Evidence tier	Definition	Typical data included	Inference strength
E1	Descriptive or associative evidence	PHA presence, %CDW, pha gene occurrence	Low
E2	Correlative evidence with partial phenotyping	PHA content with limited polymer or physiological context	Moderate
E3	Perturbation-supported mechanistic evidence	Genetic or physiological manipulation with defined outputs	High
E4	Causal cell-biological evidence	Direct demonstration of regulatory or granule-level control	Very high

POLYHYDROXYALKANOATE GRANULES AS REGULATED CELLULAR ORGANELLES

Structural organization of PHA granules

Polyhydroxyalkanoate (PHA) granules are now widely recognized as regulated, non-membranous intracellular organelles rather than passive inclusions of excess carbon. Early descriptions portrayed PHA inclusions as amorphous polymer precipitates formed spontaneously within the cytoplasm, a view shaped largely by polymer-science perspectives that emphasized chemical structure and material properties over cellular context (Sudesh et al. 2000). Advances in cell biology, imaging, and biochemical analysis have fundamentally revised this interpretation. Accumulating evidence demonstrates that PHA granules exhibit defined architecture, reproducible spatial organization, and regulated protein composition-hallmarks of functional intracellular organelles. This shift aligns with broader recognition that bacterial cells are structurally organized systems in which spatial regulation and protein localization play central roles, even in the absence of membrane-bound compartments (Thanbichler and Shapiro 2008; Rudner and Losick 2010; Errington 2013; Khursigara et al. 2018). Similar principles have been established for other non-membranous bacterial organelles, such as magnetosomes, whose ordered assembly reflects active genetic and regulatory control rather than spontaneous aggregation (Murat et al. 2010).

Structurally, PHA granules consist of a hydrophobic polyester core-most commonly poly(3-hydroxybutyrate) or related copolymers-surrounded by a proteinaceous boundary layer rather than a lipid membrane. This feature distinguishes PHA granules from classical membrane-bounded organelles while placing them within a growing class of protein-scaffolded bacterial compartments (Bresan et al. 2016). Biochemical and lipidomic analyses have confirmed the absence of phospholipid membranes, refuting earlier models proposing monolayer encapsulation of PHA inclusions (Bresan et al. 2016). Instead, granule integrity and identity are maintained by surface-associated proteins, particularly phasins and regulatory factors, which mediate both physical stabilization and regulatory integration.

Ultrastructural evidence for organized granule architecture has been provided primarily by Transmission Electron Microscopy (TEM), which consistently reveals discrete, electron-lucent spherical bodies within the cytoplasm of PHA-accumulating cells. Across diverse bacterial taxa, including *Cupriavidus*, *Pseudomonas*, and *Halomonas*, TEM studies show that granule number and size vary systematically with growth phase and physiological state rather than arising randomly (Jendrossek and Pfeiffer 2014). While TEM has been essential for establishing granule morphology and abundance, it is limited by preparation artifacts such as dehydration and staining, which can obscure fine structural features and dynamic interactions.

Cryo-Electron Microscopy (cryo-EM) has addressed many of these limitations by preserving native cellular architecture under vitrified conditions. Cryo-EM analyses confirm that PHA granules are compact, well-defined intracellular structures with stable interfaces with surrounding cytoplasmic components. Importantly, these studies reinforce the conclusion that granule boundaries are protein-based rather than lipid-based, supporting the classification of PHA granules as non-membranous organelles (Bresan et al. 2016). Cryo-EM further reveals non-random spatial relationships between granules and other intracellular structures, including the nucleoid, suggesting coordinated positioning rather than stochastic aggregation.

More recently, label-free imaging approaches have enabled quantitative analysis of PHA granules in living or minimally perturbed cells. Techniques such as optical diffraction tomography and refractive-index-based imaging allow three-dimensional visualization of intracellular polymer inclusions without fluorescent labeling or chemical fixation (Choi et al. 2021). These methods demonstrate that granule number, volume, and spatial distribution can be quantified at the single-cell level and monitored across growth phases. Importantly, label-free imaging reveals substantial heterogeneity in granule organization within clonal populations, indicating that granule architecture is a regulated yet variable outcome of cellular state rather than a fixed trait.

Together, evidence from TEM, cryo-EM, and label-free imaging highlights a critical conceptual distinction: the presence of PHA granules does not imply organized granule biology. Granule presence reflects polymer accumulation, whereas granule organization encompasses regulated features such as number control, size distribution, spatial positioning, and surface composition. Cells with similar total polymer content may differ profoundly in granule organization, with significant consequences for physiology and material properties. This distinction is often overlooked when polymer accumulation or percentage of cell dry weight is treated as a proxy for functional equivalence (Tsuge 2016; Koller 2017).

From a cell-biological perspective, organized granule architecture enables integration of storage metabolism with cellular regulation. Granule surfaces provide platforms for protein-protein interactions linking polymer synthesis and degradation to transcriptional and post-transcriptional control. Spatial organization relative to the nucleoid supports coordinated inheritance during cell division, ensuring continuity of storage capacity across generations (Galán et al. 2011). These features support interpretation of the PHA granule as a carbonosome-a specialized organelle for regulated carbon storage embedded within broader regulatory networks (Mitra et al. 2022).

Classifying PHA granules as non-membranous organelles places them alongside other bacterial compartments such as carboxysomes and protein-based microcompartments, which achieve functional compartmentalization through protein assemblies rather than lipid bilayers (Shively et al. 1973; Kerfeld et al. 2018). Unlike catalytic microcompartments, however, PHA granules function primarily as dynamic storage organelles, requiring architectures compatible with both accumulation and mobilization.

Recognition of organized granule biology has direct implications for interpreting polymer phenotype. Polymer chain-length distribution, crystallinity, and thermal behavior are shaped by the physical and regulatory environment within granules. Differences in granule size, surface composition, or turnover dynamics impose distinct constraints on polymer synthesis and remodeling, even when total polymer content is comparable. Thus, understanding granule organization is essential for interpreting polymer quality and reproducibility as physiological outputs rather than isolated chemical properties.

Within the regulatory-granule-phenotype framework, structural organization of PHA granules constitutes the foundational layer linking cellular regulation to material outcomes. By explicitly distinguishing granule presence from granule organization, this framework clarifies why polymer accumulation alone cannot account for variability in polymer properties across studies. Overall, evidence from complementary imaging and biochemical approaches demonstrates that PHA granules are regulated, non-membranous organelles whose dynamic organization underpins both cellular physiology and polymer phenotype.

Granule-associated proteins and surface regulation

The functional identity of Polyhydroxyalkanoate (PHA) granules is defined not solely by their polymeric core but by the ensemble of proteins that assemble at the granule surface. These Granule-Associated Proteins (GAPs) constitute a dynamic regulatory interface that integrates polymer synthesis, structural stabilization, spatial organization, and regulatory feedback. Rather than acting as passive coatings, GAPs actively mediate how cellular regulatory states are translated into granule architecture and polymer outcomes. This perspective represents a departure from earlier views that treated granule-associated proteins as auxiliary structural elements and instead positions them as central regulators of granule behavior.

Phasins as structural-regulatory buffers

Phasins are the most abundant and best-characterized class of GAPs and play a foundational role in granule surface regulation. These amphipathic proteins adsorb to the hydrophobic polymer surface and prevent uncontrolled coalescence of granules within the cytoplasm. Experimental manipulation of phasin abundance has repeatedly demonstrated that phasins exert direct control over granule number and size distribution. Elevated phasin levels favor the formation of multiple smaller granules, whereas reduced phasin expression leads to fewer, larger inclusions (Jendrossek and Pfeiffer 2014). This relationship indicates that phasins act as buffers that regulate the balance between granule nucleation and maturation.

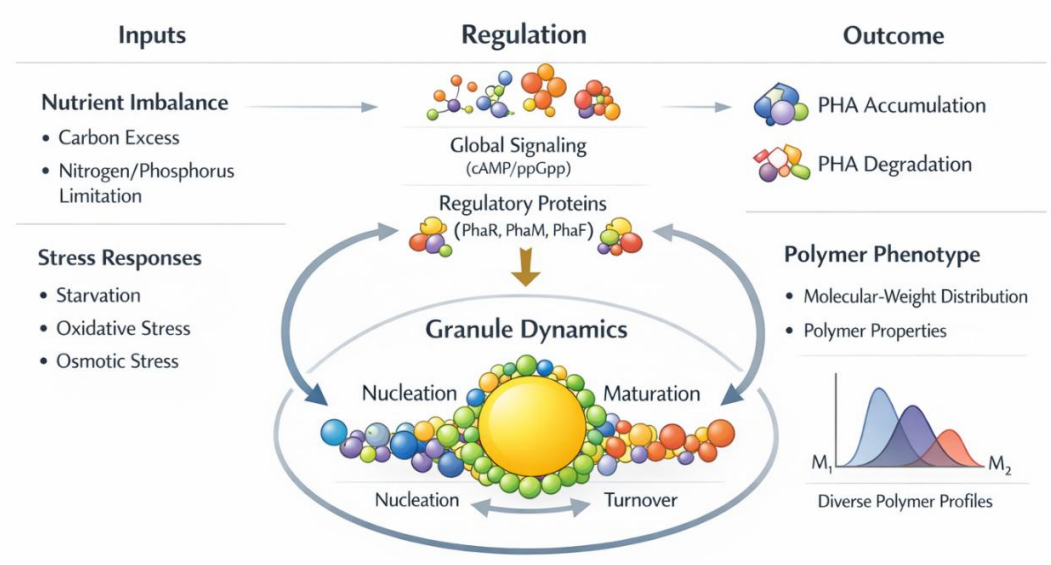

Figure 2. Conceptual framework linking cellular regulation, granule homeostasis, and polymer phenotype

Beyond structural stabilization, phasins also perform regulatory functions by modulating the access of enzymes and regulatory proteins to the polymer surface. Increased surface coverage by phasins can sterically hinder depolymerase activity, thereby stabilizing stored polymer during prolonged storage phases. Conversely, reduced phasin coverage can expose polymer chains to depolymerases, facilitating mobilization when cellular conditions shift toward growth. This dual role underscores that phasins regulate not only granule morphology but also polymer turnover dynamics (Mezzina and Pettinari 2016).

Phasins further participate in regulatory feedback loops by interacting with transcriptional regulators. In several systems, phasins sequester regulatory proteins at the granule surface, thereby linking polymer abundance to gene expression control. Such sequestration mechanisms allow the cell to sense polymer accumulation indirectly through granule surface occupancy, reinforcing the idea that granule state feeds back into regulatory networks (Pfeiffer and Jendrossek 2011). Through these combined structural and regulatory roles, phasins function as integrative buffers that stabilize granule architecture while enabling adaptive responsiveness.

Regulatory proteins at the granule surface: PhaR and PhaD

Dedicated regulatory proteins further refine granule surface control by coupling polymer presence to transcriptional regulation. PhaR is a paradigmatic example of a granule-associated transcriptional regulator whose activity depends on its affinity for PHA granules. Under conditions of low polymer abundance, PhaR binds to promoter regions of phasin genes and represses their expression. As polymer accumulates and granules form, PhaR preferentially associates with the granule surface, relieving transcriptional repression and allowing phasin expression to increase (York et al. 2001; Potter et al. 2002). This feedback loop ensures that surface protein composition adjusts dynamically to polymer load.

The PhaR-mediated regulatory circuit exemplifies how granule surface occupancy functions as a regulatory signal. Rather than relying on metabolite concentrations alone, the cell uses physical association with the granule as an information channel to coordinate gene expression with storage state. This mechanism highlights a key principle of granule biology: regulatory decisions are embedded in spatial organization and protein localization, not solely in diffusible signals.

PhaD represents another layer of regulatory integration at the granule surface. Although primarily characterized as a transcriptional activator of PHA biosynthetic genes, PhaD has also been detected in association with granules, suggesting a role in coordinating gene expression with granule maturation (Rehm 2010). Through interactions with phasins and other GAPs, PhaD may help synchronize polymer synthesis rates with granule capacity and surface composition, thereby preventing mismatches between enzymatic activity and storage architecture.

Collectively, PhaR and PhaD illustrate how regulatory proteins exploit granule surfaces as functional platforms. Their localization-dependent activity reinforces the concept of the granule surface as a regulatory interface that integrates physical state with transcriptional control, rather than as a passive boundary.

Granule-nucleoid interaction proteins: PhaM and PhaF

Granule-associated proteins also mediate interactions between PHA granules and other intracellular structures, most notably the nucleoid. PhaM and PhaF are central to this granule-nucleoid interface and play crucial roles in spatial organization and inheritance. PhaM has been shown to bind both DNA and PHA granules, effectively tethering granules to the nucleoid region (Galán et al. 2011). This tethering constrains granule movement, preventing random diffusion and promoting orderly spatial distribution within the cell.

The functional consequences of nucleoid tethering extend beyond positioning. By anchoring granules near the nucleoid, PhaM-mediated interactions facilitate symmetric granule inheritance during cell division. This spatial regulation ensures that daughter cells receive comparable storage capacity, contributing to population-level consistency in granule state. Disruption of PhaM function leads to aberrant granule localization and increased heterogeneity in granule inheritance, underscoring its role in maintaining granule homeostasis (Galán et al. 2011).

PhaF further modulates granule-nucleoid interactions and contributes to higher-order organization of the granule surface network. In Pseudomonas species, PhaF interacts with both phasins and regulatory proteins, forming multiprotein complexes that integrate structural stabilization with regulatory control (Tarazona et al. 2020). These interactions enable coordinated adjustment of granule positioning, surface composition, and regulatory signaling, reinforcing the granule as a hub of intracellular organization.

The existence of granule-nucleoid interaction proteins highlights a critical distinction between granule presence and granule organization. While polymer accumulation can occur without precise spatial control, regulated positioning and inheritance require dedicated protein machinery. PhaM and PhaF exemplify how GAPs embed granule biology within the broader cellular architecture, ensuring continuity and reproducibility across generations.

Granule-associated proteins as a regulatory interface

When considered collectively, phasins, transcriptional regulators, and nucleoid-interacting proteins form an interconnected network at the granule surface that governs nucleation, maturation, positioning, and turnover. This network enables granules to function as responsive organelles whose properties reflect cellular regulatory state rather than merely polymer quantity (Figure 3). Differences in GAP composition or interaction strength can generate distinct granule states under similar external conditions, providing a mechanistic basis for variability in polymer phenotype.

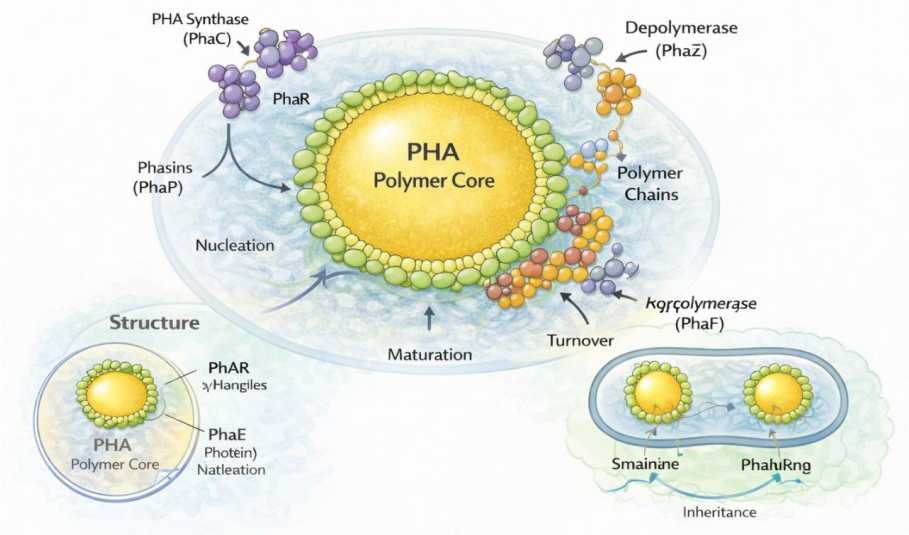

Figure 3. Granule surface-associated protein networks controlling nucleation, maturation, and turnover of PHA granules

Table 2. Comparative overview of granule-associated proteins (GAPs) involved in PHA granule organization and regulation

Granule-Associated Protein (GAP)	Primary molecular function	Localization and interaction	Regulatory role in granule biology	Phenotypic effect on granule and polymer	Representative organisms/evidence
PhaP (Phasin family)	Amphipathic surface protein; granule coating and stabilization	Binds directly to PHA granule surface; interacts with PhaC and other GAPs	Controls granule nucleation vs maturation balance through surface area regulation	Increased PhaP → more, smaller granules; altered Mw/Mn and increased polymer heterogeneity under dynamic conditions	*Cupriavidus necator*, *Pseudomonas putida* (York et al. 2001; Wahl et al. 2012; Jendrossek and Pfeiffer 2014)
PhaR	DNA-binding transcriptional regulator and granule-binding protein	Shuttles between DNA and the granule surface depending on PHA presence	Couples polymer presence to transcriptional control of phasin genes (feedback regulation)	Links storage commitment to granule surface composition and stability	*Cupriavidus necator* (York et al. 2001; Jendrossek and Pfeiffer 2014)
PhaM	Nucleoid-associated granule tethering protein	Binds both DNA and the granule surface	Ensures spatial positioning and ordered inheritance of granules	Promotes symmetric granule segregation; reduces cell-to-cell variability	*Cupriavidus necator* (Galán et al. 2011; Wahl et al. 2012)
PhaF	Structural phasin with regulatory interactions	Granule surface; interacts with PhaD and nucleoid-associated factors	Coordinates granule positioning with regulatory control	Affects granule localization, surface dynamics, and reproducibility	*Pseudomonas putida* (Galán et al. 2011; Tarazona et al. 2020)
PhaD	Transcriptional regulator of PHA biosynthetic genes	Cytosol and granule-associated complexes	Integrates transcriptional regulation with granule maturation	Indirectly modulates granule size distribution and polymer synthesis rate	*Pseudomonas putida* (Rehm 2010; Tarazona et al. 2020)
PhaC (PHA synthase)	Catalyzes polymer chain initiation and elongation	Granule surface, embedded in protein layer	Central executor of polymer synthesis; activity modulated by surface context	Determines chain growth kinetics and contributes to Mw/Mn distribution	Broadly conserved among PHA-producing bacteria (Madison and Huisman 1999; Tsuge 2016)
PhaZ (PHA depolymerase)	Intracellular polymer degradation	Granule surface or periphery	Regulates the mobilization and turnover of stored polymer	Drives polymer remodeling, Mw/Mn drift, and history-dependent phenotype	*Cupriavidus*, *Pseudomonas* spp. (Jendrossek and Pfeiffer 2014; Pagliano et al. 2021)

Note: Granule-associated proteins act collectively rather than independently. Their interactions define the *granule state*-including size distribution, surface composition, spatial positioning, and turnover history-which functions as an intermediate variable linking cellular regulatory state to polymer phenotype and reproducibility. Differences in GAP composition or dynamics can modify polymer quality without altering total PHA accumulation

Comparative analysis across taxa reveals conservation of core GAP functions alongside lineage-specific adaptations. While phasin families and regulatory circuits vary, the overarching principle of surface-mediated regulation is preserved, supporting the view that GAPs constitute a universal regulatory interface in PHA-accumulating bacteria (Jendrossek and Handrick 2002). This conversation reinforces the relevance of granule biology as a unifying framework for interpreting diverse experimental observations.

Importantly, recognizing GAPs as regulatory interfaces reframes how polymer data should be interpreted. Polymer yield or % cell dry weight alone cannot capture differences in granule surface regulation that shape polymer turnover and molecular-weight distribution. Integrating information on GAP composition and organization is therefore essential for mechanistic understanding and for improving reproducibility across studies.

In summary, granule-associated proteins define the regulatory identity of PHA granules by integrating structural stabilization, transcriptional feedback, spatial organization, and inheritance. Phasins act as structural-regulatory buffers, PhaR and PhaD link polymer presence to gene expression, and PhaM and PhaF embed granules within cellular architecture. Together, these proteins establish the granule surface as a regulatory interface that translates cellular state into granule behavior and polymer phenotype.

Granule positioning, inheritance, and homeostasis

The spatial behavior of Polyhydroxyalkanoate (PHA) granules within bacterial cells provides strong evidence that these structures are actively organized rather than passively distributed. Early models often assumed that PHA granules diffuse freely within the cytoplasm and are partitioned stochastically during cell division. In contrast, cell-biological studies now demonstrate that granule positioning is regulated and reproducible, reflecting coordinated interactions among granule-associated proteins, the nucleoid, and the cell cycle (Galán et al. 2011; Jendrossek and Pfeiffer 2014).

Granule positioning is therefore not equivalent to random diffusion. Time-lapse microscopy and ultrastructural analyses show that PHA granules frequently occupy defined intracellular regions, often closely associated with the nucleoid. This spatial arrangement is mediated by specific granule-associated proteins, such as PhaM, which bind both polymer inclusions and DNA, effectively anchoring granules to nucleoid regions (Wahl et al. 2012). Such tethering constrains granule mobility, prevents uncontrolled aggregation, and maintains ordered intracellular architecture as granule number and size change during growth and storage phases.

Regulated positioning has functional consequences beyond spatial order. By maintaining granules in predictable locations, cells ensure consistent interactions between granules and biosynthetic enzymes, depolymerases, and regulatory proteins. This spatial predictability supports coordinated control of polymer synthesis and turnover, reinforcing the view that granule organization is an integral component of PHA metabolism rather than a secondary outcome of polymer accumulation. In this sense, granule positioning acts as a stabilizing mechanism that buffers intracellular processes against stochastic fluctuations.

Inheritance of PHA granules during cell division further underscores their regulated nature. Rather than being passively partitioned, granules are typically inherited in a biased, near-symmetric manner. Cells containing multiple granules distribute them such that each daughter cell receives at least one granule or an equivalent share of storage capacity (Jendrossek and Pfeiffer 2014). This outcome is achieved not through active transport machinery but through pre-division positioning and anchoring of granules relative to the division plane.

Regulated inheritance has important physiological implications. Symmetric distribution of granules preserves continuity of storage capacity across generations, reducing abrupt differences in carbon availability or regulatory state between daughter cells. This continuity supports stable population-level behavior and limits the emergence of extreme phenotypes arising from unequal resource allocation. Conversely, disruption of positioning mechanisms results in irregular inheritance and increased heterogeneity in storage state, highlighting the functional importance of controlled granule segregation (Galán et al. 2011).

Population-level heterogeneity emerges naturally from granule positioning and inheritance dynamics. Even in clonal populations grown under uniform conditions, individual cells differ in granule number, size, and spatial arrangement. Such heterogeneity reflects regulated variability rather than noise alone, arising from differences in regulatory state, growth history, and turnover dynamics. Importantly, this variability is constrained by granule homeostasis mechanisms that limit the range of permissible granule states within the population (Uchino et al. 2007).

Granule homeostasis refers to the capacity of cells to maintain stable distributions of granule-related parameters-such as number, size, and positioning-over time despite ongoing synthesis and degradation. Homeostasis does not imply static granules; instead, it denotes dynamic stability in which granule properties fluctuate within regulated bounds. This stability emerges from feedback loops involving granule-associated proteins, regulatory networks, and spatial organization, ensuring that changes in storage state remain coordinated with overall cellular physiology.

Disruption of granule homeostasis amplifies population-level heterogeneity with direct consequences for polymer phenotype. Cells exhibiting unstable granule positioning or inheritance experience divergent turnover histories, leading to broader molecular-weight distributions and variable thermal properties of extracted polymer. Such variability complicates cross-study comparison and undermines reproducibility when polymer properties are interpreted without reference to underlying granule dynamics. Granule-state stability, therefore, provides a mechanistic link between intracellular organization and population-level consistency.

From a physiological perspective, regulated positioning and inheritance enable bacteria to balance flexibility with robustness. Cells can adjust storage capacity in response to environmental or metabolic cues while preserving organizational integrity across divisions. This balance is particularly important under fluctuating conditions that require rapid transitions between growth and storage. By embedding granule dynamics within spatial architecture and the cell cycle, bacteria ensure that storage metabolism remains an integrated component of cellular regulation.

In summary, PHA granule positioning and inheritance are governed by regulated mechanisms that distinguish granules from passive storage inclusions. Positioning is constrained by nucleoid-associated interactions rather than random diffusion, inheritance is biased toward symmetry rather than passive partitioning, and granule homeostasis limits population-level heterogeneity. Together, these features stabilize granule state and provide an organizational foundation for consistent polymer phenotype, reinforcing the central role of intracellular organization within the regulatory-granule-phenotype framework (Figure 2).

PHA granules in the context of bacterial organelle biology

Recognition of Polyhydroxyalkanoate (PHA) granules as regulated intracellular structures places them within a broader framework of bacterial organelle biology. Although prokaryotic cells lack membrane-bound organelles in the eukaryotic sense, they possess a diverse array of functionally specialized compartments that achieve spatial and regulatory segregation through protein-based architectures. Comparing PHA granules with other well-characterized bacterial organelles-such as carboxysomes, metabolosomes, and magnetosomes-highlights shared organizational principles while clarifying the distinctive role of PHA granules as carbon-storage organelles.

Carboxysomes are among the earliest described bacterial microcompartments and function as protein-shell organelles that encapsulate enzymes of carbon fixation, notably ribulose-1,5-bisphosphate carboxylase/oxygenase. Their polyhedral shells restrict diffusion, concentrate substrates, and enhance metabolic efficiency (Shively et al. 1973; Greening and Lithgow 2020). Structurally, carboxysomes are defined by ordered protein lattices that assemble into closed shells, creating discrete reaction spaces within the cytoplasm. Functionally, they represent metabolic organelles optimized for catalysis rather than storage.

Metabolosomes, including propanediol and ethanolamine utilization microcompartments, share similar architectural principles with carboxysomes. These compartments encapsulate enzyme cascades involved in the catabolism of specific substrates, isolating potentially toxic intermediates and enhancing pathway efficiency (Greening and Lithgow 2020). Like carboxysomes, metabolosomes rely on proteinaceous shells and precise assembly pathways, emphasizing that protein-based compartmentalization is a widespread and versatile strategy in bacterial cells.

Magnetosomes represent a contrasting class of bacterial organelles, distinguished by their membrane-bounded architecture and specialized function in biomineralization. Magnetosomes consist of lipid bilayers enclosing magnetite or greigite crystals, aligned along cytoskeletal filaments to confer magnetic orientation to the cell. Their biogenesis involves tightly regulated gene clusters, dedicated membrane proteins, and cytoskeletal elements that collectively control organelle nucleation, growth, spatial alignment, and inheritance during cell division (Schüler 2008; Greening and Lithgow 2020). Despite their membrane-bounded nature, magnetosomes share with PHA granules the principle of regulated positioning and coordinated inheritance, underscoring that precise subcellular organization is a conserved feature of bacterial organelle biology, independent of whether compartment boundaries are lipid- or protein-based.

Across these diverse organelles, several shared organizational principles emerge. First, bacterial organelles exhibit defined boundaries-protein-based or membrane-based-that distinguish their internal environment from the surrounding cytoplasm. Second, their assembly and maintenance are governed by dedicated protein networks that integrate organelle function with cellular regulation. Third, spatial organization and inheritance are regulated to ensure continuity across generations. These principles underscore that functional compartmentalization is a fundamental feature of bacterial cell biology rather than an exception.

PHA granules conform to these principles while exhibiting distinctive features that reflect their role as carbon-storage organelles. Unlike carboxysomes and metabolosomes, PHA granules do not encapsulate enzymatic pathways within closed shells. Instead, they store reduced carbon polymers in a hydrophobic core stabilized by a proteinaceous surface layer. This open yet regulated architecture allows dynamic access of biosynthetic and depolymerizing enzymes, facilitating reversible transitions between storage and mobilization (Jendrossek and Pfeiffer 2014).

The non-membranous nature of PHA granules further distinguishes them within the spectrum of bacterial organelles. Their boundaries are defined by protein-polymer interactions rather than lipid bilayers, enabling rapid remodeling of surface composition in response to regulatory signals. This flexibility is well-suited to the physiological demands of carbon storage, which require both stability during prolonged limitation and rapid mobilization upon nutrient repletion. In contrast, the more rigid shells of carboxysomes and metabolosomes are optimized for sustained metabolic function rather than reversible storage.

Functionally, PHA granules are unique in that they integrate metabolic buffering with regulatory memory. Polymer accumulation and turnover encode information about past physiological states, linking granule history to future cellular responses. This property has no direct analogue in catalytic organelles such as carboxysomes, underscoring the distinct conceptual category occupied by PHA granules within bacterial organelle biology.

In summary, situating PHA granules within the broader landscape of bacterial organelles highlights both shared organizational principles and functional uniqueness. Like other bacterial compartments, PHA granules are regulated, spatially organized, and integrated into cellular control networks. Unlike catalytic organelles, however, they function as dynamic carbon-storage units whose architecture supports reversible storage and regulatory memory. This comparison reinforces the classification of PHA granules as bona fide bacterial organelles and provides a conceptual bridge linking cell biology to polymer physiology.

REGULATORY GATING LINKS CELLULAR STRESS TO GRANULE DYNAMICS

Nutrient imbalance and commitment to intracellular storage

Commitment to Polyhydroxyalkanoate (PHA) accumulation represents an active physiological decision rather than a passive consequence of excess carbon availability. Classical interpretations framed PHA synthesis as a metabolic overflow that occurs when carbon flux exceeds the capacity of central metabolism. However, decades of physiological, genetic, and systems-level studies have demonstrated that PHA accumulation is tightly gated by regulatory networks that integrate nutrient status, growth potential, and stress responses (Doi 1990; Madison and Huisman 1999). This gating ensures that storage is initiated only when it provides a net adaptive benefit to the cell.

Nutrient imbalance, particularly carbon excess combined with limitation of essential nutrients such as nitrogen or phosphorus, is a recurrent trigger for storage commitment across diverse bacterial taxa. Under such conditions, anabolic pathways required for biomass expansion become constrained, while carbon and reducing equivalents continue to enter central metabolism. Rather than allowing redox imbalance or accumulation of toxic intermediates, cells actively redirect carbon into PHA synthesis as a controlled sink (Rehm 2010; Koller 2017). Importantly, this redirection is not automatic; it requires coordinated transcriptional, post-transcriptional, and enzymatic regulation that collectively define a storage-competent physiological state.

The decision to enter a storage mode reflects a fundamental growth-storage trade-off. Allocation of resources to PHA synthesis necessarily diverts carbon, energy, and enzymatic capacity away from immediate biomass production. Experimental studies consistently show that cells accumulating PHA exhibit reduced growth rates and downregulation of ribosomal and biosynthetic functions relative to actively growing cells (Jendrossek and Pfeiffer 2014; Mozejko-Ciesielska et al. 2017). This trade-off underscores that PHA accumulation is not a default metabolic pathway but a strategic reallocation of resources favoring future growth potential and survival over rapid proliferation.

Crucially, the growth-storage trade-off is reversible. Cells that commit to storage under nutrient imbalance can rapidly re-enter growth mode when limiting nutrients are replenished, mobilizing stored polymer to support biomass synthesis. This reversibility distinguishes regulated storage from terminal metabolic states and requires precise control over both synthesis and depolymerization pathways. The capacity for rapid switching further supports the view that storage commitment is an actively maintained physiological state rather than an irreversible overflow condition (York et al. 2001; Jendrossek and Pfeiffer 2014).

Evidence for regulated storage commitment extends across phylogenetically diverse bacteria. In *Cupriavidus necator*, one of the best-characterized PHA producers, nitrogen or phosphorus limitation induces a coordinated regulatory response involving phasin expression, granule formation, and altered central metabolism (Madison and Huisman 1999; Tsuge 2016). In Pseudomonas species, medium-chain-length PHA accumulation is similarly regulated through global stress responses and carbon catabolite repression systems, highlighting that storage commitment operates through conserved regulatory principles despite differences in polymer chemistry (Mozejko-Ciesielska et al. 2017). Comparable regulatory patterns have been observed in halophilic and marine bacteria, indicating that nutrient-gated storage is a widespread adaptive strategy rather than a niche-specific phenomenon.

Phosphorus limitation provides a particularly illustrative example of active storage commitment. Phosphate scarcity directly constrains nucleotide synthesis and energy metabolism, creating conditions in which continued carbon assimilation cannot be efficiently translated into growth. Under such circumstances, PHA accumulation functions as a temporally flexible reserve that preserves carbon and reduces power until biosynthetic capacity is restored. Studies across multiple taxa demonstrate that phosphorus limitation induces PHA accumulation even in the absence of extreme carbon excess, reinforcing the interpretation that storage commitment reflects integrated assessment of biosynthetic constraints rather than a single nutrient signal (Rehm 2010; Koller 2018).

The active nature of storage commitment has direct implications for granule dynamics. Entry into a storage state is accompanied by changes in granule nucleation, surface protein composition, and positioning, as discussed in Section 3. These changes are not mere consequences of polymer presence but reflect coordinated restructuring of intracellular organization in response to regulatory signals. Conversely, exit from storage involves regulated mobilization and remodeling of granules, ensuring that polymer reserves are accessed efficiently without destabilizing cellular homeostasis.

Importantly, similar nutrient imbalances do not always produce identical storage outcomes. Differences in regulatory sensitivity, timing of nutrient limitation, and integration with global stress responses can yield distinct granule states and polymer phenotypes even under comparable external conditions. This observation

underscores the necessity of distinguishing nutrient imbalance as a triggering context from regulatory gating as the determinant of intracellular response. Nutrient imbalance sets the stage, but regulatory networks decide whether, when, and how storage is executed.

In summary, nutrient imbalance initiates a regulated decision-making process that commits cells to intracellular PHA storage. This commitment embodies a reversible growth-storage trade-off that is conserved across bacterial taxa and tightly coupled to cellular regulatory state. Recognizing storage as an active physiological decision provides a mechanistic foundation for understanding how stress signals are translated into granule dynamics and, ultimately, into polymer phenotype.

Global stress response networks shaping PHA regulation

Regulation of Polyhydroxyalkanoate (PHA) metabolism is embedded within global stress response networks that coordinate cellular priorities under fluctuating environmental and metabolic conditions. These networks operate as hierarchical systems that integrate nutrient sensing, energy status, and stress signals to modulate gene expression, protein activity, and intracellular organization, thereby reallocating cellular resources to enhance stress tolerance at the expense of immediate growth when required (Tan et al. 2022). Within this hierarchy, PHA synthesis and granule dynamics are downstream outputs rather than primary drivers, reflecting broader physiological state transitions rather than isolated metabolic events (Figure 2).

The stringent response and (p)ppGpp signaling

The stringent response represents a central regulatory mechanism through which bacteria adapt to nutrient limitation and other stresses. Mediated by the alarmones guanosine tetraphosphate and pentaphosphate ((p)ppGpp), this response reprograms cellular metabolism by suppressing growth-associated processes, such as ribosome biogenesis, while promoting stress tolerance and survival pathways (Potrykus and Cashel 2008). Accumulation of (p)ppGpp therefore creates a physiological context in which diversion of carbon from growth toward storage becomes advantageous.

Multiple studies indicate that activation of the stringent response correlates with enhanced PHA accumulation across diverse taxa. In Pseudomonas species, mutants with altered (p)ppGpp metabolism exhibit significant changes in PHA synthesis, implicating stringent control as an upstream regulator of storage commitment (Mozejko-Ciesielska et al. 2017). Rather than directly inducing pha gene expression, (p)ppGpp reshapes the transcriptional landscape to favor metabolic states compatible with storage, including reduced anabolic demand and altered central carbon flux.

Importantly, the stringent response influences granule dynamics indirectly by modulating the expression and activity of granule-associated proteins. Reduced growth pressure under high (p)ppGpp conditions allows accumulation of phasins and stabilization of granule surfaces, promoting granule maturation and persistence. Conversely, relief of stringent control upon nutrient repletion facilitates depolymerase activity and granule remodeling, enabling rapid transition back to growth. This bidirectional influence reinforces the view that (p)ppGpp signaling gates storage and mobilization rather than acting as a simple on-off switch, consistent with integrated regulatory models that link global stress signaling, pha gene regulation, and granule-associated control layers (Vicente et al. 2023). Experimental evidence further demonstrates that modulation of PHA dynamics can reciprocally influence global metabolic states. Synthetic tuning of the PHA synthesis-degradation cycle in *Pseudomonas putida* has been shown to reprogram cellular metabolic states, confirming that granule turnover functions as an active regulatory node embedded within stringent-response-associated physiology rather than as a passive storage outcome (Manoli et al. 2022).

Sigma factor hierarchies and transcriptional gating

Sigma factors introduce an additional layer of regulatory hierarchy that refines global responses to stress and nutrient imbalance. By directing RNA polymerase to specific promoter classes, alternative sigma factors enable selective activation of gene sets associated with stress adaptation, stationary phase entry, and metabolic reorganization (Paget and Helmann 2003). In the context of PHA metabolism, sigma factor hierarchies influence both biosynthetic capacity and granule-associated regulation.

In several PHA-accumulating bacteria, stress-responsive sigma factors modulate the expression of pha operons, phasin genes, and depolymerases, thereby coordinating polymer synthesis and turnover with broader physiological state. In Gram-negative bacteria, this coordination is most prominently mediated by the general stress sigma factor σ^S (RpoS), which integrates nutrient limitation, oxidative stress, and stationary-phase signals into global transcriptional reprogramming (Hengge 2010). For example, sigma factors associated with the stationary phase or envelope stress can enhance the expression of granule-associated proteins, stabilizing granules during prolonged limitation (Jendrossek and Pfeiffer 2014). Conversely, growth-associated sigma factors favor expression profiles that support rapid biomass expansion and reduced storage.

Sigma factor hierarchies also contribute to the temporal structuring of storage responses. Different sigma factors may dominate at distinct stages of nutrient limitation or stress exposure, leading to phase-specific regulation of PHA metabolism. Such temporal layering allows cells to fine-tune storage commitment, avoiding premature or excessive accumulation. This dynamic regulation aligns with observations that granule number, size, and surface composition evolve rather than appearing instantaneously upon stress onset.

Critically, sigma factor-mediated control helps explain why identical nutrient imbalances can yield divergent PHA phenotypes. Variability in sigma factor activation thresholds or timing across strains or experimental conditions can produce distinct regulatory trajectories,

resulting in different granule states even under similar external conditions. Importantly, sigma factor hierarchies do not operate in isolation but intersect with other global signaling systems, including quorum sensing and second-messenger networks such as cyclic di-GMP. Cyclic di-GMP functions as a central integrator of environmental and physiological signals, coordinating transitions between motile, sessile, stress-adapted, and stationary-phase-like states by reshaping transcriptional priorities and regulatory hierarchies rather than acting on individual target genes alone (Hengge 2009). Crosstalk between quorum-sensing regulators and c-di-GMP signaling has been shown to further restructure transcriptional programs during transitions between growth, stress adaptation, biofilm-associated states, and stationary-phase-like programs, thereby modulating downstream storage-related phenotypes and resource allocation strategies (Srivastava and Waters 2012; Valentini and Filloux 2016). This integration highlights that transcriptional gating of PHA metabolism is embedded within broader regulatory circuits governing lifestyle transitions rather than being controlled by sigma factor activity alone, underscoring the necessity of considering higher-order signaling networks when interpreting PHA data and comparing results across studies.

Post-transcriptional control by small RNAs

Post-transcriptional regulation mediated by small RNAs (sRNAs) adds a further dimension of control over PHA metabolism. sRNAs modulate gene expression by affecting mRNA stability or translation efficiency, enabling rapid adjustment of protein levels without the need for new transcription, and represent a versatile and rapidly evolving regulatory layer in bacterial stress responses (Gottesman and Storz 2011). This mode of regulation is particularly well-suited to dynamic processes such as storage commitment and mobilization, which require swift responses to changing conditions.

In Pseudomonas species, sRNAs involved in carbon catabolite repression have been shown to influence PHA metabolism indirectly by regulating central carbon flux and stress response pathways (Che et al. 2025). By fine-tuning the availability of enzymes and regulatory proteins, sRNAs contribute to the precise control of granule-associated processes, including phasin abundance and depolymerase activity. Such fine-grained regulation supports reversible switching between storage and growth states.

sRNA-mediated control also enhances robustness of regulatory responses by buffering transcriptional noise. By dampening fluctuations in mRNA levels, sRNAs help stabilize protein expression profiles associated with specific physiological states. This buffering capacity is particularly relevant for granule-associated proteins, where small changes in abundance can have outsized effects on granule architecture and polymer turnover. Through this mechanism, sRNAs indirectly promote granule-state stability and reduce population-level heterogeneity.

The stringent response, sigma factor hierarchies, and sRNA-mediated control form an integrated regulatory network that channels stress signals toward specific cellular outcomes. Within this network, PHA granules act as downstream effectors whose properties reflect the cumulative influence of the global regulatory state. Changes in alarmone levels, transcriptional priorities, and post-transcriptional modulation converge to reshape granule surface composition, nucleation rates, and turnover dynamics (Figure 3). Importantly, PHAs and their degradation products are not merely passive storage forms but actively contribute to cellular stress tolerance by stabilizing proteins, mitigating oxidative damage, and supporting redox homeostasis during adverse conditions, reinforcing the role of granule turnover as an adaptive stress-response mechanism rather than a unidirectional storage process (Müller-Santos et al. 2021). These regulatory insights also provide a conceptual foundation for rational metabolic engineering approaches that leverage pathway redirection, growth modulation, and cellular organization to improve PHA production outcomes (Chen and Jiang 2017).

This integrated view clarifies why stress exposure alone cannot predict polymer phenotype. Stress signals must first be interpreted by regulatory networks, which then determine whether and how granule dynamics are modified. Consequently, polymer properties emerge from a regulatory context rather than from stress intensity per se. Global stress response networks shape PHA regulation by gating storage commitment, modulating granule-associated processes, and enabling reversible transitions between physiological states, forming a critical link in the regulatory-granule-phenotype framework (Figure 2).

Mobilization, turnover, and regulatory memory

Mobilization of Polyhydroxyalkanoate (PHA) reserves is an intrinsic component of regulated storage metabolism and should not be interpreted as a failure of storage capacity. Early production-oriented views often equated polymer degradation with yield loss or inefficiency. In contrast, physiological and cell-biological evidence demonstrates that PHA turnover is a regulated process that enables dynamic adjustment of intracellular carbon allocation in response to changing cellular demands (Jendrossek and Pfeiffer 2014). Within this framework, synthesis and degradation are coordinated processes that maintain metabolic flexibility rather than opposing outcomes.

Turnover of PHA is mediated primarily by intracellular depolymerases such as PhaZ, whose activity is tightly regulated at transcriptional, post-transcriptional, and spatial levels. During storage-committed states, depolymerase activity is suppressed or spatially constrained, allowing net polymer accumulation. Upon nutrient repletion or transition to growth-favorable conditions, depolymerase activity increases, enabling mobilization of stored polymer to support biosynthesis and energy production (York et al. 2001; Jendrossek and Pfeiffer 2014). Regulatory gating ensures that mobilization occurs in synchrony with physiological need rather than as an uncontrolled loss of reserves.

Importantly, turnover can occur concurrently with net polymer accumulation. Experimental evidence shows that partial depolymerization and re-synthesis take place even

as total PHA content increases, resulting in continuous remodeling of polymer chains within granules (Grage et al. 2009). This dynamic equilibrium challenges static models of storage and underscores that PHA granules remain metabolically active throughout their lifespan. Turnover, therefore, represents adaptive maintenance rather than inefficiency.

The concept of regulatory hysteresis provides a useful framework for interpreting mobilization dynamics. Entry into storage under nutrient limitation and exit upon nutrient repletion often follow asymmetric trajectories. Mobilization kinetics, granule restructuring, and regulatory profiles during recovery do not necessarily mirror those observed during storage commitment, reflecting dependence on prior states (Wahl et al. 2012). This hysteresis indicates that PHA metabolism exhibits regulatory memory rather than simple reversibility.

Regulatory memory arises from persistent features of granule organization, including architecture, surface protein composition, and enzyme localization, which are not immediately reset upon environmental change. Phasin abundance, granule number, and surface properties established during storage may persist into recovery phases, influencing the rate and extent of mobilization. Partial depolymerization can further modify granule surfaces, shaping subsequent rounds of synthesis. As a result, granule behavior reflects cumulative regulatory history rather than only current conditions (Mitra et al. 2022).

Polymer phenotype can thus be interpreted as encoded physiology. Molecular characteristics such as molecular-weight distribution, end-group chemistry, and crystallinity integrate information about the duration and frequency of storage and mobilization cycles. Polymers produced under prolonged, stable storage conditions differ systematically from those synthesized under repeated stress-recovery regimes, even when total accumulation levels are comparable (Koller 2017). These differences arise from the temporal structure of regulatory gating and turnover rather than from changes in polymer chemistry itself.

At the population level, regulatory memory generates controlled heterogeneity. Individual cells experience distinct micro-histories of storage and mobilization, leading to variability in granule state and polymer properties. Granule homeostasis mechanisms constrain this variability while preserving flexibility, allowing populations to distribute risk across physiological states under fluctuating conditions (Veening et al. 2008; Ackermann 2015).

Recognizing turnover as a regulated, memory-bearing process has important implications for experimental design. End-point measurements of polymer content or properties cannot capture the temporal dynamics shaping polymer phenotype. Phase-resolved sampling and integration of granule-level observations are essential to distinguish synthesis-driven accumulation from turnover-driven remodeling. Without this context, mobilization may be misinterpreted as storage failure rather than evidence of regulatory competence.

In summary, mobilization and turnover are integral components of regulated PHA storage metabolism. Through hysteresis and regulatory memory, granule dynamics encode cellular history into polymer structure, making polymer phenotype a dynamic record of past regulatory states rather than a static product of accumulation alone.

Regulatory triggers vs granule outcomes

A recurring challenge in Polyhydroxyalkanoate (PHA) research is the observation that identical or closely matched stress conditions often yield divergent polymer phenotypes. Similar nutrient limitations, redox states, or salinity regimes can produce polymers with different molecular-weight distributions, crystallinity, or reproducibility profiles, even when total accumulation levels are comparable. This inconsistency has frequently been attributed to experimental noise or strain-specific idiosyncrasies. However, evidence synthesized in this review indicates that such divergence is a predictable outcome of regulatory mediation rather than an anomaly (Doi 1990; Koller 2017).

The central principle emerging from regulatory granule biology is that stress signals do not act directly on polymer chemistry. Instead, stress is first interpreted by global regulatory networks, which then reshape granule state through changes in nucleation, surface composition, positioning, and turnover. Polymer phenotype emerges downstream of these granule-level changes. Consequently, identical stress inputs can be translated into different polymer outputs if regulatory gating or granule dynamics differ. Stress alone is therefore insufficient to predict polymer properties without explicit consideration of granule state (Jendrossek and Pfeiffer 2014).

Nutrient imbalance provides a clear illustration of this principle. Carbon excess combined with nitrogen limitation may induce PHA accumulation across multiple taxa, yet the resulting granule architectures can differ substantially depending on the timing, duration, and regulatory context of limitation. Short-term imbalance may favor rapid nucleation of multiple small granules, whereas prolonged limitation may promote maturation of fewer, larger granules. Although both scenarios can yield similar % cell dry weight values, the resulting polymers differ in turnover history and molecular-weight distribution, reflecting distinct granule states rather than differences in stress exposure (Anderson and Dawes 1990).

Global stress responses further modulate this translation process. Activation of the stringent response, alternative sigma factors, or sRNA-mediated control shapes transcriptional and post-transcriptional landscapes that influence granule-associated protein composition and depolymerase activity. Two cells exposed to the same external stressor may therefore occupy different regulatory states, leading to divergent granule dynamics and polymer outcomes. These differences are not stochastic but arise from variation in regulatory thresholds, timing, and network integration (Mozejko-Ciesielska et al. 2017; Che et al. 2025).

Granule state thus functions as a mandatory mediator between regulatory triggers and polymer phenotype. Parameters such as granule number, size distribution,

surface protein composition, nucleoid association, and turnover history collectively define the physical and regulatory environment in which polymer synthesis and remodeling occur. Without accounting for these intermediate variables, correlations between stress conditions and polymer properties remain incomplete and potentially misleading. This mediating role of granule state explains why polymer yield metrics alone fail to capture reproducible relationships between cultivation conditions and material properties (Figure 2).

The synthesis of regulatory triggers and granule outcomes clarifies a key conceptual distinction: stress conditions establish a permissive or restrictive context for storage, but they do not determine polymer phenotype directly. Instead, polymer properties reflect the granule-state trajectory shaped by regulatory gating over time. This distinction protects interpretation from environmental determinism and emphasizes mechanistic causation rooted in intracellular organization rather than external forcing.

By explicitly mapping regulatory triggers to granule-state outcomes and expected polymer phenotypes, it becomes possible to generate testable hypotheses and improve comparability across studies. Experiments that report stress conditions without granule-level characterization capture only the upstream context, whereas integration of granule-state measurements reveals the mechanistic pathway linking stress to polymer outcome. This synthesis therefore provides a conceptual scaffold for interpreting variability in the literature and for designing experiments that move beyond descriptive association.

In summary, identical stress does not guarantee identical polymer because regulatory networks and granule dynamics mediate the translation from stress signal to material outcome. Granule state is a necessary and obligatory intermediary that integrates regulatory history, spatial organization, and turnover dynamics. Recognizing this mediation resolves apparent inconsistencies in PHA studies and reinforces the central argument of this review: polymer phenotype is a physiological output of regulated granule biology rather than a direct consequence of environmental stress.

Table 3. Regulatory triggers, granule-state outcomes, and expected polymer phenotypes

Regulatory trigger	Primary regulatory pathway engaged	Immediate granule-state outcome	Secondary cellular consequence	Expected polymer phenotype (qualitative)	Key interpretive note
Nitrogen limitation (excess carbon)	Global nutrient-sensing; relief of growth-coupled repression	Increased granule nucleation; rise in granule number per cell	Carbon flux redirected from biomass to storage	High %PHA; moderate Mw; relatively narrow Mw/Mn if steady	Yield increase alone does not predict quality; granule stability matters
Phosphorus limitation	Stringent response; altered energy allocation	Fewer but larger granules; prolonged maturation phase	Slower growth; extended storage commitment	Higher Mw; potential increase in crystallinity	Often conflated with N-limitation, but granule dynamics differ
Oxygen limitation/redox stress	Redox balancing; stress-response regulators	Enhanced granule formation as an electron sink; spatial reorganization	Shift in $NADH/NAD^+$ balance	Broader Mw/Mn; increased heterogeneity	Polymer reflects redox history, not just endpoint
Salinity stress (osmotic shock)	Osmoadaptation networks; compatible solute synthesis	Competition for carbon reduces the granule growth rate	Carbon is partitioned between osmoprotection and storage	Lower Mw and/or reduced crystallinity	Storage outcome depends on regulatory prioritization
Salinity-redox cycling	Coupled stress-response gating	Repeated nucleation-turnover cycles; unstable granule states	Population-level heterogeneity	Broad Mw/Mn; reduced reproducibility	Identical means can mask divergent single-cell states
Carbon starvation after accumulation	Mobilization pathways: depolymerase activation	Partial granule erosion; surface remodeling	Maintenance metabolism supported	Mw decrease; bimodal distributions possible	Turnover is regulatory, not failure
Rapid carbon refeeding	Re-engagement of synthase complexes	Re-initiation of pre-existing granules	Memory of prior granule state retained	Path-dependent Mw/Mn (hysteresis)	Polymer encodes cultivation history
Perturbation of phasin balance	Loss of surface buffering	Irregular granule size and coalescence control	Increased stochasticity	High dispersion in Mw/Mn despite similar %PHA	Granule surface integrity underpins reproducibility
Perturbation of PhaR/PhaD regulation	Disrupted feedback between synthesis and regulation	Mis-timed nucleation or overgrowth	Decoupling of growth and storage	Unpredictable polymer quality	Regulatory control outweighs substrate supply
Stable, non-cycling limitation (control)	Homeostatic regulation	Stable granule number and size	Low heterogeneity	Consistent Mw/Mn and thermal properties	Baseline for reproducibility comparisons

POLYMER PHENOTYPE AS A PHYSIOLOGICAL OUTPUT OF GRANULE STATE

Minimal polymer descriptors and their interpretive limits

Quantitative descriptors of polymer phenotype are indispensable for comparing Polyhydroxyalkanoate (PHA) studies, yet the metrics most commonly reported provide limited insight into the biological processes underlying polymer formation. Across the PHA literature, polymer content expressed as percentage of cell dry weight (%CDW) and molecular-weight parameters such as number-average (Mn) and weight-average molecular weight (Mw) are frequently treated as proxies for physiological performance or material quality. While analytically convenient, these descriptors are often interpreted independently of intracellular granule state, leading to oversimplified or potentially misleading conclusions (Madison and Huisman 1999; Verlinden et al. 2007).

Reliance on %CDW illustrates this limitation clearly. Normalizing polymer content to biomass is useful for estimating storage capacity or process yield, but it collapses complex intracellular dynamics into a single scalar value. %CDW provides no information on granule number, size distribution, spatial organization, or turnover history-features that directly influence polymer synthesis, storage, and mobilization. Cultures with identical %CDW values may differ substantially in granule architecture, with one containing numerous small granules and another harboring fewer, larger granules subject to different regulatory constraints. These physiologically meaningful differences remain invisible in %CDW-based comparisons (Jendrossek and Pfeiffer 2014).

Moreover, %CDW conflates accumulation with stability. High polymer content may reflect recent synthesis under acute stress, prolonged storage under stable limitation, or a dynamic balance between synthesis and turnover. Without temporal or granule-level context, these scenarios cannot be distinguished. Consequently, %CDW is poorly suited for inferring regulatory competence or predicting polymer reproducibility, and treating high %CDW as an unambiguous indicator of successful storage risks misinterpreting both cellular physiology and material outcome.

Molecular-weight descriptors such as Mw and Mn provide greater resolution but suffer from analogous interpretive limitations when decoupled from granule biology. These parameters integrate information on polymer chain-length distributions shaped by enzyme activity, substrate availability, and depolymerization. However, they are typically reported as endpoint measurements on extracted polymer, abstracted from the intracellular environment in which polymer chains were synthesized and remodeled (Tsuge 2016; Koller 2017). Production-oriented studies continue to emphasize yield, substrate range, and process optimization-including the use of heterogeneous feedstocks such as food residues-while polymer quality is interpreted primarily through endpoint descriptors, with little consideration of intracellular organization or turnover history (Sabapathy et al. 2020; Täuber et al. 2025).

Mw/Mn ratios are often interpreted as intrinsic properties of a strain or cultivation condition, implying direct control by genetic background or external parameters. This interpretation overlooks granule state as an intermediate variable. Polymer chain elongation and degradation occur within physical and regulatory constraints imposed by granule architecture. Granule surface composition, accessibility of synthases and depolymerases, and turnover dynamics collectively influence chain-length distributions. As a result, identical enzymatic repertoires can yield different Mw/Mn profiles if granule state differs due to regulatory history or spatial organization (Grage et al. 2009).

The interpretive limits of Mw and Mn are particularly evident in comparisons between batch and fed-batch cultures or among different stress regimes. Polymers extracted at similar growth stages may display divergent molecular-weight profiles, not because of inherent differences in biosynthetic capacity, but due to differences in turnover timing and granule remodeling. Without granule-level information, such variability is difficult to reconcile and is often attributed to experimental noise or uncontrolled variables.

A further limitation of minimal descriptors is their inability to capture population-level heterogeneity. Extracted polymer represents an average across cells that may differ in granule number, size, and regulatory history. Heterogeneity in granule state can broaden molecular-weight distributions without indicating defects in synthesis, while narrow distributions may reflect synchronized granule dynamics rather than superior enzymatic control. These distinctions are lost when polymer metrics are interpreted in isolation.

This critique does not imply that %CDW, Mw, or Mn are uninformative. Rather, their interpretive value depends on integration with granule-level and regulatory context. When combined with data on granule organization, turnover dynamics, and regulatory state, these descriptors can contribute meaningfully to mechanistic understanding. For example, temporal changes in Mw may reveal shifts in depolymerase activity, while stable Mw under fluctuating conditions may indicate robust granule-state homeostasis.

Recognizing the limits of minimal descriptors motivates a shift from yield-centric to physiology-centric interpretation. Polymer phenotype should be understood as an outcome of regulated granule state rather than as an intrinsic material property independent of cellular organization. Such integration is essential for addressing reproducibility challenges in the PHA literature, where similar %CDW or Mw values often mask divergent underlying biological processes.

In summary, commonly reported polymer descriptors provide incomplete and potentially misleading views of PHA physiology when interpreted without granule context. Integrating %CDW and molecular-weight parameters with granule-state information is, therefore, necessary to interpret polymer phenotype as a physiological output rather than a purely technical measurement.

Table 4. Minimal polymer phenotype descriptors commonly reported in PHA studies and their physiological interpretation

Polymer phenotype descriptor	What is directly measured	Physiological meaning (what it does and does not indicate)	Major interpretive limitation if used alone	Recommended contextual information
PHA content (% cell dry weight, %CDW)	Fraction of total biomass converted into polymer	Indicates storage commitment magnitude at the population level	Cannot distinguish many small granules vs few large granules; blind to turnover and history	Granule number and size distribution; growth phase; nutrient limitation regime
Polymer titer (g L^{-1})	Total recoverable polymer per culture volume	Reflects the combined effects of biomass concentration and storage	Confounds cellular physiology with culture density; not a polymer-quality metric	Biomass yield; %CDW; cultivation mode (batch/fed-batch)
Monomer composition (e.g., PHB vs PHBV; scl vs mcl)	Relative abundance of monomer units after methanolysis	Reflects precursor availability and synthase specificity under the given regulatory state	Does not capture the chain-length distribution or remodeling history	Carbon source identity; feeding strategy; regulatory state markers
Weight-average and number-average molecular weight (Mw/Mn)	Distribution of polymer chain lengths	Integrates synthesis kinetics and turnover history	Sensitive to extraction method; uninterpretable without granule-state context	Extraction protocol; granule turnover regime; depolymerase activity
Dispersity (Đ = Mw/Mn)	Breadth of molecular-weight distribution	Proxy for heterogeneity in chain initiation, elongation, and degradation	High dispersity may reflect biology or extraction artefacts	Time-resolved sampling; granule heterogeneity metrics
Thermal properties (Tm, Tg)	Phase transition temperatures measured by DSC	Emergent outcome of monomer composition and chain organization	Cannot be mapped to a regulation without molecular-weight context	Mw/Mn; crystallinity; polymer history
Crystallinity (%)	Fraction of crystalline polymer domains	Reflects chain regularity and packing history	Influenced by post-extraction processing and thermal history	Cooling rate, annealing history, and granule maturation stage
Granule morphology (number, size, localization)	Physical organization of polymer inclusions	Direct proxy of granule state linking regulation to polymer phenotype	Rarely reported; often qualitative	Quantitative imaging; single-cell statistics
Polymer purity (protein/lipid residues)	Non-polymer components co-extracted with PHA	Indicates granule surface composition and extraction efficiency	Often misattributed solely to downstream processing	Granule surface protein profile; extraction chemistry

Note: These descriptors represent *minimal* reporting standards rather than sufficient explanations of polymer behavior. Polymer phenotype emerges from a regulated granule state; therefore, descriptors gain mechanistic meaning only when interpreted together with granule-level and regulatory context. Similar values of %CDW or titer can arise from distinct physiological states and yield polymers with divergent material properties

Granule-state determinants of polymer quality

Understanding polymer quality as a physiological output requires identification of intermediate variables that translate cellular regulation into material properties. In Polyhydroxyalkanoate (PHA)-accumulating bacteria, these variables are granule-state determinants that define the physical and regulatory context of polymer synthesis and turnover. Granule state, therefore, functions as a mechanistic link between regulatory history and polymer phenotype, influencing both polymer quality and reproducibility (Jendrossek and Pfeiffer 2014).

Granule-state determinants include granule number, size distribution, surface protein composition, spatial positioning, and turnover dynamics. These features do not directly control polymer chemistry but constrain enzyme access, reaction kinetics, and remodeling processes. As intermediate variables, they are shaped by upstream regulatory networks while directly conditioning downstream polymer outcomes. This perspective explains why identical regulatory signals or cultivation conditions can produce divergent polymer properties.

Granule number and size distribution influence polymer quality by modulating surface-to-volume ratios and enzyme accessibility. Cells with many small granules provide greater cumulative surface area for synthase and depolymerase activity, favoring dynamic turnover and broader molecular-weight distributions. In contrast, fewer and larger granules impose different kinetic constraints that may stabilize polymer chains and narrow molecular-weight profiles. These architectural differences arise from regulated nucleation and maturation rather than from polymer chemistry alone (Wahl et al. 2012; Tsuge 2016).

Surface protein composition constitutes a second major determinant. Phasins and associated regulatory proteins stabilize granule morphology, control enzyme localization, and mediate interactions with global regulatory networks. Variation in phasin abundance or composition alters granule surface properties, affecting polymer elongation and susceptibility to depolymerization. Although often subtle, these effects accumulate over extended storage or turnover periods and shape polymer phenotype (Jendrossek and Pfeiffer 2014).

Granule positioning and nucleoid association further modulate polymer quality by influencing spatial regulation and inheritance. Granules anchored to defined intracellular regions experience more consistent regulatory environments across cell cycles, promoting stability in polymer synthesis. Disrupted positioning can expose granules to heterogeneous regulatory contexts, increasing variability in polymer properties. This spatial component highlights that polymer quality depends on intracellular organization in addition to enzymatic activity.

Turnover dynamics represent a particularly influential determinant. Polymer synthesis and degradation often occur concurrently, resulting in continuous remodeling of polymer chains. The balance and timing of these processes determine average chain length, end-group composition, and crystallinity. Because turnover reflects regulatory history, polymer structure integrates information about past storage and mobilization states. Polymers produced under cycling regimes therefore differ systematically from those synthesized under prolonged, stable storage, even when total accumulation is similar (Grage et al. 2009; Koller and Braunegg 2015).

Together, these determinants shape polymer heterogeneity within and across populations. Even clonal cultures exhibit cell-to-cell variability in granule state due to differences in regulatory timing, growth history, or inheritance. This heterogeneity manifests as broader molecular-weight distributions or variable thermal properties in extracted polymer and represents a biological feature of regulated granule dynamics rather than experimental noise (Dias et al. 2008; Sindhu et al. 2021).

Reproducibility of polymer quality, therefore, depends on the reproducibility of the granule state rather than solely on the control of external parameters. Studies that standardize cultivation conditions but omit granule-level characterization may obtain consistent % cell dry weight values while observing variable polymer properties. Conversely, approaches that stabilize granule-state determinants are more likely to yield reproducible polymer phenotypes. Differences in reported polymer quality across similar studies may thus reflect unreported variation in granule architecture or turnover history rather than methodological error.

In summary, polymer quality in PHA-accumulating bacteria emerges from granule-state determinants that mediate between regulatory networks and material properties. Granule number, size, surface composition, positioning, and turnover dynamics function as intermediate variables shaping polymer heterogeneity and reproducibility. Accounting for these determinants is essential for interpreting polymer phenotype as a physiological output of regulated granule biology rather than as a direct consequence of environmental or genetic factors.

Reproducibility as a biological, not technical, property

Reproducibility of Polyhydroxyalkanoate (PHA) polymer properties has traditionally been treated as a technical issue, attributed to differences in cultivation protocols, analytical methods, or extraction procedures. While such factors undoubtedly contribute to variability, this perspective overlooks a more fundamental source of inconsistency rooted in cellular physiology. Evidence synthesized in this review supports a reframing of reproducibility as a biological property that reflects the stability and reproducibility of granule state rather than solely the precision of experimental techniques (Steinbüchel and Hein 2001).

At the core of this argument lies the equivalence between polymer reproducibility and granule-state reproducibility. Polymer chains are synthesized, remodeled, and degraded within the physical and regulatory environment defined by PHA granules. If granule architecture, surface composition, positioning, and turnover dynamics vary across experiments or populations, polymer properties will necessarily vary as well, even under ostensibly identical conditions. Conversely, when the granule state is stabilized and reproducible, the polymer phenotype becomes more consistent. This relationship underscores that polymer reproducibility is an emergent property of intracellular organization rather than a direct outcome of controlled inputs.

Granule-state reproducibility depends on the coordinated regulation of multiple intermediate variables. These include consistent timing of storage commitment, regulated nucleation and maturation of granules, stable expression of granule-associated proteins, and predictable turnover dynamics. Disruption at any of these levels can introduce variability that propagates to polymer phenotype. Importantly, many of these variables are sensitive to subtle differences in regulatory context that are not captured by standard reporting of cultivation conditions or polymer metrics.

This biological perspective helps explain why polymer properties often vary across studies using the same strain and nominally similar protocols. Differences in inoculum history, growth phase at sampling, or stress exposure timing can shift regulatory trajectories and granule-state evolution, leading to divergent polymer outcomes. Such effects are rarely documented in production-focused studies, resulting in apparent irreproducibility that is misattributed to methodological shortcomings rather than to biological variability (Chen and Jiang 2017).

Reframing reproducibility as a biological property has direct implications for cross-study comparison. Comparisons based solely on % cell dry weight, molecular-weight averages, or monomer composition implicitly assume equivalence of underlying granule states. When this assumption is violated, comparisons become confounded and potentially misleading. Integrating granule-level descriptors-such as granule number, size distribution, or surface protein profiles-would provide a more robust basis for comparison by anchoring polymer properties in a shared physiological context.

Table 5. Granule-state determinants governing polymer quality and reproducibility in PHA-accumulating bacteria

Granule-state determinant	Definition at the granule level	Primary regulatory controls	Expected impact on polymer quality	Consequence for reproducibility	Representative measurable readouts
Granule number per cell	Number of discrete PHA inclusions formed within a single cell	Phasin abundance; PhaR-mediated feedback; nucleation-maturation balance	Higher granule numbers increase total surface area, favoring shorter average chain lengths and broader Mw/Mn under dynamic conditions	Variable granule numbers amplify cell-to-cell heterogeneity	Single-cell granule counts; size-frequency distributions
Granule size distribution	Relative proportion of small versus large granules within a population	Phasin-mediated surface stabilization; duration of storage-permissive phases	Larger granules tend to support longer chains and higher crystallinity	Mixed-size distributions reduce batch-to-batch consistency	Granule diameter/volume statistics
Granule surface protein composition	Identity and abundance of granule-associated proteins (phasins, synthase, depolymerase, regulators)	Transcriptional and post-transcriptional regulation; protein-protein interactions	Modulates enzyme accessibility, chain elongation, and turnover	Surface variability leads to inconsistent polymer remodeling	Targeted proteomics; tagged-protein imaging
Granule-nucleoid positioning	Spatial localization of granules relative to the nucleoid	PhaM-mediated tethering; spatial control during the cell cycle	Promotes ordered granule inheritance and synchronized maturation	Stable positioning improves population-level reproducibility	Time-lapse localization analysis
Granule inheritance fidelity	Consistency of granule partitioning during cell division	Pre-division positioning; anchoring mechanisms	Ensures comparable storage capacity in daughter cells	Reduces divergence of polymer phenotype across generations	Inheritance symmetry metrics
Turnover history	Integrated record of synthesis and depolymerization events within granules	Regulation of depolymerase activity; stress-response signaling	Drives Mw/Mn drift, end-group variability, and crystallinity changes	Uncontrolled turnover decreases reproducibility	Time-resolved Mw/Mn; monomer profiles
Dynamic regulatory state	Temporal commitment to storage versus mobilization	Nutrient imbalance; stringent response; sigma-factor hierarchies	Determines steady-state versus cycling polymer phenotypes	Regulatory instability propagates polymer variability	Phase-resolved sampling; regulatory markers
Cell-to-cell granule heterogeneity	Distribution width of granule states within a population	Regulatory noise; asymmetric inheritance	Broadens polymer phenotype distributions	Major source of irreproducibility across replicates	Single-Cell Variance (CV, dispersion indices)

Note: Granule-state determinants function as intermediate physiological variables linking cellular regulation to polymer phenotype. Alteration of any determinant can substantially modify polymer quality without changing total polymer accumulation metrics (%CDW or titer). Consequently, the reproducibility of polymer properties depends primarily on the reproducibility of the granule state rather than on cultivation conditions alone

This reframing also shifts the burden of reproducibility from post hoc standardization to upstream experimental design. Rather than attempting to correct variability through tighter analytical control alone, researchers must consider how to stabilize granule state through controlled regulatory gating. Strategies such as phase-resolved sampling, synchronization of storage induction, or explicit manipulation of regulatory networks may prove more effective for achieving reproducible polymer phenotypes than incremental refinement of extraction or measurement techniques.

Importantly, acknowledging reproducibility as a biological property does not diminish the value of technical rigor. Instead, it complements technical control by identifying its limits. Analytical precision cannot compensate for biological variability arising from divergent granule states. Recognizing this distinction enables more realistic expectations for reproducibility and more targeted strategies for achieving it.

Finally, this perspective encourages a shift in how reproducibility is evaluated and reported. Rather than treating variability as an experimental failure, differences in polymer properties can be interpreted as informative reflections of underlying physiological diversity. When granule-state data are available, such variability becomes a source of insight rather than frustration, revealing how regulatory dynamics shape material outcomes.

In summary, the reproducibility of PHA polymer properties is fundamentally a biological phenomenon governed by the reproducibility of the granule state. Polymer phenotype emerges from regulated intracellular organization, and consistent material outcomes require stabilization of that organization. This reframing provides a coherent explanation for variability in the literature and offers a path toward more meaningful comparison and interpretation of PHA studies grounded in cellular physiology rather than purely technical metrics.

EVIDENCE SYNTHESIS FROM MANGROVE-ASSOCIATED BACTERIA

Culture-based evidence: what isolates do and do not tell us

Culture-based isolation of Polyhydroxyalkanoate (PHA)-producing bacteria from mangrove-associated environments has provided valuable insights into taxonomic diversity and biosynthetic potential. Numerous studies have reported PHA accumulation in isolates affiliated with genera such as *Cupriavidus*, *Pseudomonas*, *Halomonas*, *Bacillus*, and *Vibrio*, often emphasizing the apparent suitability of mangrove systems as reservoirs of PHA producers (Alongi 2014; Thatoi et al. 2020). Reviews focusing on production-oriented studies of *Pseudomonas* spp. further reinforce this perspective by highlighting high accumulation yields, substrate flexibility, and broad biosynthetic capacity under laboratory conditions (Mozejko-Ciesielska et al. 2019). However, while isolate-based studies are indispensable for mechanistic experimentation, they also impose interpretive constraints that must be explicitly acknowledged.

A primary limitation arises from cultivation regime choice, particularly the predominance of batch cultures in isolation-based studies. Batch cultivation is experimentally convenient and well suited for screening large numbers of isolates, but it conflates growth, storage induction, and the stationary phase into a single, temporally compressed trajectory. Under batch conditions, nutrient depletion, stress onset, and polymer accumulation occur simultaneously, making it difficult to disentangle regulatory commitment to storage from passive responses to growth arrest (Khanna and Srivastava 2005; Gutschmann et al. 2021). As a result, observed PHA accumulation may reflect transient physiological states rather than stable storage strategies.

In contrast, fed-batch or controlled continuous cultivation allows separation of growth and storage phases, enabling clearer attribution of regulatory gating and granule dynamics. Studies employing fed-batch regimes often reveal differences in granule number, size, and turnover that are not apparent in batch cultures, even when total polymer content is comparable. The relative scarcity of such approaches in mangrove-derived isolate studies limits inference about regulated storage behavior and granule-state reproducibility.

Endpoint bias represents a second major interpretive challenge. Many culture-based reports assess PHA accumulation at a single terminal time point, typically coinciding with the late stationary phase. While endpoint measurements are useful for confirming polymer presence, they obscure dynamic processes such as granule nucleation, maturation, and turnover. Without temporal resolution, it is impossible to determine whether the observed polymer represents stable storage, residual accumulation from earlier phases, or a balance between synthesis and degradation. Endpoint bias thus amplifies uncertainty when extrapolating isolate behavior to physiological interpretation.

This limitation is particularly relevant for mangrove-associated isolates, which are often inferred to possess inherently robust storage capabilities due to their environmental origin. Such inference risks environmental determinism by attributing polymer phenotype directly to habitat rather than to regulatory execution. As emphasized throughout this review, environmental context establishes selective pressures but does not dictate intracellular outcomes. Without granule-level or regulatory data, endpoint accumulation cannot be assumed to reflect adaptive storage competence.

The assumption that isolate identity guarantees polymer phenotype further complicates interpretation. Isolation confirms biosynthetic capacity-the presence of functional pha genes and the ability to accumulate polymer under at least one condition-but does not guarantee reproducible polymer quality or regulatory behavior across conditions. Isolates of the same species can display markedly different granule states depending on cultivation history, nutrient regime, and stress exposure. Conversely, taxonomically distinct isolates may converge on similar polymer phenotypes through shared regulatory strategies. Treating isolate identity as a proxy for polymer phenotype, therefore, obscures the mechanistic role of granule state (Mitra et al. 2022).

Despite these limitations, culture-based evidence remains essential when interpreted within appropriate bounds. Isolate studies provide the experimental tractability necessary to interrogate regulatory networks, manipulate granule-associated proteins, and validate mechanistic hypotheses generated from synthesis frameworks. However, their interpretive power depends on integration with granule-level observations and phase-resolved sampling rather than reliance on endpoint metrics alone.

Synthesizing culture-based reports from mangrove-associated environments highlights a consistent pattern: isolates demonstrate diverse biosynthetic potential, but polymer phenotype varies widely across studies, even for closely related taxa. This variability aligns with the regulatory-granule-phenotype framework proposed here, in which isolate capacity establishes possibility space, while regulatory execution and granule dynamics determine realized polymer outcomes. Recognizing this distinction protects interpretation from overgeneralization and aligns isolate-based evidence with broader cell-biological understanding.

In summary, culture-based isolation from mangrove-associated systems confirms that these environments harbor diverse PHA-capable bacteria. However, batch-dominated cultivation, endpoint bias, and overreliance on isolate

identity constrain physiological inference. Isolates reveal what bacteria can do under specific conditions, not what they will do reproducibly. Interpreted within a granule-centered regulatory framework, culture-based evidence provides foundational but incomplete insight into PHA physiology.

Metagenomic and gene-centric evidence: Potential vs expression

Metagenomic and gene-centric approaches have substantially expanded understanding of the distribution of Polyhydroxyalkanoate (PHA) biosynthetic potential in mangrove-associated microbial communities. Surveys of mangrove sediments consistently report widespread occurrence of phaC, phaA, phaB, and related genes across diverse bacterial taxa, suggesting that the capacity for PHA synthesis is phylogenetically and ecologically broad (Alongi 2014; Thatoi et al. 2020). Functional screening of environmental DNA has further revealed novel PHA synthases with unusually broad substrate specificity from unculturable mangrove-associated bacteria, underscoring the richness of latent biosynthetic potential beyond what is accessible through cultivation alone (Foong et al. 2018). However, the presence of biosynthetic genes does not equate to their coordinated expression or to regulated polymer accumulation.

The distinction between biosynthetic potential and regulatory execution is central to interpreting metagenomic evidence. Metagenomes capture genetic inventories aggregated across community members and time, providing insight into what microbes could do under permissive conditions. They do not reveal when, how, or to what extent genes are expressed within individual cells. Even in well-characterized model organisms such as *C. necator* H16, whose complete genome reveals a comprehensive repertoire of PHA biosynthetic, regulatory, and granule-associated genes, genomic information alone is insufficient to predict storage behavior or polymer phenotype without regulatory and organizational context (Little et al. 2019). Without transcriptional, translational, or granule-level data, gene-centric observations remain decoupled from physiological state and polymer phenotype (Rehm 2010).

This limitation is particularly salient for PHA metabolism, which is tightly regulated and context-dependent. pha gene expression is gated by global stress responses, nutrient status, and regulatory hierarchies that cannot be inferred from gene presence alone. Metagenomic detection of pha genes, therefore, establishes only the possibility of storage, not its execution. Communities rich in pha genes may exhibit little polymer accumulation if regulatory conditions do not favor storage commitment, while communities with lower apparent gene abundance may express storage phenotypes under appropriate stress regimes.

Table 6. Culture-based reports of PHA-producing bacteria isolated from mangrove-associated environments

Mangrove location (country)	Microorganism/source	Evidence class	PHA type reported	Quantitative metric reported	Notes on interpretation	Reference
Songkhla Lake and Ao Tap Lamu, Phang Nga (Thailand)	*Pseudomonas aeruginosa*, *Pseudomonas* sp., *Ralstonia* sp. (isolates)	Culture isolate (production)	mcl-PHA	PHA accumulation ≈ 35% CDW	Endpoint production metric under defined batch conditions; granule-level properties not reported	Sangkharak et al. (2020)
Vaza-Barris River mangrove, Sergipe (Brazil)	*Bacillus cereus* (isolate)	Culture isolate (production)	PHB	PHA accumulation ≈ 67% CDW	High accumulation reflects the induction regime; polymer quality and turnover are not assessed.	Guimarães et al. (2025)
Red Sea mangrove coasts (Saudi Arabia)	*Erythrobacter aquimaris*, *Tamlana crocina*, *Bacillus aquimaris*, *Halomonas halophila* (isolates)	Culture isolates (production)	PHB	PHA accumulation up to ≈ 73% CDW	Values are not directly comparable across isolates due to differing substrates and oxygen regimes	Mostafa et al. (2020a, b)
Vellar estuary, Parangipettai (India)	*Priestia flexa* (isolate)	Culture isolate (production)	PHB	PHA accumulation ≈ 80% CDW	High %CDW reported; granule number, size distribution, and regulatory state not analyzed.	Chathalingath et al. (2023)

Note: Quantitative values reported for culture isolates (%CDW, yield, or titer) represent endpoint measurements under specific laboratory conditions and should not be interpreted as intrinsic strain capacity. Metagenomic or gene-centric data (e.g., phaC abundance) indicate biosynthetic potential and are not comparable to culture-based production metrics. None of the studies listed provides systematic granule-state characterization (granule number, surface protein composition, inheritance), limiting mechanistic inference under the regulatory-granule framework

Gene-centric analyses also obscure granule-state dynamics. Even when biosynthetic genes are expressed, polymer phenotype depends on granule architecture, surface protein composition, and turnover history-features that are invisible to metagenomic sequencing. Consequently, correlations between pha gene abundance and polymer properties risk overinterpretation. Without integrating granule-level or expression data, such correlations conflate potential with outcome and may inadvertently reinforce environmental determinism. Similar limitations are evident in system-level metabolic modeling of PHA production in mixed microbial cultures, where flux-based models successfully predict yield distributions under defined constraints but remain unable to resolve polymer phenotype, regulatory execution, or intracellular storage organization, thereby treating PHA accumulation as a bulk metabolic output rather than a regulated cellular state (Dias et al. 2008). This concern reflects a broader methodological critique in microbial ecology, where 'omics'-derived inventories and model-based predictions are frequently misinterpreted as direct proxies for functional activity or ecological role, despite lacking resolution at the level of expression, regulation, and phenotype (Prosser 2015; Knight et al. 2018).

Recent metatranscriptomic studies provide partial resolution by linking gene presence to expression patterns under defined conditions. These studies demonstrate that pha gene transcription varies with nutrient limitation, redox state, and stress exposure, supporting the view that regulatory execution is conditional rather than constitutive (Tarazona et al. 2020; Che et al. 2025). However, even transcript abundance does not guarantee polymer accumulation or specific granule states, as post-transcriptional regulation and protein localization further modulate outcomes.

The interpretive challenge is compounded by community-level averaging. Metagenomic and metatranscriptomic data integrate signals across heterogeneous populations in which only a subset of cells may be storage competent at any given time. Polymer accumulation and granule dynamics occur at the single-cell level, whereas gene-centric data represent ensemble averages. This mismatch in scale limits the ability to infer polymer phenotype or reproducibility from community-level gene surveys.

Despite these constraints, metagenomic evidence remains valuable when framed appropriately. It delineates the ecological distribution of storage potential and identifies candidate taxa and pathways for further investigation. When combined with culture-based experiments, single-cell imaging, or granule-level analyses, gene-centric data can guide hypothesis generation and contextualize physiological findings. However, such integration is essential to avoid conflating genetic capacity with functional outcome.

In summary, metagenomic and gene-centric studies demonstrate that mangrove-associated microbial communities harbor widespread PHA biosynthetic potential. Yet potential alone does not determine polymer phenotype. Regulatory execution, granule-state dynamics, and cellular context mediate the translation from gene to polymer. Recognizing this distinction safeguards interpretation from overreach and reinforces the central argument of this review: polymer phenotype emerges from regulated intracellular organization rather than from gene presence alone.

Why mangrove systems are biologically informative but not deterministic

Mangrove ecosystems provide a biologically informative context for examining regulated intracellular storage systems, including Polyhydroxyalkanoate (PHA) metabolism. These environments are characterized by fluctuating salinity, dynamic redox gradients, and high organic matter availability, all of which impose recurrent physiological constraints on resident microbial communities (Alongi 2014; Thatoi et al. 2020). Such conditions favor regulatory flexibility and make mangrove-associated bacteria useful models for studying stress-mediated cellular responses. However, the informative value of mangrove systems lies in their capacity to expose regulatory mechanisms, not in their ability to shape polymer phenotype deterministically.

Interpreting mangrove origin as a causal driver of polymer properties risks environmental determinism. While environmental stress can trigger regulatory pathways that enable storage, it does not directly dictate intracellular outcomes. As demonstrated throughout this synthesis, stress signals must be interpreted by cellular regulatory networks and translated into specific granule states before polymer synthesis occurs. The same environmental conditions can therefore yield different polymer phenotypes depending on regulatory execution, timing, and granule dynamics. Attributing polymer properties directly to mangrove conditions bypasses this critical mediating layer.

Mangrove systems are best understood as stress-rich contexts that recurrently challenge microbial physiology, thereby increasing the likelihood that regulatory storage pathways are engaged. This makes them valuable sources of storage-capable bacteria and of physiological diversity relevant to PHA studies. However, the presence of stress does not guarantee uniform regulatory responses across taxa or even within populations. Variation in regulatory thresholds, network architecture, and cellular history ensures that responses remain contingent rather than predetermined.

The anti-deterministic stance adopted here aligns with broader perspectives in microbial ecology that emphasize context-dependent expression of functional traits. Just as gene presence does not guarantee expression, environmental exposure does not guarantee specific phenotypes. Mangrove-associated bacteria illustrate this principle by demonstrating wide variation in PHA accumulation and polymer properties under comparable environmental conditions, both in situ and in culture (Rehm 2010).

Recognizing mangroves as informative rather than deterministic also clarifies the scope of inference from mangrove-based studies. Observations derived from these

systems should be interpreted as evidence of regulatory capacity and flexibility, not as proof of environmentally encoded material properties. This distinction is essential for avoiding overgeneralization and for maintaining mechanistic rigor in cross-system comparisons.

In summary, mangrove ecosystems serve as valuable natural laboratories for studying stress-regulated PHA metabolism, offering insight into how bacteria manage intracellular storage under fluctuating constraints. Their value lies in revealing regulatory possibilities rather than imposing deterministic outcomes. By treating mangroves as stress contexts rather than causal drivers, this synthesis preserves focus on intracellular regulation and granule biology as the primary determinants of polymer phenotype.

TESTABLE PREDICTIONS, FALSIFIABILITY, AND EXPERIMENTAL ROADMAP

Explicit predictions derived from the regulatory-granule framework

A central strength of the regulatory-granule framework is its capacity to generate explicit, testable predictions that can be evaluated independently of strain identity or environmental origin. Rather than correlating stress conditions directly with polymer yield, the framework posits that granule state mediates polymer phenotype. This mediation yields falsifiable predictions at the level of granule organization, surface regulation, and turnover dynamics, which are summarized conceptually below.

P1. Identical stress regimes will not produce identical polymer phenotypes unless granule-state trajectories converge.

Under comparable nutrient limitation or salinity-redox cycling, polymer properties will diverge when granule number, size distribution, or surface protein composition differ. Convergence of polymer phenotype is predicted only when granule-state readouts-such as stabilized granule counts and consistent phasin coverage-also converge (Tsuge 2016).

P2. Manipulation of granule-associated proteins will alter polymer phenotype without changing total polymer content.

Perturbation of phasin abundance or regulatory proteins at the granule surface is predicted to modify molecular-weight distributions, turnover rates, and reproducibility, even when % cell dry weight remains constant. This prediction directly challenges yield-centric interpretations and is falsified if polymer quality remains invariant despite altered granule surface regulation (Jendrossek and Pfeiffer 2014).

P3. Granule positioning and nucleoid association will correlate with inheritance symmetry and polymer reproducibility.

Cells exhibiting regulated granule-nucleoid association are predicted to show more symmetric granule inheritance and reduced intergenerational variability in polymer phenotype. Disruption of positioning mechanisms should increase population-level heterogeneity without necessarily reducing average polymer accumulation (Wahl et al. 2012).

P4. Cycling stress regimes will generate polymers with signatures of regulatory memory.

Repeated transitions between storage and mobilization are predicted to generate polymers with distinct chain-length distributions and end-group patterns that reflect turnover history rather than cumulative stress alone (Grage et al. 2009). Experimental comparisons of acute versus chronic stress in indigenous cyanobacteria show that identical stress factors applied under different temporal regimes produce markedly different polyhydroxybutyrate accumulation patterns, demonstrating that storage outcomes depend on stress history (Samadhiya et al. 2023). Similar findings in psychrophilic Arctic bacteria indicate that polyhydroxyalkanoate accumulation enhances survival under repeated cold and nutrient stress rather than maximizing yield, supporting a reversible, stress-conditioned storage strategy consistent with regulatory-memory effects (Grzesiak et al. 2024).

P5. Environmental origin will predict regulatory flexibility but not polymer phenotype per se.

Mangrove-associated isolates are predicted to display broader regulatory response ranges and granule-state plasticity, but polymer phenotype will depend on experimental execution rather than habitat origin. Failure to observe this decoupling would falsify the framework by supporting environmental determinism (Alongi 2014; Thatoi et al. 2020).

These predictions emphasize that meaningful testing of PHA physiology requires granule-resolved measurements rather than endpoint polymer metrics alone. Importantly, each prediction is falsifiable: observation of invariant polymer phenotype despite divergent granule states, or consistent polymer outcomes under divergent regulatory execution, would directly challenge the framework. Conversely, validation across taxa and cultivation regimes would support granule state as a necessary mediator linking regulation to material outcome.

Experimental designs and validation logic

Validation of the regulatory-granule framework requires experimental designs that resolve intracellular dynamics rather than relying solely on endpoint measurements. Conventional production-oriented experiments, which typically sample cultures at late stationary phase and report aggregate polymer metrics, are poorly suited to test granule-mediated mechanisms. Instead, experiments must be structured to capture temporal transitions in regulatory state, granule organization, and polymer remodeling through phase-resolved sampling (Tsuge 2016).

Table 7. Mechanistic predictions of PHA granule surface regulation under mangrove-like salinity-redox cycling.

Prediction/hypothesis	Dynamic input variables (controlled)	Mechanistic rationale (granule-surface logic)	Falsification criterion	Minimum readouts to test (phase-resolved)	Key references
P1. Duty cycle sets a granule number-size trade-off: higher-frequency, shorter storage-permissive phases yield more, smaller granules (nucleation-dominant), while longer permissive phases yield fewer, larger granules (maturation-dominant).	Oxygen/redox waveform (period, amplitude, duty cycle); salinity waveform; carbon input held constant; limitation regime (N/P) held constant.	Granule nucleation and maturation are filtered by surface occupancy of GAPs (phasins and regulators). Increased switching frequency favors repeated nucleation events and stabilizing surface coats that prevent coalescence; longer permissive phases permit maturation and coalescence control toward larger inclusions.	At matched mean carbon input and matched growth limitation, granule number and size distributions do not shift systematically with duty cycle (no consistent trend across ≥3 duty cycles).	Single-cell granule imaging (counts/cell; size/volume distributions; localization); optional tagged-phasin abundance on granules.	(Wahl et al. 2012; Jendrossek and Pfeiffer 2014; Mezzina and Pettinari 2016; Choi et al. 2021)
P2. Cycling induces turnover pulses that broaden Mw/Mn even at similar endpoint %PHA: repeated permissive-restrictive transitions cause Mw drift and increased dispersity, despite comparable endpoint accumulation.	Redox switching with defined phase length; refeeding vs no refeeding; salinity co-cycling vs constant salinity; identical extraction/measurement protocol.	Granule-surface regulation gates depolymerase access and synthase re-engagement. Cycling increases episodes of partial mobilization and re-synthesis, encoding "polymer history" into chain-length distributions without necessarily changing endpoint %CDW.	Cycling vs steady controls show no reproducible change in Mw/Mn (or dispersity) after controlling for extraction chemistry, standards, solvent, and sampling time.	Time-resolved Mw/Mn (SEC/GPC) at ≥3 phases (pre-cycle baseline; mid-cycle; post-cycle); endpoint %PHA; monomer composition; granule imaging to distinguish nucleation vs turnover.	(Jendrossek and Pfeiffer 2014; Tsuge 2016; Pagliano et al. 2021; Mitra et al. 2022)
P3. Hysteresis (state memory) under reoxygenation/refeeding: forward and reverse transitions are path-dependent, so granule and polymer trajectories do not retrace the same route.	Step-up/step-down oxygen; step-up/step-down salinity; limitation relief (N/P restoration) with identical timing across replicates.	Regulatory sequestration at the granule surface (e.g., PhaR-phasin feedback; nucleoid-tether interfaces) creates persistence of surface composition and localization, generating non-symmetric entry vs exit dynamics (granule "state memory").	For the same perturbation magnitude, granule metrics and polymer metrics show superimposable trajectories in forward vs reverse transitions (no history dependence).	Multi-cycle phase-locked sampling; granule number/size/localization time series; Mw/Mn time series; optional targeted quantification of key GAPs (immunoblot/proteomics or tagged proteins).	(Galán et al. 2011; Pfeiffer and Jendrossek 2011; Wahl et al. 2012; Tarazona et al. 2020)
P4. Salinity-redox co-cycling amplifies population heterogeneity relative to single-input cycling by forcing allocation trade-offs (osmoadaptation vs storage).	Combined oscillatory salinity + oscillatory oxygen/redox; amplitude and frequency matrix; constant carbon input; matched mean growth limitation.	Osmoadaptation competes for carbon and reducing equivalents; co-cycling increases regulatory switching noise and variability in granule-surface composition across cells, expanding the distribution of granule states and	Variance (cell-to-cell dispersion) in granule metrics and polymer phenotypes under co-cycling is not higher than single-input cycling controls at matched mean limitation.	Single-cell granule metrics (variance/CV in number and size); population-level polymer phenotype panel (Mw/Mn, dispersity; monomers); at least one osmoadaptation proxy (compatible solutes or expression marker where feasible).	(Mezzina and Pettinari 2016; Thatoi et al. 2020; Mitra et al. 2022)

		downstream polymer phenotypes.			
P5. Surface-regulatory bottlenecks predict predictable "failure modes" of reproducibility: when GAP regulation is perturbed, reproducibility drops even if yield remains high.	Genetic/physiological perturbation of surface regulators (phasins; PhaR/PhaD; nucleoid-tether components); cycling vs steady controls; constant cultivation and extraction protocols.	Polymer reproducibility is a readout of granule-state reproducibility. Disrupting surface buffering (phasins) or regulatory feedback (PhaR/PhaD) destabilizes granule number/size and turnover, widening polymer distributions without necessarily lowering %PHA.	Perturbations that measurably disrupt granule organization do not increase dispersion in polymer phenotype (Mw/Mn and/or thermal metrics remain equally tight as wild-type controls).	Replicate-to-replicate variability (CV) in Mw/Mn and key thermal descriptors (Tm/Tg/crystallinity) plus granule imaging; include yield and %PHA to show decoupling from quality.	(Rehm 2010; Jendrossek and Pfeiffer 2014; Bresan and Jendrossek 2017; Tarazona et al. 2020)

Phase-resolved sampling entails collecting biological material at defined physiological stages, such as pre-storage growth, early storage commitment, mature storage, and mobilization. These phases can be operationally defined using nutrient availability, growth rate changes, or regulatory markers rather than arbitrary time points. By aligning sampling with physiological transitions, researchers can distinguish polymer synthesis driven by storage commitment from remodeling driven by turnover. This temporal resolution is essential for testing predictions related to hysteresis, regulatory memory, and granule-state stability (Grage et al. 2009).

Granule imaging should be integrated with polymer profiling to link intracellular organization with material outcomes. Imaging modalities such as transmission electron microscopy, cryo-electron microscopy, or label-free optical tomography provide complementary information on granule number, size distribution, and spatial positioning. When combined with polymer analyses-including molecular-weight distribution and thermal properties-these approaches enable direct correlation between granule state and polymer phenotype. Importantly, imaging and polymer profiling must be performed on matched samples from the same physiological phase to preserve interpretive coherence.

Minimal but decisive experiments are those that perturb a single granule-state determinant while holding other variables constant. For example, controlled modulation of phasin expression can alter granule surface composition without changing external stress conditions or overall polymer content. If polymer properties shift in response to this perturbation, the granule-mediated mechanism is supported; if not, it is falsified. Such targeted designs provide stronger mechanistic inference than complex multifactorial experiments that confound interpretation.

Similarly, comparative experiments using identical cultivation conditions but different phase histories can test the role of regulatory memory. Cultures exposed to sustained nutrient limitation versus cyclic limitation-repletion regimes can be sampled at equivalent polymer content yet different granule states. Differences in polymer phenotype under these conditions would support predictions regarding hysteresis and turnover-driven remodeling. Absence of such differences would challenge the framework.

Importantly, validation does not require comprehensive omics integration or large-scale screening. Focused experiments combining phase-resolved sampling with granule imaging and polymer profiling can yield decisive evidence with modest resource investment. This emphasis on parsimony enhances reproducibility and facilitates cross-laboratory comparison, addressing a persistent challenge in the PHA literature.

Finally, experimental logic must align with falsifiability. Designs should be structured such that failure to observe predicted granule-polymer relationships would clearly refute, rather than ambiguously weaken, the framework. Explicit articulation of expected outcomes and alternative explanations is therefore essential. By prioritizing granule-resolved, temporally structured experiments, researchers can rigorously evaluate whether granule state is indeed the necessary mediator linking regulation to polymer phenotype.

Limits of current evidence and conditions for falsification

Although the regulatory-granule framework provides a coherent mechanistic interpretation of Polyhydroxyalkanoate (PHA) metabolism, its validity is constrained by the current structure of available evidence. Most existing studies emphasize polymer accumulation or biosynthetic capacity, while only a limited subset explicitly resolves regulatory state, granule organization, and polymer history within the same experimental system. Consequently, much of the support for granule-mediated regulation remains inferential rather than demonstrably causal (Tsuge 2016).

A primary limitation is the scarcity of experiments that decouple external stress signals from intracellular granule state. In many studies, stress exposure, regulatory

activation, granule formation, and polymer phenotype change concurrently, making it difficult to identify necessary intermediates. Without controlled perturbations that selectively alter granule organization while holding upstream regulatory signals constant, alternative explanations-such as direct enzymatic control or metabolic flux effects-cannot be fully excluded (Jendrossek and Pfeiffer 2014). This evidentiary gap restricts the strength of causal claims.

The framework would be falsified under several clearly defined conditions. First, if polymer phenotype consistently changes in response to stress or nutrient imbalance while granule number, size distribution, surface protein composition, and turnover dynamics remain unchanged, the granule-state mediator hypothesis would be invalid. In such a scenario, polymer properties would need to be explained by regulation acting directly on polymerizing enzymes or metabolic precursors, independent of granule organization.

Second, falsification would occur if direct perturbation of granule-state determinants-such as phasin abundance, granule-nucleoid tethering, or controlled alteration of granule number-fails to produce corresponding changes in polymer phenotype. If polymer molecular-weight distribution, crystallinity, or reproducibility remain invariant despite demonstrable shifts in granule architecture, the proposed causal role of granule state would be undermined.

Third, the framework would be challenged if polymer reproducibility proves independent of granule-state reproducibility. If experiments demonstrate stable polymer phenotype across populations exhibiting high granule-state heterogeneity, this would contradict the central claim that granule organization constrains polymer consistency. Such evidence would imply that granule-level variability is biologically inconsequential for material outcomes.

Finally, falsification would be supported if temporal history proves irrelevant. If polymers extracted before and after defined storage-mobilization cycles exhibit indistinguishable molecular signatures despite documented differences in regulatory state, the concept of polymer history as encoded physiology would require rejection (Grage et al. 2009).

By articulating these falsification criteria explicitly, the framework avoids immunization against contradictory evidence. Instead, it defines the empirical conditions under which it must be revised or abandoned. This openness to refutation strengthens the framework's scientific value and aligns it with rigorous standards of mechanistic explanation rather than narrative coherence alone.

IMPLICATIONS FOR REPRODUCIBILITY AND TRANSLATION

Reproducibility in Polyhydroxyalkanoate (PHA) research has long been framed as a technical issue related to cultivation conditions, analytical protocols, or downstream processing. However, evidence synthesized from mechanistic, culture-based, and gene-centric studies shows that variability in polymer phenotype often persists even when such technical factors are rigorously controlled. Within a regulatory-granule framework, this inconsistency is better understood as a biological phenomenon arising from differences in intracellular organization rather than as experimental noise or methodological deficiency (Ackermann 2015).

Across studies, comparable PHA yields or similar percentages of cell dry weight frequently correspond to divergent molecular-weight distributions, crystallinity, and thermal properties. Such discrepancies are difficult to reconcile under models that treat polymer accumulation as a direct outcome of metabolic flux alone. Interpreting polymer phenotype as a downstream consequence of granule state provides a unifying explanation: unless regulatory gating and granule organization are reproduced, polymer properties are not expected to converge, even under nominally identical external conditions (Jendrossek and Pfeiffer 2014). Reproducibility should therefore be evaluated not only in terms of process inputs and outputs but also at the level of intracellular structure and regulatory history.

This perspective has immediate implications for cross-study comparison. Studies that report polymer metrics without information on granule number, size distribution, turnover dynamics, or regulatory state implicitly assume cellular equivalence. Such assumptions are rarely justified and likely underlie many apparent contradictions in the literature. Recognizing granule-state reproducibility as a prerequisite for polymer reproducibility offers a principled basis for explaining why results diverge across laboratories, strains, or cultivation regimes without invoking unmeasured environmental or genetic idiosyncrasies.

From a translational standpoint, this framework reframes key challenges in PHA biotechnology. Efforts to improve polymer quality, consistency, and process robustness have largely focused on strain selection, feedstock optimization-including increasing use of lignocellulosic and other renewable substrates-reactor control, and process scaling, often motivated by broader bioeconomy and sustainability goals and by the wide range of biomedical and industrial applications attributed to PHAs (Dietrich et al. 2017; Raza et al. 2018; Sathya et al. 2018; Estévez-Alonso et al. 2021; Ghosh et al. 2021; Vigneswari et al. 2021; Gautam et al. 2024). Classical fed-batch strategies under carbon-limited conditions, such as medium-chain-length PHA production in *P. putida* via controlled substrate feeding, have demonstrated that precise manipulation of external parameters can substantially enhance yield and productivity (Sun et al. 2007). More aggressive innovations, including open, unsterile, and continuous cultivation systems using halophilic producers, further show that industrial-scale PHA production can be achieved with reduced operational constraints and high volumetric productivity (Tan et al. 2011).

At the same time, advances in rational strain engineering increasingly emphasize that robust and reproducible PHA production requires coordinated manipulation of metabolic pathways, regulatory circuits, and intracellular organization, rather than optimization of

yield or recovery efficiency alone (Borrero-de Acuña and Poblete-Castro 2023). These insights highlight that process-level control is insufficient when biological reproducibility remains unconstrained.

These technological developments occur within a broader global context marked by escalating plastic waste accumulation and the limitations of conventional waste-management strategies, which have intensified demand for truly biodegradable and biologically derived polymers (Pilapitiya and Ratnayake 2024). Recent reviews of PHA-based alternatives to conventional plastics emphasize that scalability, performance consistency, and process controllability remain central challenges limiting wider industrial adoption, despite advances in microbial production and feedstock diversification (Hadri et al. 2025). Sustainability assessments further indicate that the environmental performance of bio-based polymers, including PHAs, is highly sensitive to assumptions about production consistency, material equivalence, and system boundaries, underscoring that variability in polymer quality and reproducibility undermines sustainability claims rather than merely affecting economic efficiency (Hottle et al. 2013). Consistent with this view, system-level analyses show that large-scale transitions toward bio-based plastic packaging can generate complex trade-offs between climate mitigation and biodiversity conservation when biological production stages are insufficiently constrained. Together, these findings position reproducibility as a central bottleneck for industrial translation rather than a secondary technical concern (Mahato et al. 2023).

Downstream processing introduces an additional and often underappreciated source of variability. Comparative analyses of extraction methods from mixed microbial cultures demonstrate that solvent choice, thermal exposure, and recovery conditions can substantially alter molecular-weight distributions and polymer integrity, even when upstream cultivation metrics are comparable (Samorì et al. 2015). In parallel, extensive efforts to chemically or post-synthetically modify PHAs have expanded their application space by tailoring flexibility, hydrophobicity, and functional groups (Hazer and Steinbüchel 2007). While such approaches remain valuable for customization, they operate largely independent of the biological processes governing polymer formation and therefore cannot resolve variability originating from unstable granule organization or inconsistent regulatory execution. Engineering strategies that explicitly target granule-associated proteins, nucleation-maturation balance, or turnover regulation offer a more direct route to stabilizing polymer phenotype (Mezzina and Pettinari 2016).

Importantly, granule biology should not be viewed as an obstacle to translation but as an underutilized control layer. Treating granule state as an engineerable intermediate variable enables polymer quality to be decoupled from fluctuating cultivation conditions and aligns with broader trends in synthetic and systems biology that emphasize intracellular organization as a determinant of functional output (Greening and Lithgow 2020).

In summary, reproducibility and translational reliability in PHA research are inseparable from cell biology. Polymer phenotype cannot be expected to reproduce unless the regulatory and structural states that generate it are also reproduced. By foregrounding granule-state control, the regulatory-granule framework provides a biologically grounded foundation for more predictable, interpretable, and sustainable PHA biotechnological development.

CONCLUDING REMARKS

This review advances a cell-biological reinterpretation of Polyhydroxyalkanoate (PHA) accumulation by framing PHA granules as regulated intracellular organelles rather than passive repositories of excess carbon. By integrating mechanistic, culture-based, and gene-centric evidence, the synthesis demonstrates that polymer phenotype-encompassing molecular-weight distribution, heterogeneity, and reproducibility-emerges as a physiological output shaped by regulatory gating and granule state. A key contribution lies in explicitly distinguishing observation, inference, and causation, thereby resolving long-standing ambiguities in how PHA production data are interpreted. The regulatory-granule-phenotype framework highlights several core insights. Storage commitment is an active and reversible physiological decision embedded within global regulatory networks, not a simple overflow response. Granule-associated proteins and spatial organization act as critical intermediaries translating regulatory state into polymer outcomes. Consequently, polymer quality and reproducibility are best understood as biological properties dependent on granule-state stability rather than as technical artifacts of cultivation or extraction. This perspective explains why polymer quantity alone fails to predict polymer quality across studies.

Mangrove-associated bacteria are used as a biologically informative context to illustrate how conserved intracellular storage mechanisms operate under recurrent physiological constraints, without invoking environmental determinism. This framing keeps analytical focus on intracellular processes that are broadly applicable across bacterial systems. Looking ahead, the framework defines a clear research agenda emphasizing integrated analyses of regulatory state, granule organization, and temporally resolved polymer properties. Experimental designs that capture transitions into, within, and out of storage states will be essential for testing hypotheses related to regulatory memory and turnover-dependent remodeling. Treating granule state as an engineerable intermediate variable opens new opportunities to stabilize polymer properties through biological control, positioning PHA granules as a tractable model for understanding how cells encode material properties through regulated intracellular organization.

Future research should prioritize integrative experimental frameworks that simultaneously quantify regulatory states, granule architecture, and polymer characteristics, using standardized and comprehensive polymer analytics. Applying such approaches across controlled stress regimes and environmentally adapted

bacteria will be essential to test the regulatory-granule-phenotype framework and to improve predictive control of PHA production for biotechnological applications.

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