Control of Product Molecular Weight in the Enzymatic Synthesis of a Biodegradable Polyester, Polyhydroxybutyrate

by

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To my father
for pointing the way
and
Gabriella
for seeing me through
ACKNOWLEDGEMENTS

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ABSTRACT

Polyhydroxybutyrate (PHB) is a polymer accumulated by many bacterial species to store carbon and reducing equivalents. PHB is packaged intracellularly in inclusions termed “granules” that contain the polymer in an amorphous state, surrounded by a protein coat. The molecular weight ($M_W$) of PHB is typically in the range of $1-2 \times 10^6$ Da, and it shows the striking characteristic of low polydispersity, (narrow $M_W$ distribution), implying that PHB synthesizing organisms have a mechanism to control PHB $M_W$. Several approaches have been undertaken in this work to uncover this mechanism of $M_W$ control. An in vitro investigation was conducted, using purified PHA synthase, the native substrate (hydroxybutyryl-coenzyme A), and substrate analogs. The size of PHB synthesized in vitro was found to be dependent on the size of the substrate cofactor and that when hydroxybutyryl-N-acetylcysteamine was used as a substrate, the product was PHB covalently linked to N-acetylcysteamine. Preliminary experiments supported a model whereby substrate cofactor in thiolate form (e.g. $\overset{-}{S}$-CoA) participates in a chain transfer reaction at an internal PHB ester within the enzyme, and the accessibility of the site at which this reaction takes place changes during the course of polymerization. In an attempt to understand what this change was and how its timing was governed, a variety of in vivo studies were performed. Transcriptional profiling of the genes known to be involved in PHB biosynthesis was performed in order to identify candidates that may be involved in these processes, and phaP, known to be involved in granule structure formation was singled out for further investigation. Analysis of a PhaP overproducing C. necator strain revealed that it was indeed important for $M_W$ determination, which was underscored by observations in recombinant Escherichia coli harboring phaP in combination with the PHB synthesis genes. Additionally, it was found that levels of the PHB polymerizing enzyme (PHA synthase), PHB $M_W$ and granule formation were linked in recombinant E. coli. These results led to a model of a physical mechanism whereby the size of the elongating PHB chain governed accessibility to the site of the chain transfer reaction.

Thesis Advisor: Anthony J. Sinksey
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### Control of Product Molecular Weight in the Enzymatic Synthesis of a Biodegradable Polyester, Polyhydroxybutyrate

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Chapter 1: Polyhydroxyalkanoate (PHA) Homeostasis

1.1 Introduction. Polyhydroxyalkanoates (PHAs) are naturally occurring polyoxoesters of the form \((\text{COCH}_2\text{CHR} \text{CO})_n\) where \(R\) is typically an alkyl chain from 1 to 5 carbons and \(n \geq 2500\) (scheme 1.1) [1]. PHAs are found in a wide variety of bacterial species when grown under nutrient limitation where they serve as a storage form of carbon and reducing equivalents [2]. They are also transiently accumulated during exponential growth, although the metabolic basis for their production under these conditions has not been elucidated [3]. Research into the biology of PHAs in the last quarter century has been driven by the fact that PHAs are derived from renewable resources and can be made into biodegradable plastics with properties ranging from thermoplastics to elastomers [1]. In addition, PHA biology serves as a simple model system in which to study problems common to all nontemplate-dependent polymerizations, such as starch, glycogen, polyphosphate, or rubber biosynthesis [3]. An extensive review of PHA homeostasis has recently been published [3], so this review will focus on aspects of PHA biology relevant to this study.

![](image)

**Scheme 1.1**

1.2 PHA Homeostasis. Most research into the biology of PHAs has focused on either metabolic engineering to maximize their intracellular production in bacteria [4, 5] or plants [6], biochemical characterization of the enzymes responsible for PHA production [7, 8], or examination of extracellular PHA degradation [9]. More recent research tends to regard PHA biology as a cyclic process of synthesis and mobilization, involving multiple proteins throughout the PHA lifecycle [10-12]. PHA production begins with the synthesis of monomeric hydroxyacyl-Coenzyme A substrates from carbon metabolism intermediates. This aspect of PHA metabolism will not be reviewed here except to note that the precursor of the simplest and most abundant PHA, polyhydroxybutyrate (PHB), is synthesized in two steps from acetyl-CoA. Beta-ketothiolase (PhaA) catalyzes the condensation of two acetyl-CoA monomers to acetoacetyl-CoA, which is reduced to hydroxybutyryl-CoA (HB-CoA) by acetoacetyl-CoA reductase (PhaB). Polymerization of hydroxyacyl-CoA substrates is carried out by the PHA synthase enzyme. PHAs are stored in large intracellular inclusion bodies termed “granules,” and at least one class of proteins, the phasins, is known to influence granule properties. When carbon or reducing equivalents are required, PHAs are degraded by the actions of PHA depolymerases and oligomerases.

1.2.1 PHA synthase. PHA synthase carries out the final committed step of PHA synthesis, catalyzing the polymerization of hydroxyacyl-Coenzyme A substrates to high \(M_w\) PHAs. Purified PHA synthase can carry out this reaction \textit{in vitro} [13] and is the only enzyme that has shown to be required to perform the reaction \textit{in vivo} [14].
1.2.1.1 Occurrence of PHA synthases. PHA synthases have been identified in a broad range of both gram-positive and gram-negative bacteria, as well as in several archaea. A review in 2003 reported that 59 synthases had been cloned [7], but given the acceleration in sequencing of bacterial genomes and the development of PCR-based assays for cloning of PHA synthase structural genes [15], that number is certainly a gross underestimation of currently available sequences.

1.2.1.2 Classification of PHA synthases. Steinbuchel et al. described three classes of PHA synthases based on subunit composition and substrate specificity [16]. This has since been extended to four classes by McCool et al. [17]. Class I synthases are a single polypeptide (denoted PhaC) of ~65 kDa and were originally believed to be constrained to polymerization of monomers with short-chain length (scl) sidechains (HB- or hydroxyvaleryl-CoA). The Class II synthases have similar primary structures as members of Class I, but produce PHAs with longer sidechains (medium-chain length or MCL) (scheme 1.1, R=C_{3}H_{7} to C_{6}H_{13}) utilizing ≥C6 substrates. Class II synthases occur as two isozymes (denoted PhaC1 and PhaC2) and organisms containing Class II synthases typically encode both PhaC1 and PhaC2. Class III and IV synthases are both active as heterodimers of two polypeptide subunits and produce scl-PHAs [18, 19]. For both Class III and IV, one ~40 kDa subunit (PhaC) contains the active site while a second subunit (~40 kDa, denoted PhaE for Class III, and ~20 kDa, denoted PhaR for Class IV) is required for enzyme activity. Synthase genes are typically found in a pha locus containing other genes relevant to PHA homeostasis (figure 1.1). The best characterized synthases belong to Class I (PhaC_{Cn} from Cupriavidus necator) and Class III (PhaEC_{Av} from Allochromatium vinosum). Cupriavidus necator was previously known as Wautersia eutropha [20], Ralstonia eutropha[21] Alcaligenes eutrophus [22] and Hydrogenomonas eutrophus [23], while Allochromatium vinosum was previously known as Chromatium vinosum [24].

PhaC_{Cn} from C. necator has recently been shown to be able to utilize longer sidechain substrates both in vitro [25] and in vivo [26]. In addition, the PHA synthase from Aeromonas caviae readily polymerizes both scl and mcl PHAs even though it is classified as a Class I synthase based on sequence homology [27], and the Class III enzyme from Thiocapsa pfennigii also has a substrate range that incorporates both scl and mcl PHAs [28]. These enzymes draw into question the relevance of substrate specificity in the classification system; nevertheless, the substrate preferences of most synthases studied to date are accounted for by the current classification system.

1.2.1.3 Cofactor requirement in Class III and Class IV. Both Class III and Class IV synthases have a PhaC subunit of ~40 kDa (vs. 60-70 kDa for Class I and II) which contains the conserved cysteine, histidine, and aspartate residues that have been shown to be important for catalysis as described in section 1.3.2. In addition, Class III and IV synthases require a second subunit in addition to PhaC for high activity.

In the case of the Class III synthase, this subunit is denoted PhaE and is a ~40 kDa polypeptide with no significant homology to other available sequences. In all known cases, the coding region for phaE is found immediately upstream of phaC and is thought to be co-transcribed and co-translated (figure 1.1) [29], however, no studies have been performed on a Class III synthase isolated from its native host.

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When isolated from recombinant *E. coli*, the Class III synthase from *A. vinosum* is active as a 1:1 heterodimer of PhaE<sub>AV</sub> and PhaC<sub>AV</sub> [30]. It was initially reported that the PhaC<sub>AV</sub> subunit is completely inactive on its own [18], but subsequent investigation found low, but detectable, activity from isolated His-tagged PhaC<sub>AV</sub> [30]. Addition of purified His-PhaE<sub>AV</sub> was able to restore much of the catalytic activity; however, ten equivalents of PhaE<sub>AV</sub> had to be added, suggesting that most of the PhaE<sub>AV</sub> was either misfolded or inactive. Recently, chimeric enzymes were constructed using PhaE and PhaC from *Thiocapsa pfennigii* with PhaC and PhaE (respectively) from *A. vinosum*, and the chimeras were shown to produce wildtype levels of PHB *in vivo*, which was not surprising given the high degree of homology (85% identical for PhaC and 53% identical for PhaE) [28].

For the Class IV synthase, originally cloned from *Bacillus megaterium*, the second subunit is denoted PhaR<sub>Bm</sub> and is 22 kDa [17]. PhaR<sub>Bm</sub> has no apparent homology to PhaE<sub>AV</sub> (or other PhaEs) but it is worth noting that PhaEs are poorly conserved. The *Synechocystis* sp. PhaE, in particular, exhibits only 14.5, 17, and 13% identity, respectively, to the PhaE’s from *A. vinosum*, *Ectothiorhodospira shaposhnikovii*, and *Thiocystis violacea*, which is similar to the homology of PhaR<sub>Bm</sub> and the PhaE sequences (13-14%).

The first group to study a Class IV synthase found that in recombinant *B. megaterium* the subunits (PhaC<sub>Bm</sub> and PhaR<sub>Bm</sub>) were not present in a 1:1 stoichiometry and that the synthase was only active when isolated from PHA producing cells expressing both PhaC<sub>Bm</sub> and PhaR<sub>Bm</sub>, leading the authors to claim that active synthase could not be reconstituted *in vitro* from the two polypeptides [17]. However, the authors point out that the former finding (stoichiometry ≠ 1:1) may be an artifact of their sample preparation. The interpretation of the latter finding (no activity *in vitro*) is suspect given their use of PhaC-gfp translational fusions and the findings of another group that looked at *E. coli* overexpressing PhaC<sub>Bsp</sub> and PhaR<sub>Bsp</sub> from *Bacillus* sp. INT005, which are highly homologous to the proteins from *B. megaterium* (72 and 47% identity, respectively) [19]. This second group found that addition of lysate from *E. coli* expressing PhaC<sub>Bsp</sub> to lysate from *E. coli* expressing PhaR<sub>Bsp</sub> produced a mixture with high PHA synthase activity, while each individual lysate had none. In addition, they found that when they purified His-PhaC<sub>Bsp</sub> from cells co-expressing His-PhaC<sub>Bsp</sub> and PhaR<sub>Bsp</sub>, an approximately equal amount of PhaR<sub>Bsp</sub> was copurified, similar to what is seen during the preparation of recombinant PhaEC<sub>AV</sub>. Thus the Class IV PhaR appears to be an orthologous replacement for the Class III PhaE, differing primarily in genetic organization and size.

1.2.2 Intracellular organization of PHA. As mentioned above, PHAs are found as large hydrophobic inclusion bodies termed “granules” (figure 1.3). The size and number of granules vary by strain, but in *C. necator*, transmission electron microscopy (TEM) has shown that granules are typically ~0.5 μm in diameter, with 8-12 granules per cell produced under nutrient limited conditions [31]. During growth on rich medium, fewer granules are observed [32].

The early stages of PHA granule formation have been proposed to involve the self-assembly of micelles formed from the amphiphilic PHA/PHA synthase complex (figure 1.2) [33]. In this model, (I) soluble synthase (II) begins to polymerize
hydroxyacyl-CoA, extruding a hydrophobic PHA chain. (III) As free PHA/PHA synthase complexes come into contact, the hydrophobic tails associate, forming a micelle structure. (IV) These micelles aggregate into nascent granules and recruit other proteins, such as the phasin protein described in section 1.2.2.1, to the granule surface. Additional models, including a membrane-budding model [8] and assembly on a protein scaffold [32] have also been proposed.

**1.2.2.1 Components of the granule surface.** Griebel et al. reported the first analysis of isolated PHB granules, from *Bacillus megaterium*, which they found to be 97.7% PHB, 1.9% protein and 0.4% lipid [34]. However, the granule purification protocol they utilized produced crystalline PHB which, as described below, suggests that some of the granule coating had been lost and consequently other groups have questioned these figures, particularly the lipid component [35].

SDS-PAGE of the granule associated proteins from *Rhodococcus ruber* identified a 15.5 kDa polypeptide (PhaPRb) as the major protein component of the granule [36], whose levels were correlated with PHB levels. Immunoelectron microscopy localized the protein to the granule surface in whole cells [37]. In *C. necator*, a 24 kDa (PhaPCn) protein was identified as the major granule associated protein and found to complement a PHB leaky mutant that produced low amounts of PHB [38]. As with the 15.5 kDa *R. ruber* protein, levels of PhaPCn were correlated with PHB and it was localized to the granule surface. Analysis of granule associated proteins from a wide variety of gram-positive and gram-negative strains subsequently revealed that small abundant granule associated proteins, dubbed phasins, are widespread [39].

Phasins are thought to act as emulsifiers, preventing the coalescence of granules [38]. This hypothesis was derived from the observation that disruption of the *phaPCn* locus in *C. necator* led to the production of one large granule per cell, while overexpression of PhaP caused the accumulation of many small granules (figure 1.3) [38]. Recently, Tian et al quantitated the amount of PhaP on the granule surface under nutrient rich conditions, by quantifying protein in a culture via immunoblotting and calculating the granule surface area in that culture volume by stereological analysis of TEM images and cell counting [11]. Relying on an assumption of a globular structure for PhaP, they calculated that 27-54% of the granule surface was covered by PhaP at the timepoint they examined. If the granule bound structure of PhaP is not globular, the actual coverage could be very different.

Recently, isolated PHB granules have been imaged using atomic force microscopy after a minimal purification protocol [40]. This analysis revealed several interesting surface features which will be described in detail. The authors noted the presence of 35 nm globular structures with a central pore, which are connected by a network of 4 nm thick linear structures. Treatment of these isolated granules with the detergent sodium dodecyl sulfate resulted in rapid disappearance of the surface structures noted above, followed by the apparent dissolution of a 4 nm thick boundary layer which overlaid a layer of parallel arrays with 7 nm spacing, which the authors hypothesize is PHB. The authors presented a model for the granule surface whereby the interior polymer is completely encapsulated by a 4 nm thick lipid monolayer, on top of which reside 35 nm wide polymerization/depolymerization centers, with a central pore that allows access to the granule across the lipid layer in a porin-like fashion. The linear
structures joining the 35 nm pore structures are proposed to be made of PhaP and to form a protein scaffolding system that the authors view as analogous to the cytoskeleton and that holds in place the polymerization depolymerization centers [40].

Other proteins are associated with the granule surface, including PHA synthase [33], intracellular PHA depolymerase [41], and regulatory proteins [42].

1.2.2.2 Amorphous nature of PHAs. Purified PHB readily forms lamellar crystals, and it was originally believed that PHB was in a crystalline state within the cell as well, because purified granules exhibited crystallinity [43]. However, whole-cell $^{13}$C-NMR experiments demonstrated that in C. necator, PHB is in an amorphous state [44], a finding that was subsequently confirmed by wide-angle X-ray diffraction [45]. Differential scanning calorimetry was used so show that the mcl-PHA produced by Pseudomonas putida is in an amorphous state [46], indicating that the observations in C. necator were neither unique to that organism or that polymer.

Given the propensity of purified PHAs to crystallize, a number of mechanisms have been proposed to account for the formation of amorphous granules in vivo, including the incorporation of water as a plasticizer [47] and a physical-kinetic model [48]. The former model was based on fourier transform infrared spectroscopic measurements of purified granules that showed the presence of water, and the further observation that removal of the water increased crystallization [47]. However, it was not demonstrated that water was actually inside the granule. The latter model relied on the assumption that newly synthesized PHB is amorphous and that individual particles will undergo crystallization at a rate dependent on the rate of spontaneous nucleation [48]. Sanders and coworkers calculated, based on known nucleation parameters of pure PHB, that for a granule with a diameter of 0.25 µm at 30°C, in the absence of any perturbation, the rate of nucleation will be $2.0 \times 10^{-11} \text{s}^{-1}$, or less than once in 1000 years. In addition, the same group was able to synthesize “biomimetic” granules that were amorphous, small, and coated with a detergent to prevent contact with nucleating agents which underwent no crystallization in the ten months between the experiment and publication of their findings [49]. Furthermore, Sanders et al. found that removal of the detergent coat resulted in rapid crystallization, which is presumably analogous to what occurs to native granules during purification.

1.2.2.3 Regulation of phasin expression. Phasin (PhaP$_{cn}$) is the most abundant protein in C. necator PHB-producing cells [38], and its levels are correlated with PHB production [50]. In Paracoccus denitrificans the phasin (PhaP$_{hn}$) is a 16 kDa protein which was identified as the most abundant protein on isolated PHB inclusions [42]. Sequence walking revealed another gene immediately downstream of phaP$_{hn}$, which was similar to genes in other PHB-producing bacteria and to stdC of Comamonas testosteroni, a steroid inducible transcription factor, and which was denoted phaR$_{hn}$ [42]. Maehara et al. observed lower levels of the PhaP$_{hn}$ protein in an E. coli XL1-Blue strain harboring a plasmid encoding both phaP$_{hn}$ and phaR$_{hn}$, and so they proposed that PhaR$_{hn}$ is a negative regulator of phaP$_{hn}$ transcription. Maehara and coworkers subsequently showed that PhaR$_{hn}$ bound to the promoters of phaP$_{hn}$ and phaR$_{hn}$ and that it repressed transcription of phaP$_{hn}$ in vitro [51]. They proposed a model (figure 1.4) whereby PhaR$_{hn}$ binds to its own promoter and the promoter of phaP$_{hn}$ in the absence of PHB (I),
but is titrated to the surface of the PHB granule upon PHB synthesis, allowing transcription of those two genes (II). As PhaP\textsubscript{prn} accumulates, it competes with PhaR\textsubscript{prn} for binding sites on the granule surface, resulting in PhaR\textsubscript{prn} binding at the two promoters and repression of the respective genes (III).

Meanwhile, York et al. provided a parallel line of evidence from \textit{C. necator} implicating PhaR\textsubscript{crn} in repression of \textit{phaP}_{crn} in a manner that depended on PHB. Immunoblotting was used with anti-PhaP\textsubscript{crn} antibodies and it was demonstrated that PhaP\textsubscript{crn} was at higher than wildtype levels in a \textit{C. necator} ΔphaAR\textsubscript{crn} strain and was undetectable in both a \textit{C. necator} ΔphaAC\textsubscript{crn} strain and in a strain containing a catalytically inactive synthase (PhaC\textsubscript{crn} C319A mutant) [52, 53]. In addition, York and coworkers showed by immunoblotting that PhaP protein was not present in \textit{E. coli} harboring both \textit{phaP}\textsubscript{crn} and \textit{phaR}\textsubscript{crn} coding regions, but that upon introduction of a plasmid encoding \textit{phaCAB}_{crn}, which allowed the strain to accumulate PHB, PhaP\textsubscript{crn} protein also accumulated, strongly suggesting that regulation of PhaP\textsubscript{crn} expression depended on PHB [53]. The evidence argues that PhaR\textsubscript{crn} is a PHB-responsive negative regulator of transcription from the \textit{phaP}_{crn} locus. Furthermore, York et al. found that PHB levels in \textit{C. necator} were reduced by deletion of either \textit{phaP}\textsubscript{crn} or \textit{phaR}\textsubscript{crn} and were increased in \textit{E. coli} by the addition of \textit{phaP}\textsubscript{crn} [50, 53], although the basis of this phenomenon is unknown.

1.2.3 Intracellular PHB depolymerases and oligomerases. PHAs serve as storage polymers during nutrient limitation, and so mechanisms must exist to utilize the PHAs upon cessation of limited conditions. Several putative intracellular depolymerases have been identified in \textit{C. necator}, and unraveling their functions will undoubtedly reveal a complex story.

The first PHB depolymerase was cloned from an \textit{E. coli} cosmid library of \textit{C. necator} chromosomal DNA by assaying cell lysates from the library for depolymerase activity against artificial amorphous PHB granules [41]. The protein, PhaZ\textsubscript{1}a (originally denoted PhaZ), was purified via a His-tag and showed low but detectable activity against artificial amorphous granules. Disruption of the gene produced a strain that accumulated more PHB in nutrient rich medium, but this strain was still capable of degrading PHB, suggesting the existence of other depolymerases. Two more putative depolymerases, \textit{phaZ}1\textsubscript{b} and \textit{phaZ}1\textsubscript{c} (originally denoted \textit{phaZ}2 and \textit{phaZ}3) were cloned using a degenerate PCR strategy and of these, evidence that PhaZ1b was a depolymerase was obtained by examination of a Δ\textit{phaZ}1\textsubscript{a} Δ\textit{phaZ}1\textsubscript{b} strain, which exhibited no PHB degradation under carbon limited cultivation [54]. Two additional \textit{phaZ}1\textsubscript{a} homologues (\textit{phaZ}1\textsubscript{d} and \textit{phaZ}1\textsubscript{e}, originally \textit{phaZ}4 and \textit{phaZ}5) have also been identified by sequence homology, but no evidence has been presented as to their function [55, 56]. The catalytic triad of PhaZ1a was identified by site-directed mutagenesis and the active residues aligned with the catalytic triad from the \textit{A. vinosum} synthase, suggesting that the PHB synthases and depolymerases may share a common origin [57].

Abe et al. have recently cloned a novel putative PHB depolymerase from \textit{C. necator}, denoted PhaZd, based on its homology to an extracellular depolymerase from \textit{Ralstonia pickettii} [58]. The gene product was found to be expressed in PHB producing cells and localized to both the granules and the cytosol, but was not found to be excreted from the cell. This depolymerase was shown to have > 100-fold greater activity towards
artificial amorphous granules than PhaZ1a, and it also had low activity towards oligomers of hydroxybutyrate. Even so, deletion of this gene had little effect on the cells’ ability to degrade PHB, as the strain only accumulated slightly more PHB than wildtype and was still capable of PHB degradation.

In addition to the depolymerases, two distinct oligomer hydrolases have been identified in \textit{C. necator}, so named because they exhibit a higher activity towards oligomers of hydroxybutyrate (HB₃,₅) than towards artificial amorphous granules. Saegusa and coworkers cloned \textit{phaZb} (also originally named \textit{phaZ2}, as \textit{phaZ1b}) by DNA hybridization to a fragment of the gene encoding an extracellular oligomer hydrolase from \textit{R. pickettii} and showed that the gene coded for a protein with the ability to hydrolyze trimers of hydroxybutyrate [59], although it also showed activity towards artificial amorphous granules. A double deletion mutant (\textit{ΔphaZ1a ΔphaZb}) was found to accumulate more PHB during growth on rich medium than the wildtype strain or either single deletion mutant, but PHB degradation was still observed [60]. Subsequently, Saegusa et al. cloned \textit{phaZc} based on its homology to an intracellular oligomer hydrolase from \textit{Acidovorax} sp. Strain SA1 [61]. It too was shown to contribute to PHB mobilization in a deletion mutant, but oddly, the double deletion mutant \textit{ΔphaZb ΔphaZc} accumulated less PHB than the single \textit{ΔphaZb} mutant.

What is emerging here is an extraordinarily complex picture of PHB mobilization in \textit{C. necator}. As many as eight putative depolymerases or oligomers have been identified to date, some with seemingly redundant functions. Examination of expression and localization of these gene products has so far yielded surprising results, that suggest that some of these enzymes perform their function during polymerization rather than degradation [10, 11, 58], while examination of deletion mutants has failed to yield a clear picture [41, 54, 58]. A detailed understanding of the roles played by these enzymes waits on the development of robust methods for biochemical characterization and analysis of \textit{in vivo} functions [3].

1.3 Enzymology of the PHA Synthase. Purification of soluble recombinant PHA synthase was first accomplished by Gerngross et al [62], by reengineering the 5' \textit{phaCn} sequence to contain a strong \textit{E. coli} ribosome binding site, placing this sequence downstream of the IPTG inducible \textit{trc} promoter, and using this construct to express PhaCn in \textit{E. coli} UT5600, which doesn’t produce the \textit{ompT} gene product, a membrane bound protease. In addition, the authors added 0.05% of the detergent Hecameg, which was found to substantially increase recovery and reduce elution volumes during ion exchange chromatography [62]. As discussed below, PhaCn purified in this manner exhibits a significant lag phase before reaching maximum turnover rate. In addition, the specific activity of purified PhaCn is highly variable, as reviewed in [25]. The Class III synthase, PhaECₐ, also exhibits multiphase kinetics, but in its case, the initial phase of substrate turnover is more rapid, followed by a slower phase [63].

The complicated behavior of these PHA synthases \textit{in vitro} has made it challenging to study the \textit{in vitro} kinetics of these enzymes and may be related to the multiple stages of PHB synthesis discussed below: initiation, elongation, and termination. Alternatively, the kinetic phases may be related to the apparent requirement for enzyme dimerization or the fact that the synthase is carrying out an interfacial catalysis on the
boundary layer of the PHB granule. Despite this complexity, however, a great deal has been learned through *in vitro* studies.

![Scheme 1.2](image)

1.3.1 Mechanism of initiation. Purified recombinant PHA synthase is able to catalyze the polymerization of PHA de novo using acyl-CoA substrates (scheme 1.2) [13, 64], demonstrating that the synthase is capable of carrying out an initiation reaction, but it is unclear whether or not the initiation reaction is the same as what occurs *in vivo*. Polymerization catalyzed by recombinant PhaC<sub>Cn</sub> exhibits a lag phase [62], which is not seen in synthase partially purified from *C. necator* [65], suggesting that synthase from the native host may be primed. NMR analysis of PHB from *C. necator* has shown that the hydroxy-terminal OH is present in substoichiometric proportion to the carboxy-terminal COOH in PHB synthesized just after the onset of a rapid accumulation phase, which also suggests that the PHB chain may begin with some priming molecule [66]. It has been found that the lag phase can be diminished *in vitro* through the addition of (HB)<sub>n</sub>-CoA, where n=2-4, or of a substrate analog, saturated trimer-CoA (sT-CoA), which consists of a CoA thioester of a trimer of hydroxybutyrate with the terminal OH replaced by H (scheme 1.3) [63]. Additionally, repeated rounds of addition of small amount of HB-CoA (350 monomer/enzyme) abolishes the lag phase [67] while greatly increasing specific activity [13].

![Scheme 1.3](image)

Dimerization of the synthase has been proposed to play a role in the proposed priming step [62]. Recombinant PhaC<sub>Cn</sub> is present as a mixture of monomer and dimer [63]. Addition of substrate or one of the *in vitro* priming molecules (HB<sub>n</sub>-CoA or sT-CoA), either of which reduces the lag phase, also shifts the equilibrium towards dimerization of the synthase [63]. Dimerization can also be driven by the addition of high levels (>50% v/v) of multihydroxyl compounds such as glycerol or fructose [67]. In these preparations, no lag phase is observed, although the peak turnover rate is lower. Isolated PhaC<sub>Cn</sub> dimer has higher activity than the monomeric enzyme, which is conceivably a result of eliminating the lag phase [63, 67].

The Class III synthase PhaEC<sub>E</sub> from *Ectothiorhodospira shaposhnikovii* also shows a lag phase, but the lag phase can be removed by high salt concentrations (1.5M) or by priming with HB-CoA [68]. The Class III synthase PhaEC<sub>A</sub>, which is highly homologous (PhaE subunit, 39% identical; PhaC subunit 68% identical) to the enzyme from *E. shaposhnikovii*, has not been reported to exhibit a lag phase.
1.3.2 Mechanism of elongation. Early experiments demonstrated that the elongation reaction involved a sulfhydryl [34]. Several residues are conserved across Class I and Class III synthases [16], three of which have been shown to be involved in the elongation reaction by use of site-directed mutagenesis as described below [30, 62, 69, 70]. Sequence alignment of a diverse set of PHA synthases revealed that only one cysteine was absolutely conserved, and that it was found in a lipase box motif [N-X-X-G-X-C-X-G-G] where the canonical serine was replaced by cysteine [18]. Mutation of this residue to alanine in PhaCn (C319) and PhaECv (C149) produced inactive enzyme [30, 62]. Of two conserved histidines, mutation of one (PhaCn H495, PhaECv H331) to glutamine produced inactive enzyme [69, 70], suggesting that this residue was responsible for activating the cysteine for nucleophilic attack on the HB-CoA thioester (figure 1.5). In addition to this catalytic dyad, a nucleophile is required to activate the 3′-hydroxyl group of the incoming hydroxybutyrate moiety for nucleophilic attack on the thioester of the acylated cysteine in the active site, a role proposed for a conserved aspartate (D480 in PhaCn, D302 in PhaECv) (figure 1.5) [69, 70]. This role for D302 is supported by the observation that mutation of this residue reduces enzyme activity by several orders of magnitude. A threading model of the A. vinosum PhaC subunit, constructed based on homology to the Pseudomonas cepacia lipase, places these three residues near the active site [69].

Further support for the proposed mechanism was derived from experiments involving sT-CoA. Incubation of PhaCn and PhaECv with 3H-sT-CoA followed by trypsin digestion and peptide analysis, showed that each enzyme was labeled with 3H at the catalytic cysteine [30, 63]. In PhaCn, ~0.5 equivalents of label were observed per enzyme, suggesting that there was one active site per enzyme, which would explain the requirement for dimerization during the priming step discussed above. Although the same was not observed for PhaECv, 0.5 equivalents of CoA were released in a burst upon incubation with sT-CoA, followed by a much slower phase of CoA release [70], suggesting that the enzyme was initially labeled with 0.5 equivalents but that subsequent hydrolysis released the sT, freeing up the enzyme to load another sT. The second phase of slow release was not observed with the D302N mutant, which is consistent with the proposed role of this residue described above [70].

A final piece of evidence for the active dimer hypothesis was derived from number average molecular weight (Mn) analysis of PHB formed in vitro from PhaECv-catalyzed polymerization of HB-CoA [68]. The authors extracted PHB produced at substrate to enzyme ratios varying from 5,000 to 20,000 and found that the observed Mn closely matched the Mn predicted based on the assumption of an active dimer and no termination of the polymerization reaction.

The requirement for dimerization led to the hypothesis that each synthase monomer contributes one cysteine residue to the active site (figure 1.5, Panel A) [69]. Elongation is initiated with the loading of hydroxybutyrate at each cysteine. Elongation proceeds as the 3′-hydroxyl on one hydroxybutyryl-enzyme intermediate is activated for nucleophilic attack on the second acyl-enzyme intermediate by the active site aspartate. The chain is transferred back via a transthioesterification reaction and a new hydroxybutyrate monomer is loaded. An alternative hypothesis was recently proposed in which HB-CoA is non-covalently bound at the active site and its hydroxyl group activated for nucleophilic attack at the acyl-enzyme thioester, producing a non-covalently

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bound acyl-CoA intermediate (figure 1.5, Panel B) [3]. This model is attractive because the lipase-based structure model does not seem to allow for the two active sites to come into close enough proximity to carry out the dimer based mechanism, however, no evidence for this non-covalent model has been presented.

1.3.3 Mechanism of Termination. A recent quantitative analysis of PHB accumulation in C. necator has calculated the stoichiometry of PHB chains to PHA synthase molecules to be 60:1 during growth on rich medium [11]. Additionally, it has been shown that the $M_W$ of PHB reaches a maximum during the early phase of PHB accumulation and either remains constant or decreases slightly throughout later stages of accumulation [71]. These data are generally interpreted as evidence that the PHA synthase is capable of terminating synthesis of one PHB chain and re-initiating synthesis of a new chain.

![Scheme 1.4](image)

Doi et al. proposed that chain termination occurs via a transesterification of the PHB chain from the active site cysteine to activated water (scheme 1.4) [71]. Other groups have proposed that hydroxybutyrate [72] or Coenzyme A [64] functions as the chain transfer agent in the chain termination reaction in vivo. A number of reports have been published which are interpreted as supporting the chain transfer model, where the production of low $M_W$ PHB in C. necator has been promoted by the addition of hydroxyl-containing compounds (e.g. glycerol, polyethylene glycol, etc.) [72, 73]. In some cases this compound is subsequently detected as a covalent modification on the carboxylic acid end of the polymer by NMR [66, 72, 74], although the same authors did not always observe that phenomenon [73]. Although these observations are consistent with the chain transfer model, it is also possible that the compounds become associated with the PHB granule during the long scale of fermentation and are undergoing transesterification at an internal PHB ester.

PhaC$_C^n$ may be capable of terminating the elongation reaction in vitro in a manner that depends on enzyme concentration. Gerngross and Martin [13] measured the $M_W$ of PHB produced during in vitro polymerization of HB-CoA by PhaC$_C^n$, and found that when the enzyme concentration was held constant, PHB $M_W$ remained constant as well if either the substrate concentration was varied or the reaction was stopped at different times. Each of these findings is consistent with termination and re-initiation occurring during the reaction. However, there were many fewer PHB chains produced than there were PHA synthase molecules present, so it is possible that each active synthase produced one chain of a fixed length, and that the percentage of active synthases varied with substrate concentration or time. In contrast, the authors did observe a reduction in $M_W$ when the enzyme concentration was increased.
There is also evidence that the Class III synthase PhaEC$_{Av}$ can carry out termination in vitro. Josses et al. showed that >10 PHB molecules were produced per PhaEC$_{Av}$ in an in vitro polymerization reaction, and that the $M_W$ of this polymer was independent of synthase concentration [64]. Furthermore, the PHB had a $M_W$ of 1.6 x 10$^6$, which is very close to the $M_W$ of PHB produced by PhaEC$_{Av}$ in recombinant E. coli.

In addition, Tian et al recently provided additional evidence that PhaEC$_{Av}$ can carry out termination using SDS-PAGE autoradiography of products from the polymerization of [1-14C]HB-CoA which will be described in detail [75]. The authors found that polymerization reactions at low substrate to enzyme ratios (S/E) produced several species that could be resolved by SDS-PAGE autoradiography, and showed that they could be chased into high apparent $M_W$ species, demonstrating the chemical competence of these species. Two species are of particular note: species I which had an apparent $M_W$ of ~40 kDa and species II with a higher, broad distribution of $M_W$. Tian and coworkers found that species II was produced at high levels at S/E = 45 when the polymerization reactions were quenched with Laemmli buffer immediately after the reaction completed (30 s), but that if the same reaction were allowed to incubate for several minutes, the intensity of species II decreased, and species I increased.

To demonstrate that species II was in fact becoming species I, they ran a reaction for 10 s with cold HB-CoA (S/E = 5) and then for 10 s with [1-14C]HB-CoA (S/E = 5); nearly all the product was species II, the higher $M_W$ product. The reaction was repeated, except the second incubation step was for 300 s. In this case, species II had nearly disappeared and species I was present at high levels. Quantitative analysis of the phosphorimage revealed that only ~20% of the radioactivity found in the first reaction was still present in the second reaction. In an earlier work, the authors presented evidence that species I is PhaC$_{Av}$ covalently modified with HB, where n = 3-10, based on the observation that during in vitro polymerization of [1-14C]HB-CoA with a PhaEC$_{Av}$ D302A mutant at low substrate to enzyme ratios, species I was the only product [76]. When the products from this reaction were trypsin digested and separated by HPLC, several fragments containing the catalytic cysteine (C149) were radiolabelled, and the fragments' mass was determined to be equal to the predicted peptide fragment plus 3-10 HB units [76]. These observations led them to propose that they were observing a termination reaction in the shift from species II to species I [75].

In order to account for the observation that the product of the termination was PhaC$_{Av}$-HB$_{3-10}$, the authors proposed the model shown in figure 1.6 [75]. The PHB chain is postulated to leave the enzyme through a “product exit channel” which is distinct from a “substrate entrance channel” through which substrate enters the active site. A nucleophile on the surface of the enzyme catalyzes a chain transfer reaction, leaving behind primed enzyme (figure 1.6, panel A). The chain is then hydrolyzed from the surface exposed residue (panel B) and the PHB chain is released from the enzyme (panel C). They also propose an alternative model (not shown) whereby the nucleophile catalyzes a chain transfer directly to water. Their model is based on the solved structure of the thioesterase domain of the surfactin synthase, which carries out the cyclization of a lipopeptide through formation of an ester bond using a $\beta$-hydroxy fatty acid [77].

1.4 PHA Biosynthesis in Recombinant Organisms. Metabolic engineering of both native PHA producers and E. coli has led to advances in PHA yield, reduction in
feedstock costs, and generation of novel PHAs, all of which are reviewed elsewhere [4, 78]. Results relating specifically to the $M_w$ and intracellular organization of the homopolymer PHB are discussed below.

1.4.1 $M_w$ of PHB Produced in Recombinant Organisms. A variety of factors have been shown to influence the molecular weight of PHB produced in vivo, including genetic factors (discussed below), media components (described in section 1.3.3) and pH [79, 80]. In C. necator it has been shown that changes to the PHA synthase can result in changes to PHB $M_w$. Libergesell et al. introduced plasmids encoding the synthases from several purple sulfur bacterial species into the C. necator strain PHB-4, which lacks a functional PHA synthase, and analyzed the polymer produced [81]. In three of the strains analyzed, harboring the synthase from Allochromatium vinosum, Thiocystis violacea, or Thioecaspse pffenigii, they found that the PHB produced had higher $M_w$ than that produced from PhaC$_{Cn}$. They were able to compare the polymer to that made in the native host for A. vinosum and T. violacea, and observed that the PHB $M_w$ was similar in both the native host and the respective recombinant C. necator strain, implying that $M_w$ was under control of the synthase. Recently, it has also been shown that mutation of the PhaC$_{Cn}$ synthase can cause changes to PHB $M_w$ [82, 83]. For example, Tsuge et al. showed that a single amino acid substitution at a conserved alanine (Ala510) in PhaC$_{Cn}$ was sufficient to dramatically alter PHB $M_w$ in C. necator [82], with up to a three-fold increase observed depending on the substitution.

One of the most striking observations regarding the $M_w$ of PHB produced in recombinant bacteria has been the production of PHB with ultra-high molecular weights in E. coli. This phenomenon was first noted by Sim et al. who observed that PHB $M_w$ was inversely correlated with synthase activity in E. coli DH5α harboring the synthase from Cupriavidus necator, PhaC$_{Cn}$ [84]. Through production of PHB in cells with very low synthase activity they were able to produce PHB with a $M_w$ of $2.5 \times 10^7$ Da, ~25 fold higher than what is typically seen in C. necator [85]. Kusaka et al. extended on this finding, showing that $M_w$ in E. coli was pH dependent, with the highest $M_w$s achieved at lower pH [86]. They hypothesized that E. coli is able to synthesize a chain transfer agent in a pH-dependent fashion, although they were not able to identify that compound. A recent kinetic study by this group showed that PHB $M_w$ reached an early maximum and subsequently remained constant [79], as is seen in C. necator. This last experiment in particular indicates that recombinant Class I synthase is capable of termination and re-initiation in E. coli.

Despite the fact that these findings were published eight years ago and several papers have been published using recombinant C. necator, no reports have been made in the literature of the production of ultrahigh $M_w$ PHB in that organism. In contrast, the only author who to my knowledge addresses the question found that modulation of PHA synthase activity had no effect on $M_w$ [87], although the PHA synthase in their recombinant strain was the Class I synthase from Aeromonas caviae rather than from C. necator itself. From this one may infer that some mechanism for $M_w$ control exists in C. necator that is absent in E. coli. The phasin protein (section 1.2.2.1) may be just such a factor, as it was shown that inclusion of the phasin gene from Paracoccus denitrificans in E. coli XL1-Blue affected the $M_w$ of PHB produced from the P. denitrificans synthase in...

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that heterologous system, however the authors did not investigate the mechanism further [42].

1.4.2 Intracellular organization of PHB in Recombinant Organisms. Limited information is available in the literature pertaining to the organization of PHB in recombinant C. necator or E. coli. As discussed in section 1.2.2.1, a C. necator strain lacking PhaPCn was shown to produce one large granule per cell, while a PhaPCn overexpressing strain produces more and smaller granules than the wildtype strain. Changes in granule size in C. necator were also observed by TEM in the work of Liebergesell et al described above [81], where it appeared that the strains that were producing the highest $M_w$ PHB (harboring the synthases from A. vinosum, T. violacea, and T. pfennigii) produced larger granules than the wildtype strain and the other strains examined.

A few reports have examined PHB organization by TEM in recombinant E. coli, particularly the strain XL1-Blue. Lee et al inaugurated these studies by an examination of the relative yields of PHB from several E. coli strains harboring a plasmid encoding the PHB biosynthetic operon from C. necator grown in LB 2% glucose flask cultures [88]. XL1-Blue was one of the highest yielding strains and was selected for further examination. TEM showed that the PHB was organized into large (~0.5 μm dia) granules by 22 h [88]. This was also shown to be the case in XL1-Blue producing ultra-high $M_w$ PHB ($6.6 \times 10^6$ Da), which was achieved by maintaining the pH at 6.0 [89]. The authors also showed that this PHB was in an amorphous state as is the case in C. necator as discussed in section 1.2.2.2. Another group found that using a different vector for expression of the PHB biosynthetic operon, only one granule per cell was produced, but the size of these granules is unknown as a scale bar was omitted from the images [90]. Interestingly, XL1-Blue harboring the PHB biosynthetic operon from Paracoccus denitrificans produces more and smaller granules than those observed by Lee et al, and these granules were shown by SDS-PAGE to bind large amounts of beta-lactamase [42].

The phasin protein has been shown to effect PHB organization in E. coli as well. Seo et al. recently used recombinant E. coli to study the effect of PhaP and PhaR on granule size and enzyme activity in E. coli [90]. They constructed E. coli strains harboring plasmids bearing combinations of phaCAB, phaP, and phaR from C. necator, and presented TEM images showing that more and smaller granules were produced in the presence of phaPCn. In these experiments, PHB accumulation was increased by the presence of both phaPCn and phaRCn, and to a lesser extent by either phaPCn or phaRCn alone, an observation previously made by York and coworkers in recombinant E. coli DH5α [53]. Seo et al extended on York et al’s work by showing that PHA synthase activity was unchanged by the presence of phaPCn or phaRCn [90]. Maehara and coworkers also used TEM to show that phasin promotes the production of more and smaller granules in XL1-Blue, using plasmids encoding the P. denitrificans PHB biosynthetic operon and phaPm [42], while Pieper-furst et al showed that the phasin from Rhodococcus ruber was able to induce granule formation in XL1-Blue harboring the C. necator PHB biosynthetic operon by electron microscopy [91].

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1.5 Scope of the current work. PHB and other PHAs have primarily been of interest as environmentally-friendly replacements for petroleum based plastics. Research into PHB has been driven by the need to engineer strains for economical production through development of cultivation techniques for inexpensive feedstocks, metabolic engineering for production of high-value polymers, or strain improvement for high yield [4, 5]. As such, some of the most interesting biological questions associated with PHB homeostasis have not been examined in detail. In the current work, I will focus on one of the problems I find most interesting, the mechanism of $M_w$ determination in the synthesis of PHB.

As noted above, many chains of PHB are polymerized per synthase during PHB production, implying that the polymerization undergoes several rounds of termination and re-initiation. Yet the elongation reaction does not terminate until the PHB is $> 1 \times 10^6$ Da. Therefore, mechanisms must exist both to terminate PHB synthesis and to control the timing of the termination reaction, in effect, sensing the length of the PHB chain. This thesis presents a detailed study of these interlinked phenomena.

In chapters 2 and 3, I focus on the termination mechanism through *in vitro* experiments using purified PhaECAv and various substrate analogs, leading to a model for the termination reaction presented in section 3.4.1. Chapter 4 presents an analysis of the transcription and expression of genes related to PHB biosynthesis in *C. necator* in an attempt to identify proteins that may play a role in the processes of $M_w$ determination and granule formation. In chapter 5, I investigate effects of genetic manipulations involving the phasin protein on PHB $M_w$ and granule size in *C. necator*. Finally in chapter 6, I explore PHB $M_w$ and granule formation in recombinant *E. coli*, and propose an explanation for the basis of $M_w$ determination.
1.6 Figures

**Class I:** *Cupriavidus necator*

```
phaC -> phaA -> phaB
```

**Class II:** *Pseudomonas oleovorans*

```
phaC1 -> phaZ -> phaC2 -> phaD
```

**Class III:** *Allochromatium vinosum*

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phaC <-> phaE -> phaA -> ORF4 -> phaP -> phaB
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**Class IV:** *Bacillus megaterium*

```
phaP <-> phaQ -> phaR -> phaB -> phaC
```

**Figure 1.1:** Genetic organization of PHA biosynthetic genes from four archetypal PHA producing strains. *phaA*: beta-ketothiolase; *phaB*: acetoacetyl-CoA reductase; *phaC*: PHA synthase; *phaD*: unknown function; *phaE*: PHA synthase coenzyme; *phaP*: phasin; *phaQ*: transcription factor; *phaR* (*B. megaterium*): PHA synthase coenzyme; *phaZ*: PHA depolymerase; ORF4*: unknown function.
Figure 1.2: Micelle model of granule initiation. (I) Synthase (orange) exists as soluble enzyme prior to initiation of polymerization. (II) Synthase polymerizes short stretches of PHB, producing hydrophobic tails (pink). (III) Hydrophobic tails associate, resulting in assembly of micelle. (IV) As polymerization proceeds, water is excluded, producing a dense granule (pink) that recruits phasins (green) and other proteins as it grows.
Figure 1.3: Granule phenotypes associated with PhaP in *C. necator*. (A) wildtype; (B) ΔphaP; (C) ΔphaR (PhaP overproducer). TEM images courtesy of Dr. Gregory York.
Figure 1.4: Model of PhaR mediated regulation of phaP transcription. Only phaP is shown, although it has been proposed that same mechanism applies tophaR [51]. (I) PhaR (red) binds the promoter of phaP, blocking transcription. (II) PhaR binds newly synthesized PHB (grey) allowing transcription of phaP, and PhaP (green) accumulates and binds to the granule surface. (III) All available binding sites on the granule are occupied, and PhaR binds again at the phaP promoter, blocking transcription.
Figure 1.5: Proposed mechanism of the PHA synthase initiation and elongation. (A) Two cysteine model. His activates Cys for attack on thioester of HB-CoA, resulting in loading of hydroxybutyrate (HB) at first Cys. S-CoA leaves the active site and HB-CoA enters, which is loaded at the second Cys. Asp activates the hydroxyl on second HB-Cys for nucleophilic attack at the thioester of the first HB-Cys, elongating the PHB chain. Cys thiolate then attacks at the thioester of the acyl-Cys, transferring the PHB chain. The next step of elongation begins as S-CoA leaves the active site and HB-CoA is loaded (not shown). (B) Non-covalent intermediate model. As in (A), but the role of the second Cys is played by non-covalently bound CoA.
Figure 1.6: Model for chain termination from Tian et al. Adapted from [75] (A) Surface exposed nucleophilic amino acid (:Nu) on PHA synthase (orange) catalyzes chain transfer reaction at internal PHB ester (B) PHB is hydrolyzed from nucleophilic residue and (C) dissociates from enzyme, leaving behind primed synthase. Only one synthase of putative dimer shown.
1.7 References


69. Jia, Y., Kappock, T.J., Frick, T., Sinskey, A.J., and Stubbe, J., Lipases provide a new mechanistic model for polyhydroxybutyrate (PHB) synthases:


Chapter 2: In vitro analysis of the chain termination reaction in the synthesis of poly-(R)-β-hydroxybutyrate by the Class III synthase from Allochromatium vinosum [1]

This work has previously been published.

2.1 Introduction. Polyhydroxyalkanoic acids (PHAs) are polyesters produced by a wide range of microorganisms as intracellular storage materials when limited for a nutrient other than carbon [2, 3]. Over 125 different constituents have been identified in PHAs produced under a variety of growth conditions in vivo [2, 4]. These biodegradable materials exhibit a range of properties from elastomers to thermoplastics [5]. In the past decade efforts have focused on generation of PHAs in a biorenewable source such as bacteria or plants [6]. More recently efforts have focused on identifying a specialty niche for these materials in tissue engineering or coatings and paints [7-9]. Understanding the chemistry and biology of PHA production in vitro and in vivo is an essential step in making these materials in a useful form, including controlling the molecular weight and polydispersity of the polymers.

PHA synthase catalyzes the formation of insoluble PHA from soluble (R)-3-hydroxyacyl-CoA substrates, and the product accumulates in the cytoplasm of cells in the form of insoluble inclusions or granules [3]. Four classes of PHA synthase have been identified, their classification being based on their subunit composition and substrate specificity [10, 11]. Class I and Class III synthases, share a substrate preference for HB-CoA and 3-hydroxyvaleryl-CoA, but the Class I synthase consists of a single ~65 kDa subunit (PhaC) while the Class III synthase is made of two subunits, each ~40 kDa (PhaC and PhaE, referred to subsequently as PhaEC) [10, 12]. The mechanism of elongation (scheme 2.1) in the prototypical Class I synthase PhaC_C from Cupriavidus necator (formerly Ralstonia eutropha [13]) and Class III synthase PhaE_C, from Allochromatium vinosum (formerly Chromatium vinosum [14]), both involve a covalent intermediate 2 where the synthase is acylated at an active site cysteine residue [15-17] and have been proposed to involve a non-covalent intermediate 1 [12]. Little is known about the mechanism of termination.

It has long been known that chain termination and re-initiation occurs in vivo in wildtype Cupriavidus necator [18] and we have recently performed a quantitative analysis showing that ~350 PHB molecules are synthesized per synthase when cells are cultured to produce high amounts of PHB [19]. The literature contains only one model for the mechanism of termination (scheme 2.2), in which a nucleophilic “chain transfer agent” attacks at the active site thioester of 2, thereby releasing PHB and terminating elongation [18]. Activated water [18, 20], CoA [21, 22] and hydroxybutyrate [23] have been proposed to act as agents to terminate the elongation reaction via chain transfer in vivo.
Scheme 2.1

Supporting evidence for this model has come from the observation that addition of alcohols (polyethylene glycol [24], glycerol [23], 1,3-propanediol [23] and others) to C. necator grown under PHB producing conditions results in PHB with a lower molecular weight ($M_W$) compared to PHB from fermentations lacking these compounds. Furthermore, the polymers produced in the presence of these alcohols have been isolated and characterized by NMR and shown to be attached covalently to the carboxy terminus of the PHB chain [23, 25, 26]. These alcohols are proposed to act as a more efficient chain transfer agents than the candidates for termination in vivo under normal growth conditions (e.g. water, hydroxybutyrate [18, 23]). An increased rate of termination by chain transfer accounts for the observed decrease in $M_W$.

Scheme 2.2

In contrast to the observations in the wildtype organism, when the C. necator Class I synthase is heterologously expressed in Escherichia coli, the $M_W$ of PHB depends on the PhaC$\text{C}_{\text{n}}$ activity [27]. In addition, enzyme concentration influences molecular weight in vitro [28]. These data suggest that this synthase is not capable of carrying out chain termination by itself, and that the chain transfer model needs to be reevaluated.

The Class III synthase, PhaEC$\text{A}_{\text{V}}$, has not been studied in vivo. In vitro, however, the $M_W$ of PHB produced from PhaEC$\text{A}_{\text{V}}$ has been reported to be independent of substrate to enzyme ratio, suggesting that this enzyme is capable of performing chain termination [12, 21, 29] and may be a good system in which to study the chain termination reaction.
In the present communication we report the use of a substrate analog (R)-3-hydroxybutyryl-N-acetylcysteamine (HB-NAC), in which the CoA moiety has been replaced by N-acetylcysteamine (NAC). As we recently reported [30], this analog is a substrate for both Class I and III synthases with turnover numbers 1/5000 and 1/100, respectively, of those observed with HB-CoA. Initial studies with PhaEC$_{AV}$ and HB-NAC on the termination process in vitro are presented.

2.2 Materials and Methods

2.2.1 Materials. PhaEC$_{AV}$ was isolated as previously described [17] and had a specific activity of 140 µmol/min/mg. HB-CoA and HB-NAC were prepared as described by Yuan et al. [30]. The concentration of the former was determined using ε$_{260nm}$ = 16.4 cm$^{-1}$ mM$^{-1}$ (pH 7) and the latter using ε$_{234nm}$ = 5.33 cm$^{-1}$ mM$^{-1}$ (pH 7).

2.2.2 In vitro PHB production from HB-NAC or HB-CoA and PhaEC$_{AV}$. All reactions were carried out at 30°C in 25 mM sodium phosphate (pH 7.8), 22 mM NaCl with variable concentrations of HB-NAC or HB-CoA (ranges are listed in table legends). The reaction was initiated by the addition of 1.0 µM PhaEC$_{AV}$. The substrate consumption was monitored using Ellman's reagent and the discontinuous assay method [17]. Under the assay conditions the synthase was shown to retain enzymatic activity after 16 h (data not shown).

2.2.3 Preparation of PHB from in vitro reactions for Mw analysis. When the reaction reached completion (6 h for HB-NAC, 10 min for HB-CoA), Proteinase K was added to remove PhaEC$_{AV}$ (40U per mg PhaEC$_{AV}$) and the reactions were incubated at 37°C for 16 h. Samples were frozen at -80°C and lyophilized. The residue was extracted with 2 ml CHCl$_3$ by refluxing for 16 h. CHCl$_3$ was removed with N$_2$, and the residue was redissolved in 2,2,2-trifluoroethanol (TFE) (Aldrich, St. Louis, MO) to give a concentration of 0.25 mg/ml for HB-CoA samples or 1.0 mg/ml for HB-NAC samples. Samples were then filtered through a 0.2 µm PTFE membrane ( Pall, East Hills, NY) and used for gel permeation chromatography (GPC).

2.2.4 Preparation of PHB from in vitro reactions for NMR. When the reaction was complete (6 h), the sample was centrifuged (5000 x g, 30 min) and the pellet was dried in vacuo. The pellet (6 mg PHB) was dissolved in 700 µl CDCl$_3$ (Cambridge Isotope Laboratories, Inc., Andover MA) by refluxing for 2 h, cooled and analyzed by NMR spectroscopy. Subsequent to the first analysis by NMR, the sample was repurified twice by precipitation with 3 volumes of cold methanol (-20°C). The precipitant was collected by centrifugation (5000 x g, 30 min) and dissolved in CDCl$_3$ (reflux 2 h). This repurified sample was then reanalyzed by NMR spectroscopy.

2.2.5 Molecular weight determination of PHB. Coupled multi-angle light scattering (LS) and GPC were used to determine the $M_w$ of PHB. LS was performed using a DAWN-EOS (λ = 690 nm) multi-angle laser photometer (Wyatt Technology Corporation, Santa Barbara, CA). GPC was carried out on a Knauer HPLC (Berlin, Germany) attached to PLgel 10 µm Mixed-B (for in vitro PHB samples) or 20 µm Mixed-
A (for PHB from *E. coli*) column (Polymer Laboratories, Amherst MA). A Wyatt Optilab DSP differential refractometer was used for quantitation of PHB (dn/dc = 0.144). TFE was used as a mobile phase at a flow rate of 1.0 ml/min and 100 µl were injected. Narrow molecular weight poly(methyl-methacrylate) (PMMA) (18.2 kDa) (cat. no. 602, Scientific Polymer Products, Ontario, NY) was used to normalize LS detector and other PMMA standards in the range of 20 – 2,000 kDa were used to confirm calibration (cat. no. STD-4) (dn/dc = 0.172). The calculation of molecular weight from light scattering was performed with ASTRA 4.9 software (Wyatt Technology) employing the Zimm formalism.

2.2.6 NMR end Group Analysis of PHB. PHB (6 mg) was isolated as described above from a polymerization reaction containing 7.5 mM HB-NAC and 1.0 µm PhaEC_Av and analyzed by NMR spectroscopy. \(^1\)H-NMR spectra were recorded at room temperature on a VARIAN Inova-500 spectrometer (Varian, Palo Alto, CA). Chemical shifts were determined relative to CHCl₃ (δ = 7.27 ppm): δ = 1.23 (d, 3H, J = 6.4 Hz, CH₃CHOH), 1.28 (d, 3H, J = 6.1 Hz, OCH(CH₃)CH₂), 1.98 (s, 3H, NHCOC₂H₃), 2.62 and 2.48 (dd, J₁ = 7.3 Hz, J₂ = 15.7 Hz, dd, J₁ = 5.7 Hz, J₂ = 15.7 Hz, 2H, OCH(CH₃)CH₂), 2.81, (dd, 2H, J₁ = 8.0 Hz, J₂ = 15.7 Hz, OCH(CH₃)CH₂COS), 3.05 (td, 2H, J₁ = 1.2 Hz, J₂ = 6.3 Hz, CH₂NH), 3.43 (qd, 2H, J₁ = 2.5 Hz, J₂ = 6.3 Hz, SCH₂CH₂), 4.19 (m, 1H, CH₃CHOHCH₂), 5.26 (sex, 1H, J = 6.1 Hz, OCH(CH₃)CH₂), 6.16 (m, 1H, NH).

2.2.7 Production of PHB in recombinant *Escherichia coli*. PHB was produced in *E. coli* DH5α (New England Biolabs, Beverly, MA) harboring the plasmid pAcT41 [31] (phaCABₙ operon in pUC18 (Invitrogen, Carlsbad, CA)) or pJOE7 which is identical to pAcT41, except the phaCₙ ORF has been precisely replaced by the phaECₙ ORFs. pJOE7 was constructed by ligating the 2.2 kb BamH₁/PstI fragment of pGY53 [32] into the 5.3 kb BamH₁/PstI fragment from pAcT41. For PHB production, a fresh transformant was picked into 5 ml LB ampicillin (100 µg/ml) and grown at 37ºC for 6 h. This preculture (1 ml) was used to inoculate duplicate cultures of 100 ml LB ampicillin + 2% glucose (w/v) either with or without 1 % (w/v) 1,3-propanediol in 500 ml baffled flasks. Cultures were grown 24 h at 37ºC with shaking at 140 rpm (Infors CH-4103 incubator, Bottmingen, Switzerland).

2.2.8 Isolation of PHB from *E. coli*. At 24 h, 10 ml of each culture (described above) were pelleted (5,000 x g, 5 min) in a test tube, resuspended in 10 ml cold (4ºC) distilled water, pelleted again (5,000 x g, 5 min), and resuspended in ~0.5 ml of residual supernatant. Samples were then frozen at ~80ºC and lyophilized. Samples were extracted in their original test tubes by refluxing with 5 ml CHCl₃ for 4 days with stirring, with solvent added as needed to replace solvent lost to evaporation. Samples were then brought up to 5 ml and transferred to a fresh vial where they were dried under N₂ and then redissolved in 5 ml TFE. Samples were diluted to 0.25 mg/ml and filtered through a 0.2 µm PTFE membrane (Pall, East Hills, NY). \(M_w\) analysis was carried out as described above.

2.3 Results
2.3.1 PhaEC_A produces PHB of low $M_W$ from HB-NAC. In vitro reactions were carried out varying the molar ratio of substrate to enzyme (S/E) from 1500 to 15,000 (table 2.1). PHB was extracted and $M_W$ was characterized by coupled gel permeation chromatography and multi-angle light scattering (GPC/LS). At all molar ratios of S/E above 1500 the $M_W$ was $\sim$75 kDa. Since all the HB-NAC was consumed and incorporated into PHB, termination or transfer must be occurring $\sim$20 times during the course of substrate consumption at S/E = 15,000.

2.3.2 $^1$H-NMR shows that PHB-NAC is produced from HB-NAC. Since the polymer was small (<1000 monomer units), previous results [33] suggested that the polymer ends could be resolved by $^1$H-NMR. At the hydroxy terminus we expected to see a multiplet at $\delta = 4.19$ for the indicated proton (CH$_3$CHOHCH$_2$), and comparison of its integration to the main chain protons could be used to determine $M_N$. We observed the expected multiplet at $\delta = 4.19$ (figure 2.1c) and integration of this peak relative to the internal PHB peaks (figure 2.1b, $\delta = 1.28, 2.48$ and 2.62, 5.26) indicated a degree of polymerization of $\sim$700, or $M_N$ of $\sim$6.1 x 10$^4$ Da, which agrees well with our observed $M_W$ of 7.34 x 10$^4$ Da.

In addition, we observed a set of resonances close to, but not identical to, the NAC moiety of HB-NAC (figure 2.1c, $\delta = 1.98, 3.05, 3.43, 6.16$). These resonances were present in stoichiometric proportions to the peak at $\delta = 4.19$. After repurification of the sample and repetition of the experiment failed to alter the peak ratios, we concluded that we had synthesized PHB covalently modified with NAC (PHB-NAC). We ruled out contamination with HB-NAC by spiking the sample with HB-NAC, which produced resonances that did not overlap with those assigned to PHB-NAC (data not shown).

2.3.3 Exogenous NAC does not promote chain termination in vitro. Since we had synthesized PHB-NAC instead of PHB, we reasoned that NAC in the thiolate form may be a more efficient chain transfer agent (nucleophile) than water, and thereby promote premature termination of the elongation reaction. To test this model, we carried out in vitro polymerizations using both HB-CoA and HB-NAC substrates with and without added NAC. Little or no change was observed in the $M_W$’s of the PHB derived from either substrate in the presence of NAC (table 2.2 Reactions A vs. B & D vs. E). This indicates that exogenous NAC does not act as a chain transfer agent.

2.3.4 1,3-propanediol does not promote chain termination in vitro, but does promote chain termination in E. coli. Madden et al. reported that adding 1 g/l 1,3-propanediol to fermentations of Cupriavidus necator produced a $\sim$50% reduction in PHB $M_W$ [23]. We performed in vitro polymerizations using HB-NAC or HB-CoA in the presence of 1,3 propanediol. As with exogenous NAC, little or no effect on $M_W$ was observed (table 2.2, reaction A vs. C & D vs. F).

None of the experiments in the literature showing the production of reduced $M_W$ PHB in vivo from fermentations in the presence of exogenous alcohols had been carried out with an organism containing a Class III synthase. Therefore, we performed the following control experiment. PHB was produced in recombinant Escherichia coli harboring the plasmid pAET41, which contains the phaCAB$_{Cn}$ operon from C. necator (encoding Class I PHA synthase, $\beta$-ketothiolase and reductase, respectively), or the plasmid pJOE7, which is identical to pAET41 except the phaC$_{Cn}$ ORF is precisely
replaced by the cotranscribed OFRs for phaeA and phaC. Cells were cultured in media with and without 1,3-propanediol and PHB was extracted and characterized by GPC/LS.

PHB produced in the presence of propanediol had a much lower $M_w$ in each strain (table 2.3). To our knowledge, this constitutes the first observation of this phenomenon in an organism that does not naturally produce PHB. We also performed an analogous experiment in wildtype C. necator and in a C. necator strain in which the Class I synthase PhaCCh was replaced by PhaECCh, and saw similar results (data not shown).

**2.3.5 HB-NAC promotes premature chain termination during heteropolymerization of HB-CoA and HB-NAC.** Finally, we carried out a heteropolymerization reaction using both HB-CoA and HB-NAC (table 2.2, reaction G). The rate of thiolate release was biphasic in these reactions, with a rapid phase followed by a slow phase (data not shown). The $M_w$ of PHB produced in this reaction was 430 kDa and showed a bimodal distribution (figure 2.2). The elution volume of the later eluting peak was close to the elution volume of the peak of PHB from a HB-NAC homopolymerization reaction, indicating a similar $M_w$. In contrast, the earlier eluting peak eluted between the elution volume of PHB produced from HB-CoA and HB-NAC homopolymers. The intermediate $M_w$ of the earlier peak is likely due to some incorporation of HB-NAC monomers that are able to promote premature termination during the fast phase of predominantly HB-CoA polymerization. The results of this mixed polymerization shows that only when delivered to the active site during polymerization is NAC (or HB-NAC) able to terminate the growing PHB chain.

**2.4 Discussion.** The mechanism of PHA polymerization has been shown to involve covalent catalysis [15, 17]. According to these models, a dimer of PHA synthase catalyzes both the initiation and elongation of PHB chains. Here we provide evidence that the Class III PHA synthase carries out a chain termination reaction and propose models for how this might occur.

In this report, we show that low $M_w$ PHB is produced from HB-NAC (table 2.1). As reviewed in the introduction, the prevailing model of chain termination involves transfer of the elongating PHB to a nucleophile (water [18], hydroxybutyrate [23] or CoA [21]) in the active site by attack at the thioester of 2, regenerating unmodified enzyme (scheme 2.2). We show that each PHB chain synthesized from HB-NAC ends in a NAC moiety (figure 2.1), so if chain transfer is occurring, it must be NAC that is the chain transfer agent. However, increasing the concentration of exogenous NAC or adding NAC to HB-CoA polymerizations has little or no effect on $M_w$ (table 2.2). $M_w$ is, however, altered in heteropolymerization reactions of HB-NAC and HB-CoA, and two populations of PHB chains are produced (table 2.2, figure 2.2). The early eluting population has an intermediate $M_w$ relative to PHB produced in homopolymerization reactions of each substrate. We believe that this intermediate $M_w$ population is produced during the early phase of polymerization, when all of the HB-CoA is consumed and that incorporation of some HB-NAC derived monomers during this phase increases the rate of chain termination.

HB-CoA and HB-NAC are both substrates for the synthase and consequently can function as competitive inhibitors of one another. The initial rates of substrate incorporation will be governed by the formula $v_0 = v_{max}[S]/(K_m' + [S])$, where $K_m' = (K_m$
\( \times (1 + [I]/K_i) \) and \( v_{\text{max}} = k_{\text{cat}}[E] \). Given a \( K_m \) and \( k_{\text{cat}} \) of 0.13 mM and 3920 min\(^{-1}\) for HB-CoA and 8.6 mM and 39 min\(^{-1}\) for HB-NAC and [PhaEC\(_{Av}\) dimer] = 0.5 \( \mu \)M, \( v_0 \) for each substrate will be 1960 \( \mu \)M/min for HB-CoA and 0.29 \( \mu \)M/min for HB-NAC, a 6700-fold difference. As the concentration of HB-CoA declines, the rate of HB-NAC consumption will increase and the rate of HB-CoA consumption will decrease slightly so that the average difference in \( v_1 \) throughout the course of HB-CoA consumption will be \( \sim 3500 \)-fold. The \( M_w \) of the PHB fraction produced during this fast phase was not determined by LS since it overlaps with the later eluting fraction, but based on elution volume it can be estimated to be around 400 kDa, which corresponds to a degree of polymerization of 5000 or less depending on the sample’s polydispersity. If we are correct that HB-NAC incorporation is responsible for the observed premature termination, then it is extraordinarily efficient as a terminator in this reaction. Yet, on its own, HB-NAC will produce polymer that contains an average of 700 monomers. This implies there is a change in the system during polymerization of HB-NAC that allows termination to occur only after several hundred monomers have been incorporated. The existence of this change from a highly processive to a less processive state is also suggested by the fact that reports of PHB polydispersity in the literature are typically low. Understanding this process is a problem itself that we are currently investigating, and is distinct from determining the mechanisms of termination, which we address in this study.

Scheme 2.2 shows the prevailing model for the mechanism of chain termination. We recently reported observations consistent with an alternative mechanism of termination by chain transfer [29], distinct in that after chain transfer, the enzyme is left covalently linked to an HB oligomer at the active site cysteine. Performing SDS-PAGE and autoradiography on in vitro reactions of PhaEC\(_{Av}\) and \(^{14}\)C-HB-CoA at S/E = 45, we saw the time-dependent formation of a small uniformly labeled species well after the polymerization reaction had finished. This species appeared to be generated from a larger labeled species. This suggested a model similar to that of the polyketide synthases [34], in which chain transfer occurs, but at an internal ester bond within 2 rather than at the thioester linkage itself (scheme 2.3). Hydrolysis would thereby release most of the PHB chain, but leave behind enzyme primed with a short HB oligomer.

\[
\text{Scheme 2.3}
\]

If chain termination is in fact occurring by nucleophilic attack of NAC in the thiolate form at either the active site thioester or an internal PHB ester, it only occurs when NAC is released in the active site during polymerization of HB-NAC. Exogenous NAC, added to the reaction, is unable to promote termination. NAC exiting the active site after donating its HB moiety to the growing PHB chain is either in a favorable position to attack the active site thioester or it passes through an exit channel that is not solvent accessible and can attack the PHB chain during exit. However, both of these
explanations are at odds with the observation that molecules as large as PEG-1000 can influence PHB $M_w$ in vivo [24].

A third model to explain our results is based on a polymerization model that includes a non-covalent intermediate 1 (Scheme 2.4). In this model, substrate binds at the active site, and the hydroxyl is activated for nucleophilic attack on the PHB chain bound to the active site cysteine, transferring the chain to the non-covalently bound substrate. Either chain elongation continues as PHB is transferred back to the active site cysteine (scheme 2.1), or termination occurs as 1 is released from the active site (scheme 2.4). In this model, the $K_d$ of 1 affects the partitioning between the elongation and termination reactions, thereby influencing $M_w$. Chain length would be independent of substrate to enzyme ratio and independent of exogenous thiolate concentration in the reaction.

![Scheme 2.4](image)

Presumably, PHB-CoA (1) would have a higher $K_d$ than PHB-NAC (identical to 1, but CoA replaced by NAC), similar to the difference in $K_m$ of the respective substrates (HB-CoA $K_m = 0.14$ mM; HB-NAC $K_m = 8.6$ mM). The differences in $K_d$ would account for the $M_w$s observed in our work. In this model, every chain produced from HB-NAC would be modified with NAC as we showed. Additionally, reactions with mixed polymerizations would be expected to produce intermediate chain lengths, since addition of HB-NAC to a long, HB-CoA derived PHB chain would produce a non-covalent intermediate with a much higher $K_d$ and early chain termination.

None of the models discussed explain the observation that propanediol does not increase the rate of chain termination in vitro although it readily causes production of lower $M_w$ PHB from the Class III synthase in E. coli (tables 2.2 & 2.3). Since we were able to produce this $M_w$ reduction in E. coli, we are able to rule out the possibility that propanediol reduced $M_w$ in C. necator by activating a PHB depolymerase. Based on our in vitro findings, we believe that alcohols are not in fact involved in a chain termination reaction in vivo. Instead, we propose that the alcohols examined become associated with the PHB granule and undergo a transesterification reaction with PHB during the long time scale of fermentation, hydrolyzing the PHB chain. This would be consistent with observations of much higher polydispersities in alcohol supplemented media than are typically observed for PHB from enzymatic polymerization.

Currently, this disparity between in vivo and in vitro remains unexplained and the mechanism of termination is unclear. We are carrying out further experiments to distinguish among the models proposed. We believe that by probing this process in vitro we are gaining insights that may be obscured in in vivo experimentation and that the mechanism for chain termination will ultimately prove to be quite different from the model in the literature.
2.5 Tables and Figures

Table 2.1: $M_w$ of PHB from HB-NAC

<table>
<thead>
<tr>
<th>S/E$^a$</th>
<th>$M_w$ (Da)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1500</td>
<td>$5.84 \pm 0.12 \times 10^4$</td>
</tr>
<tr>
<td>3000</td>
<td>$7.40 \pm 0.01 \times 10^4$</td>
</tr>
<tr>
<td>6000</td>
<td>$7.97 \pm 0.04 \times 10^4$</td>
</tr>
<tr>
<td>12,000</td>
<td>$7.36 \pm 0.09 \times 10^4$</td>
</tr>
<tr>
<td>15,000$^c$</td>
<td>$7.34 \times 10^4$</td>
</tr>
</tbody>
</table>

$^a$All reactions carried out with 1 $\mu$M PhaEC$_{Av}$ and the appropriate concentration of HB-NAC. S/E = molar ratio of HB-NAC to PhaEC$_{Av}$ dimer. The PHB synthase has been shown to be active as a dimer of two PhaEC$_{Av}$ [15].

$^b$Results are from duplicate reactions, except$^c$.

$^c$Sample used for NMR analysis.

Table 2.2: PHB from mixed polymerizations.

<table>
<thead>
<tr>
<th>Reaction$^a$</th>
<th>Substrate</th>
<th>Addition</th>
<th>$M_w$ (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>6 mM HB-NAC</td>
<td>none</td>
<td>$6.87 \pm 0.13 \times 10^4$</td>
</tr>
<tr>
<td>B</td>
<td>6 mM HB-NAC</td>
<td>10 mM NAC</td>
<td>$6.44 \pm 0.10 \times 10^4$</td>
</tr>
<tr>
<td>C</td>
<td>6 mM HB-NAC</td>
<td>10 mM 1,3-propanediol</td>
<td>$6.85 \pm 0.07 \times 10^4$</td>
</tr>
<tr>
<td>D</td>
<td>7 mM HB-CoA</td>
<td>none</td>
<td>$1.71 \pm 0.06 \times 10^6$</td>
</tr>
<tr>
<td>E</td>
<td>7 mM HB-CoA</td>
<td>10 mM NAC</td>
<td>$1.69 \pm 0.02 \times 10^6$</td>
</tr>
<tr>
<td>F</td>
<td>7 mM HB-CoA</td>
<td>10 mM 1,3-propanediol</td>
<td>$1.76 \pm 0.04 \times 10^6$</td>
</tr>
<tr>
<td>G</td>
<td>7 mM HB-CoA + 7 mM HB-NAC</td>
<td>none</td>
<td>$4.31 \pm 0.40 \times 10^5$</td>
</tr>
</tbody>
</table>

$^a$All reactions carried out with 1$\mu$M PhaEC$_{Av}$. Results are from duplicate reactions.
Table 2.3: $M_w$ of PHB from *E. coli* DH5α harboring PHA biosynthetic genes

<table>
<thead>
<tr>
<th>1,3-Propanediol</th>
<th>pAeT41$^b$</th>
<th>pJOE7$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>$3.83 \pm 0.10 \times 10^6$</td>
<td>$1.72 \pm 0.04 \times 10^6$</td>
</tr>
<tr>
<td>1%</td>
<td>$6.04 \pm 0.66 \times 10^5$</td>
<td>$1.22 \pm 0.04 \times 10^5$</td>
</tr>
</tbody>
</table>

$^a$Cultivated in LB with 2% glucose and 100 µg/mL ampicillin.

$^b$Encodes *phaC*n, *phaA*n, and *phaB*n.

$^c$Encodes *phaEC*Av, *phaA*n, and *phaB*n.
Figure 2.1: NMR analysis of PHB-NAC. (a) Structure of PHB-NAC with protons labeled. (b) $^1$H-NMR spectrum of PHB-NAC. Numbers beneath scale indicate relative integrated areas of each peak. (c) Blowup of regions containing resonances for endgroup protons $a, j, h, g, b$, and $i$ ($\delta = 1.23$ (CH$_2$CHOH), 1.98 (NHCOC$H_3$), 3.05 (CH$_2$NH), 3.43 (SCH$_2$CH$_2$), 4.19 (CH$_3$CHOHCH$_2$), and 6.16 (NH), respectively). Numbers beneath scale indicate relative integrated areas of each peak.
Figure 2.2: GPC of PHB from *in vitro* reactions utilizing different substrates. HB-CoA (---), HB-NAC (----), and HB-CoA and HB-NAC (-----). Concentration axis of each sample was normalized to produce similar sized peaks.
2.6 References


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Chapter 3: Preliminary evaluation of chain termination models using additional substrates with the Class III synthase, PhaEC_{Av}

3.1 Introduction. In section 1.3.3, I described two models for the mechanism of chain termination during polyhydroxybutyrate (PHB) synthesis. The first, the “chain transfer model” that was put forth by Doi and others (section 1.3.3; scheme 1.4) [1-3], proposes that the elongation reaction is terminated when a nucleophile hydrolyzes the PHB from the active site thioester. Tian et al developed an alternative model to explain results described in section 1.3.3, where they observed that PhaC_{Av} was apparently modified by HB_n subsequent to termination [4]. If Tian et al’s interpretation of these results is correct, hydrolysis of the PHB chain must be occurring at an internal ester within the PHB chain, rather than at the thioester active site. Consequently, they proposed a model (“termination site model”) in which a catalytic nucleophile located at an active site for the termination reaction either within an exit channel, where the PHB chain is leaving the synthase, or on the synthase surface, carries out either a one-step or two-step transfer of the PHB chain to water (described in detail in section 1.3.3 & scheme 1.4) [4].

Either of these models would predict that slowing the rate of elongation would produce a lower \( M_w \) polymer, as observed in chapter 2 during the in vitro polymerization of the substrate analog, hydroxybutyryl-N-acetylcyesteamine (HB-NAC) (table 2.1) [5]. However, the chain transfer model is not sufficient to explain the observations I presented where only HB-NAC or N-acetylcyesteamine (NAC) delivered to the active site as a part of HB-NAC were able to promote chain termination, while the termination site model fails to account for the observation that PHB is covalently modified with NAC when HB-NAC is used a substrate.

In section 2.4, I presented two additional models in order to explain these new observations. In the first, (“non-covalent model”) a non-covalent intermediate is present in the elongation reaction and termination occurs when this intermediate dissociates from the active site. This model does not directly account for the observations of Tian and coworkers [4], but neither is it contradicted by those observations. In the second (“thiolate model”), the substrate cofactor (e.g. CoA or NAC) in the thiolate form can exit the active site through the exit channel proposed by Tian et al and hydrolyze the PHB chain at an internal ester, releasing PHB covalently modified with the cofactor from the synthase and leaving behind the HB_{n} modified PhaC_{Av} observed by Tian and coworkers. In this model, the difference in size of the PHB produced from HB-CoA and from HB-NAC could either be due to differences in the rate of elongation with no difference in the rate of termination (as in the chain transfer and termination site models), or due to the degree of steric hindrance that the thiolate cofactor must overcome in order to access the exit channel.

In this chapter, I describe initial experiments designed to discriminate between the models presented in section 2.4. These data appear to show that termination is also observed in vitro using the native substrate, HB-CoA (as has been previously reported), that there is no non-covalent intermediate during elongation, and that polymer \( M_w \) depends on the size of the thiolate cofactor. As such, these preliminary findings favor the thiolate model detailed in section 3.4.1, involving chain transfer to the cofactor thiolate at an internal PHB ester within the exit channel at a rate governed by the steric hindrance.
encountered by the thiolate. Additional support for this model could be obtained from the experiments described in section 7.2.

### 3.2 Materials and Methods

#### 3.2.1 Materials

PhaEC$_{AV}$ was isolated as previously described [6] and had a specific activity of 140 μmol/min/mg. HB-CoA and hydroxybutyryl-pantheine thioester (HB-Pant) were prepared as described by Yuan et al. [7]. The concentration of the former was determined using $\varepsilon_{260nm} = 16.4$ cm$^{-1}$ mM$^{-1}$ (pH 7). HB-Pant was a gift of Dr. Wei Yuan and was used as provided. Hydroxybutanol Coenzyme A thioether was a gift of Dr. Wei Yuan and its concentration was determined using $\varepsilon_{260nm} = 16.4$ cm$^{-1}$ mM$^{-1}$ (pH 7).

#### 3.2.2 In vitro PHB production from HB-CoA or HB-Pant and PhaEC$_{AV}$, purification and characterization of the reaction product

Reactions were carried out as described in section 2.2.2 [5], except that parallel samples were not prepared; however, very little difference was observed between parallel samples in chapter 2. Variable concentrations of substrate and enzyme were used as described in the text. PHB was purified and characterized by GPC/LS as described in chapter 4 [5].

### 3.2 Results

#### 3.2.1 Termination in vitro using HB-CoA as a substrate

In order to study termination in vitro with the native substrate, I needed to determine appropriate substrate and enzyme concentrations to observe termination. To that end, I performed in vitro polymerization reactions containing 5 mM HB-CoA and variable amounts of PhaEC$_{AV}$. As shown in table 3.1, $M_W$ increased as the substrate to enzyme (PhaEC$_{AV}$ dimer [8]) ratio was increased from 50,000 to 200,000 HB-CoA. In each reaction 100% of the substrate was turned over, and the mass observed in the refractive index detector was ~ 20 μg, indicating 80% recovery.

Our analytical system does not accurately measure number average molecular weight ($M_N$) which is required for calculating the stoichiometry of PHB chains:synthase, however, $M_N$ is by definition lower than $M_W$, so using $M_W$ to calculate the number of chains per synthase identifies a lower limit to the degree of termination and re-initiation. In the reaction at S/E = 200,000, that number is 3.7 chains of PHB per synthase dimer, indicating that termination and reinitiation takes place in vitro during HB-CoA polymerization.

#### 3.2.2 Hydroxybutanol-Coenzyme A thioether does not promote premature termination during the polymerization of HB-CoA

Hydroxybutanol-Coenzyme A thioether (Scheme 3.1, I) which contains a C3'-hydroxyl group, but no hydrolysable thioester linkage, is an inhibitor of the PHA synthase, reducing the turnover of HB-CoA by ~50% when it is present in a 1:1 stoichiometry with HB-CoA. If the non-covalent model proposed in section 2.4 is correct, and assuming that I acts through competitive inhibition, then introduction of this inhibitor should result in the generation of a non-covalent intermediate which stalls the elongation reaction, resulting in the accumulation
of non-covalent intermediates and the production of lower $M_W$ PHB, as outlined in Scheme 3.1.

![Scheme 3.1](image)

Reactions were carried out containing HB-CoA at 5 mM, PhaEC$_{AV}$ at 50 nM (S/E = 200,000) and I at either 0, 0.5 or 5.0 mM in the same manner as the reactions in 3.3.1. Reactions were complete within 4 h and 98, 92, and 64%, respectively, of the available HB-CoA was turned over, based on free thiol at 4 h. Recovery after GPC, measured as PHB mass observed by refractive index divided by mass expected based on turnover in each reaction, was ~80%, 75% and 100% respectively. As shown in table 3.2, all $M_W$s were near $5 \times 10^6$ Da, contrary to the prediction of the non-covalent model.

3.2.3 Termination *in vitro* using hydroxybutyryl-pantetheine thioester (HB-Pant) as a substrate. In chapter 2, chain termination was observed at a higher frequency *in vitro* using the substrate analog hydroxybutyryl-$N$-acetylcysteamine than was observed with HB-CoA, and I was interested in whether the rate of termination in general depended on substrate structure, or whether the HB-NAC results were an anomaly of that particular substrate. PhaEC$_{AV}$ had previously been determined to have a $k_{cat}$ and $K_m$ for the compound hydroxybutyryl-pantetheine thioester similar to its $k_{cat}$ and $K_m$ for HB-NAC [7], so the $M_W$ of PHB was investigated. As shown in table 3.3, the $M_W$ was intermediate to that produced from the other two substrates. It should be noted, however, that Yuan et al reported that this compound has limited solubility in aqueous buffer. If the rate of elongation using this substrate was limited by the rate of solubilization, then the $k_{cat}$ may have been understated in their report. As such, this experiment should ideally be repeated with an alternate substrate as described in section 7.3.

3.4 Discussion. During *in vitro* polymerization of HB-CoA by PhaEC$_{AV}$, termination and reinitiation was observed (table 3.1), repeating the findings of Jossek et al who carried out *in vitro* polymerization of HB-CoA with PhaEC$_{AV}$ and noted the formation of ~25 PHB chains per synthase. However, in the current work, an inverse correlation between $M_W$ and synthase concentration was observed. As the concentration of enzyme increased from 50 to 200 nM, the $M_W$ decreased from $4.6 \times 10^6$ Da to $2.8 \times 10^6$ Da. This is in contrast to Jossek and coworkers’ results [9]. They analyzed polymer produced under two conditions, using either 110 nM or 2.8 μM PhaEC$_{AV}$ with 13.4 mM HB-CoA (S/E = 240,000 and S/E = 9,000 respectively) and found that in each case the PHB $M_W$ was $1.6 \times 10^6$ Da. It seems possible that their results were an artifact of their analytical technique. The columns they used (Waters HR 3, HR 4, HR 5, and HR 6, in series) contain 5 μm beads, and I have observed a high degree of polymer shearing using a column with 10 μm beads (Polymer Labs Mixed-B), although we used different solvents (chloroform or 2,2,2-trifluoroethanol, respectively), so our techniques are not directly comparable. My results are reminiscent of what was observed *in vivo* by Sim et al. using the *C. necator*
synthase in *E. coli* where, as discussed in section 1.3.3, PHB *M*<sub>n</sub> was found to be inversely correlated with enzyme activity [10]. In chapter 6, I will show that the observation of Sim et al. can be extended to *E. coli* harboring PhaEC<sub>Av</sub>, lending further credence to the idea that there was an error in Jossek et al.’s measurements.

One model for the termination process requires a non-covalent intermediate during the elongation reaction (non-covalent termination model; scheme 2.4), so I performed the experiment outlined in scheme 3.1 that was designed to test for a non-covalent intermediate. Hydroxybutyryl-Coenzyme A thioether (scheme 3.1, I) inhibits the polymerization of HB-CoA, and based on its structure it is expected to function as a competitive inhibitor of HB-CoA. If there is a non-covalent intermediate during polymerization, I would be expected to load at the active site, attack at the thioester linkage of the acylated cysteine and form a non-covalent intermediate. Since the thioether would not be able to be hydrolyzed, the reaction would stop and this intermediate would either stay in the active site, inactivating the enzyme, or dissociate from the enzyme, producing low *M*<sub>n</sub> polymer. In either case, the acyl-I complex would be detectable by GPC/LS subsequent to proteinase K digestion of the enzyme, and it would be expected to be smaller than polymer produced in the absence of I.

Instead, the results in table 3.2 show no change in *M*<sub>n</sub> of polymer produced in the presence of I, even when it is present in quantities sufficient to reduce the polymerization rate by nearly 50% (table 3.2, 5 mM I). It is possible that either I functions as a non-competitive inhibitor, or that it is able to bind in the active site but that either the lack of the carbonyl prevents proper positioning of the 3′-hydroxyl group for attack at the acylated cysteine or the *pK*<sub>a</sub> of the hydroxyl group is sufficiently higher than that of the 3′-hydroxyl in HB-CoA to prevent its activation. Neither of these explanations seems particularly likely, however, particularly given the broad range substrates that PhaEC<sub>Av</sub> is able to polymerize [7]. It seems more likely that there is no non-covalent intermediate during the termination reaction. This experiment does not address the possibility that there is a non-covalent intermediate during initiation or the earliest stages of elongation, as very short chains (<~100 kDa) would not have been detected using this method.

As discussed in section 3.1, several of the potential models for chain termination imply that PHB *M*<sub>n</sub> should be correlated to elongation rate. The results in chapter 2 are consistent with this, in that during polymerization of hydroxybutyryl-N-acetylcycteamine (HB-NAC), the rate of elongation is slowed drastically and the *M*<sub>n</sub> of the PHB produced is very low. In order to test whether this was in fact what was taking place, I produced polymer *in vitro* from substrate for which PhaEC<sub>Av</sub> has a lower turnover number than it does for HB-NAC, namely hydroxybutyryl-pantetheine thioester (HB-Pant). As shown in table 3.3, the *M*<sub>n</sub> of polymer produced was in fact intermediate between that of polymer produced from HB-CoA and HB-NAC, providing evidence that the results in chapter 2 are not simply due to a reduced rate of elongation. It appears instead that there is a correlation between the size of the substrate cofactor (CoA, Pant, or NAC) and the size of the polymer produced (compare data in table 3.3 with the space filling models in figure 3.1).

### 3.4.1 Working model for the chain termination reaction in the enzymatic polymerization of PHB

These preliminary experiments point the way to a working model for termination which must contain the following. First, the polymer must be
covalently modified with the cofactor after termination (chapter 2). Second, termination must depend on cofactor size and not elongation rate (chapter 2 & 3). Third, there must be no non-covalent mechanism involved (chapter 3). Fourth, the termination reaction must leave behind a primed synthase, covalently modified by HBₙ, where n = 3-10, as observed in recent work by Tian et al. [4]. Fifth, the model must explain the efficiency of HB-NAC as a terminator in mixed HB-CoA HB-NAC polymerizations (chapter 2). Sixth and finally, it must explain the narrow polydispersity of enzymatically produced PHB (multiple sources including [11]).

The first four of these observations can be explained by a modification of the model proposed recently by Tian et al. [4], which is based on the solved structure for the thioesterase domain of the surfactin synthase [12]. In their model, as described in section 1.3.3 & figure 1.6, the elongating PHB is extruded from the enzyme active site through an exit channel which is separate from the entrance channel for substrate. Termination occurs when a nucleophilic residue, in the exit channel or on the surface of the enzyme, catalyzes a chain transfer reaction at an internal ester linkage on the PHB chain, leaving behind a primed synthase. The chain transfer reaction is either to the nucleophilic residue itself, from which it is subsequently hydrolyzed, or to water, either of which results in the ultimate release of the polymer chain.

The proposed modification to this model is that the cofactor itself functions as the chain transfer agent to terminate the elongation reaction. As illustrated in figure 3.1, the favored reaction after an individual elongation step would be for the cofactor in the thiolate form to dissociate from the active site through the entrance channel (Panel A). However, a low frequency of departure through the exit channel takes place, allowing access to the PHB chain (Panel B). The departing thiolate in the exit channel would be capable of catalyzing a transthioesterification reaction at an internal PHB ester, releasing the bulk PHB chain from the synthase (Panel C). Alternatively, there may be a residue within the exit channel which acts as a general base catalyst to activate the cofactor in the thiol form for nucleophilic attack on an internal PHB ester, in effect forming a second active site on the enzyme for the termination reaction.

This model accounts for the first through fourth observations above. The terminated chain is covalently modified with the thiol cofactor. The exit channel would function as a size-based selectivity filter, with smaller cofactors encountering less steric hindrance in accessing the termination site, and thus being able to carry out termination more frequently. In addition, the rate of termination would be independent of elongation rate because a termination reaction could only occur after an elongation step. No non-covalent intermediate is required. Hydrolysis of the PHB at an internal ester leaves behind an HBₙ primed synthase.

In the remainder of this work, I will attempt to develop a model to account for the fifth and sixth observations described above, regarding the efficiency of HB-NAC as a terminator during HB-CoA polymerization and the low polydispersity of PHB. Both these phenomena relate to the mechanism by which the timing of the termination reaction is controlled, i.e. the mechanism of PHB Mₘ control. In the following chapters, I will present observations on gene expression during batch fermentation of C. necator in an attempt to identify proteins which may be important for PHB Mₘ control, experiments relating manipulations of PhaP to PHB Mₘ in C. necator, and an analysis of PHB Mₘ in

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recombinant *E. coli* which will lead finally to a working model for the mechanism of $M_W$ control presented in sections 6.4.2 and 6.4.3.

### 3.5 Tables and Figures

**Table 3.1:** Results from *in vitro* polymerization of HB-CoA

<table>
<thead>
<tr>
<th>[PhaEC] (nM)</th>
<th>S/E $^b$</th>
<th>Theoretical $M_W$ (Da)$^c$</th>
<th>Observed $M_W$ (Da)</th>
<th>PHB chains per synthase $^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>50,000</td>
<td>$4.3 \times 10^6$</td>
<td>$2.8 \times 10^6$</td>
<td>1.5</td>
</tr>
<tr>
<td>100</td>
<td>100,000</td>
<td>$8.6 \times 10^6$</td>
<td>$3.6 \times 10^6$</td>
<td>2.4</td>
</tr>
<tr>
<td>50</td>
<td>200,000</td>
<td>$17.2 \times 10^6$</td>
<td>$4.6 \times 10^6$</td>
<td>3.7</td>
</tr>
</tbody>
</table>

$^a$Reactions contained 5 mM HB-CoA in 20 mM KPi, 22 mM NaCl, pH 7.8. $^b$S/E = molar ratio of HB-CoA to PhaEC$_{AV}$ dimer. The PHB synthase has been shown to be active as a dimer of two PhaEC$_{AV}$ [8]. $^c$Assuming polydispersity = 1 ($M_N = M_W$).

**Table 3.2:** Hydroxybutanol CoA thioether does not promote chain termination

<table>
<thead>
<tr>
<th>[Inhibitor] mM</th>
<th>$M_W$ (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>$4.7 \times 10^6$</td>
</tr>
<tr>
<td>0.5</td>
<td>$5.5 \times 10^6$</td>
</tr>
<tr>
<td>5</td>
<td>$5.2 \times 10^6$</td>
</tr>
</tbody>
</table>

$^a$Reactions contain 5 mM HB-CoA, 100 nM PhaEC$_{AV}$ and inhibitor in 20 mM KPi, 22 mM NaCl, pH 7.8

**Table 3.3:** $M_W$ of PHB does not correlate with rate of turnover of substrate

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$M_W$ (Da)</th>
<th>$k_{cat}$ (min$^{-1}$)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 mM HB-NAC$^c$</td>
<td>$6.9 \times 10^4$</td>
<td>49</td>
</tr>
<tr>
<td>6 mM HB-Pant</td>
<td>$3.0 \times 10^5$</td>
<td>19</td>
</tr>
<tr>
<td>7 mM HB-CoA$^c$</td>
<td>$1.7 \times 10^6$</td>
<td>&gt;2000</td>
</tr>
</tbody>
</table>

$^a$Reactions contain 1µM PhaEC$_{AV}$ and substrate in 20 mM KPi, 22 mM NaCl, pH 7.8. $^b$Yuan et al. [7]. $^c$table 2.2 [5].
Figure 3.1: Space filling models of cofactors. (A) Coenzyme A; (B) pantetheine; (C) N-acetylcysteamine. Drawn in ACD ChemSketch 8.0.
Figure 3.2: Model for chain termination. PHA synthase (orange) is shown immediately after carrying out elongation step, while cofactor in thiolate form (:Nu) is still in active site. Either (A) :Nu dissociates from active site through substrate entrance tunnel allowing new round of elongation, or (B) :Nu enters product exit channel and attacks PHB chain at internal ester linkage followed by (C) PHB:Nu dissociates from enzyme, leaving behind acylated synthase (m = 2-9).
3.6 References


Chapter 4: Transcriptional analysis of Cupriavidus necator genes related to poly-(R)-3-hydroxybutyrate homeostasis during batch fermentation [1]

This work has been previously published in: *Applied Microbiology and Biotechnology*, 2005. 68(5): p. 663-672, “Transcriptional analysis of Ralstonia eutropha* genes related to poly-(R)-3-hydroxybutyrate homeostasis during batch fermentation,” Adam G. Lawrence†, Joerg Schoenheit†, Aimin He, Jiamin Tian, Pinghua Liu, JoAnne Stubbe, and Anthony J. Sinskey. © Springer-verlag 2005. With kind permission of Springer Science and Business Media. *Cupriavidus necator* was formerly known as *Ralstonia eutropha* [2]. †These authors made equal contributions to this work.

4.1 Introduction. Polyhydroxyalkanoates (PHAs) are a class of polyesters accumulated by a wide variety of bacteria as storage media for carbon and reducing equivalents [3]. Widespread interest in investigating PHAs originally arose due to their biodegradable properties and due to the fact that they were derived from biological sources, and are potentially a renewable replacement for fossil-fuel based polymers [4]. The simplest naturally occurring PHA is poly-(R)-3-hydroxybutyrate (PHB). The gram-negative soil bacterium *Cupriavidus necator* can accumulate PHB up to 90% of total cell mass when limited for a nutrient other than a carbon source.

PHA metabolism is also of interest as a model system for studying a complex class of biological processes we term non-template polymerizations, that in general are poorly understood [5]. Starch, polyphosphate, and cyanophycin biosynthesis are other examples of these processes. These polymerizations involve a phase transition from soluble monomers to insoluble polymers and formation of structured inclusion bodies. In most cases, while the enzymes that carry out the polymerization reaction have long been known, and *in vitro* polymerization reactions demonstrated, the assembly of the structured inclusions *in vitro* has proved difficult or impossible.

In *C. necator*, there is phenotypic evidence for the involvement of eight gene products in PHB homeostasis. PHB is synthesized directly from acetyl-CoA by the enzymes β-ketothiolase (encoded by *phaA*), acetoacetyl-CoA reductase (encoded by *phaB*), and PHA synthase (encoded by *phaC*), which are thought to be co-transcribed [6-8]. Intracellular PHB is found in large (up to ~ 500 nm diameter) inclusion bodies termed “granules” [9], whose size and number *in vivo* is correlated with expression of the phasin protein (encoded by *phaP*). PhaR (encoded by *phaR*) has been shown to regulate accumulation of the phasin [10-12]. The gene products of *phaZ1a, phaZ1b, and phaZ2 (PhaZ1a, PhaZ1b and PhaZ2, respectively) have been shown to be involved in PHB degradation [13-15]. In addition, there are several genes that have recently been identified by sequence homology to either *phaP* or *phaZ1a* (*phaP2, phaP3, phaP4, phaZ1c, phaZ1d, and phaZ1e*) [14, 16]. Of these, the PhaP homologues have been shown to be expressed and localized to the granule, while nothing has been reported regarding PhaZ1d and PhaZ1e [16]. Unfortunately, these sequences (except for *phaZ1c*) were not available when we began our study. Note that an alternate system of nomenclature has been proposed where *phaZ1a, phaZ1b*, etc. are numbered *phaZ1, phaZ2*, etc. and our *phaZ2* is named *phaY* [17].
In vivo, synthase, phasin and depolymerase have all been shown to be localized on the surface of granules [13, 18, 19], and understanding how the synthesis and degradation of these proteins correlates with granule assembly and breakdown will be crucial to building accurate models of the dynamic granule architecture. In addition, several regulatory genes that affect transient and stress-related PHB metabolism have been identified in a variety of organisms (recently reviewed in [5]) including, among others, phbR in Azotobacter vinelandii [20], aniA in Rhizobium etli [21]. To date only phaR has been identified as an important regulatory gene in C. necator, but it seems likely that factors are missing based on the complexity of systems in other organisms.

In order to understand the complex process of PHB granule assembly, we have studied time-resolved PHB metabolism in C. necator in both complex medium and defined media that promote PHB production (high fructose, no ammonium) and PHB utilization (no carbon, high ammonium) [22, 23]. In the current work, we have examined both the wildtype strain and a phaC deletion strain during three stage cultivation (cell growth, PHB production, and PHB utilization) in defined media. We describe the results of reverse transcriptase quantitative PCR (RT-qPCR) analysis of mRNA transcripts from eight of the genes described above (phaA, phaB, phaC, phaP, phaR, phaZ1a, phaZ1b, and phaZ1c) and compare the results with quantitative Western blotting for PhaP and PhaZ1b.

4.2 Materials and Methods

4.2.1 Bacterial strains and cultivation conditions. These experiments were performed with Cupriavidus necator strains H16 (ATCC 17699), Re1034, Re1052, Re1097, Re1107, and Re1110 [11, 14]. C. necator pre-cultures were cultivated aerobically at 30°C in tryptic soy broth-dextrose free (TSB) medium (Becton Dickinson, Sparks, MD). Cell growth and PHB production were examined in minimal medium [7] supplemented with 2% fructose and 0.1% ammonium chloride (PHB-GP medium); PHB utilization was examined in minimal medium supplemented with 0.25% ammonium chloride (PHB-U medium). All media contained 10 µg/ml gentamicin.

Each cultivation was done in duplicate. For each fermentation, 5 ml TSB medium containing gentamicin were inoculated with a single colony from a fresh TSB plate and incubated for 17 to 24 h. This pre-culture (1 ml) was transferred into a 1 l baffled flask containing 100 ml TSB medium and gentamicin and grown to an OD<sub>600</sub> between 1-2 (Genesys® 20, Thermo Spectronic Inc., Rochester, NY). Cells were harvested and washed in sterile 0.85% saline and resuspended in 500 ml PHB-GP medium in 0.5 l fermentors (Sixfors, Infors AG, Bottmingen, Switzerland) to OD<sub>600</sub> = 0.05. The cells were grown at 30°C with stirring at 500 rpm. The pH was maintained at 6.8 ± 0.2 with 2 M HCl and 2 M NaOH and the pO<sub>2</sub> was maintained above 60% by sparging with air at 1 to 4 l of air/min as needed. Cell growth and PHB production were carried out in PHB-GP media for 40 h, at which time ~160 ml of cells were harvested from each production fermentor, washed once in 25 ml PHB minimal salts (13 mM sodium phosphate, 2.6 mM potassium sulfate, pH 6.8) at 4°C, and resuspended in 500 ml PHB-U in a 0.5 l fermentor to OD<sub>600</sub> = 7.0. The washing procedure took 20 min. Utilization fermentation was carried out for 120 h.
At each timepoint, a large enough volume was taken from the fermentor to perform all analyses and then split into aliquots. Not all analyses were performed at each timepoint. A total volume of less than 250 ml was removed for samples from the production fermentor and less than 100 ml from the utilization fermentor.

### 4.2.2 Analytical techniques.

Fructose was quantified as follows: 1 ml of culture was pelleted in a microcentrifuge; the supernatant was filtered through a 0.2 μm Acrodisc (Pall, East Hills, NY) filter and analyzed by HPLC immediately using an Aminex HPX-87H column (Bio-Rad, Hercules, CA) under the following conditions: column temperature, 40°C; gradient, isocratic; mobile phase, 5 mM sulfuric acid; flow rate, 0.6 ml/min; 25 μl injection. Fructose eluted at 9.9 min, and was detected by change in refractive index and was quantified by comparison to a standard curve in the range of 0.1 to 2.0 % (w/v).

For PHB and cell dry weight (CDW) measurements, between 10 and 2 ml (depending on culture density) were pelleted in dried, weighed test tubes as described previously [14]; the supernatant was stored at -20°C for ammonium quantification. PHB and CDW were quantified precisely as described previously [14, 24]. PHB for the standard curve was obtained from Sigma (St. Louis, MO). Residual cell mass (RCM) was calculated as CDW minus mass of PHB.

Ammonium was quantified employing Berthelot’s method as previously described [25], except all volumes were reduced 5-fold. Samples were diluted ~1000-fold in MilliQ water in order to be in the linear range of the assay. The formation of indophenol was monitored at 600 nm and quantification was performed by comparison to a standard curve in the range of 500 to 10 μg/l.

### 4.2.3 Isolation of RNA.

Total RNA was extracted from *C. necator* using an RNA extraction kit (RNasey mini kit, Qiagen, Valencia, CA) employing the manufacturer’s protocol (RNasey Mini Handbook, Qiagen, 06/2001). OD$_{600}$ was used to approximate cell count. During the growth phase, an OD$_{600}$ of 1.0 was determined to correspond to ~8 x 10$^8$ cells/ml using a hemocytometer (Petroff-Hausser-counter, Hauser Scientific, Horsham, PA). During the PHB production and utilization phases, RCM showed little change, and it was assumed that cell count did not change significantly. Aliquots of culture (1.5 ml) were centrifuged at 4°C (60 s, 14,000 x g); the pellet was immediately frozen in liquid nitrogen, and stored at -80°C to prevent RNA degradation. The frozen pellet was resuspended in TE (10 mM Tris, 1 mM EDTA, pH 8.0) to an OD$_{600}$ of 5.0, which contained 4 x 10$^9$ cells/ml during the growth phase. During PHB production phase, the OD$_{600}$ increased due to PHB accumulation rather than cell growth, so correspondingly fewer cells were used for extractions. Cell suspension (100 μl) was transferred to a new tube to which 5 μl lysozyme (Sigma Cat. No. L-6876, St. Louis, MO) at 1 x 10$^3$ U/ml was added. The reaction was incubated at 22°C for 20 min with gentle agitation on a Nutator (Becton Dickenson, Sparks, MD). A control experiment described in the Results section showed that mRNA integrity was largely unaffected by 20 min lysozyme treatment. Buffer RLT (350 μl) (RNasey mini kit) was added, the sample was vortexed, and transferred to a 2 ml Microtube (Sarstedt Inc., Newton, NC), containing 50 mg of glass beads (Sigma G-4649 Lot 110K5310). Cells were lysed by milling twice (45 sec each) in a Savant Fastprep FP 120 bead mill (Savant Instruments
Inc., Holbrook, NY) at 4°C. After centrifugation (5 sec, 14,000 x g), the supernatant was transferred to a fresh tube and mixed with 250 μl iced ethanol. The mixture was then added to the RNeasy mini column and purification was carried out as described in the RNeasy Mini Handbook (Qiagen, 06/2001), with the addition of the optional, on-column DNase I digest described in Appendix D (RNase-free DNase Set, Qiagen, cat no. 79254). RNA was eluted with 30 μl RNase free water and stored at -20°C until use as the template for reverse transcriptase quantitative PCR (RT-qPCR).

Eluted total RNA was quantified using A_{260nm} (ε=(40 μg/ml)^{-1}) (Cary 50 UV-spectrophotometer, Varian Inc., Walnut Creek, CA). The integrity of RNA was confirmed employing the Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA) by inspecting the electropherogram for degradation of the 16S or 23S rRNA.

4.2.4 Reverse transcriptase quantitative PCR (RT-qPCR). Primers for the mRNA of interest (table 4.1) were designed to amplify fragments between 100 and 150 bp employing the Whitehead Institute’s web based Primer3 software (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3 www.cgi [May 14th 2004]) [26]. Primer specificity was confirmed by PCR using genomic DNA or fresh bacterial colonies as template. Reactions were performed using Qiagen HotStarTaq polymerase as described in the manufacturer’s protocol, with 0.1 μM of each primer, 0.2 mM of each dNTP, and 20% Q solution. All other conditions followed Qiagen’s protocol, including the thermocycler program which used a 53°C annealing step.

DNA standards were generated by PCR (as above) using the primer pairs in table 4.1 with C. necator genomic DNA as template. PCR products were separated on a 1.2% agarose gel and DNA was extracted from agarose gel slices using the Qiagen Gel Extraction kit (Qiagen, Valencia, CA) following the manufacturer’s protocol with elution into 50 μl TE buffer. DNA was quantified with the Pico Green® dsDNA Quantitation Kit (Molecular Probes, Eugene, OR) in a Hitachi FT4500 Fluorometer (Hitachi Instruments Inc.) by comparison to a standard curve of λ DNA.

RT-qPCR was performed using the QuantiTect SYBR green RT-PCR one-step kit (Qiagen) in a PTC-200 Cycler with a CFQ-3200 Detector (MJ Research, Waltham, MA). All reactions and reagents were kept on ice until RT-qPCR was started. Reactions were set up as described in the QuantiTect handbook (11/2003), using 0.4 μM of each primer and either 100 ng for H16 or 1μg for Re1034 total RNA template or varying amounts of PCR standards in a 50 μl reaction. RT-qPCR was carried out in MJ Research 96-well microplates (Cat. No. HSP-9665) sealed with MJ Research 8-strip caps (Cat. No. TCS-0803). One microplate was used for each primer pair; each well contained a different template. The thermocycler program was set as described in the QuantiTect handbook with a 53°C annealing temperature and 37 amplification cycles. Fluorescence was measured after every elongation step with λ_{ex} = 494 nm and λ_{em} = 521 nm. A cycle threshold (C_{T}) of 0.0016 was empirically determined to be in the linear range for all samples and was used to determine the cycle number (C_{A}). To measure genomic DNA contamination, a control reaction was prepared for each RNA template where QuantiTect Reverse Transcriptase Mix was omitted. The fluorescence signal observed from this control was assumed to be from genomic DNA.

To generate a cDNA standard curve for each gene studied, qPCR was also performed using a dilution series of the qPCR standards described above as template in
reactions without reverse transcriptase. On each microplate, six wells were loaded with between 0.2 fg and 2 pg of qPCR standards. A plot of the standards’ mass vs. \( C_\text{d} \) was used to generate a cDNA standard curve. The standard curve was used to determine the DNA in each sample and control.

The quantity of genomic DNA in each control reaction was subtracted from the amount of DNA in each sample to yield the quantity of cDNA. Genomic DNA contamination in the individual samples varied from 0.01 to 0.05 fg. cDNA levels were only considered significant if they were more than twice the maximum level of contaminating genomic DNA (0.05 fg). Consequently, cDNA levels below 0.05 fg were treated as below the limit of detection.

4.2.5 Quantitative Western blotting. A frozen sample pellet (collected as described under RNA extraction above) was thawed and resuspended in an appropriate volume of Lysis buffer (50 mM Tris, 1 mM EDTA, 2% SDS, pH=8) to yield an OD\(_{600}\) of either 5, 10 or 20, depending on the size of cell pellet. The cell suspension was sonicated for 30 s (0.8 s on, 0.2 s off) and heated at 95°C for 20 min before being spun down at 13000 rpm for 6 min. Quantitative Western blotting was carried out precisely as described elsewhere [22].

4.3 Results

4.3.1 Cupriavidus necator cultivation. We sought to develop a batch fermentation strategy that would minimize metabolic changes in the cell unrelated to poly-(R)-3-hydroxybutyrate (PHB) homeostasis. Cells were grown in a defined medium based on media previously used for the growth of \( C.\) necator in this lab [7]. Quantities of fructose and ammonium were changed, however, to allow for a growth phase with minimal PHB production (growth phase), followed by a nitrogen limited phase, in which PHB would be accumulated (production phase). Figure 4.1a shows the results from two duplicate fermentations run in parallel. During the growth phase, cultivations were characterized by an increase in residual cell mass (RCM), with constant levels of ~15% PHB as a percentage of cell dry weight (CDW). At 15.5 h in fermentor 1 and 16.5 h in fermentor 2, when ammonium was exhausted, RCM stopped increasing and PHB was accumulated up to 5 mg/ml (~75% CDW) over 20 h. Linear consumption of fructose was observed during this production phase.

After the production phase, cells were washed to remove residual fructose and used to inoculate fresh medium lacking carbon to undergo a utilization phase. Cells were diluted ~three-fold to yield an initial OD\(_{600}\) of 7. Over 60 h in the utilization fermentor, 50% of the PHB was degraded (figure 4.1b). No further degradation was observed out to 120 h. The initial rate of degradation was ~0.2 mg\(\cdot\)ml\(^{-1}\)\(\cdot\)h\(^{-1}\) and slowed throughout the period of cultivation. A control experiment was performed in which cells were transferred to fresh utilization medium after the utilization phase. No further decrease in PHB was observed, demonstrating that the cessation of PHB degradation was not due to nutrient limitation (data not shown).

4.3.2 RNA extraction. RNA extractions during the growth phase at 12, 13 and 15.5 h yielded ~65 μg RNA/10\(^9\) cells (figure 4.2a). During the production phase, the RNA yield
decreased steadily, reaching 6 µg RNA/10⁹ cells at 36 h. This number at 36 h was insufficient to perform RT-qPCR. During the utilization phase (figure 4.2b), the RNA yield increased to 18 µg RNA/10⁹ cells. In order to determine RNA yield per cell, cell count was determined only at OD₆₀₀ = 1.0, where it was found to be 8 × 10⁸ cells/ml. Cell count at other time points was assumed to be proportional to residual cell mass, which underwent negligible change after the growth phase (figure 4.1).

In order to ensure that degradation of the transcripts of interest was not occurring during our cell lysis procedure, a control experiment was performed in which transcript levels for phaC, phaR, phaP, phaZIa, phaZIb, and phaZIc were quantified from RNA that was extracted as described in the Materials and methods section (which included a 20 min lysozyme treatment at 22°C) or with a shorter, 5 min lysozyme treatment. Relative transcript levels were quantified at one timepoint during growth, one timepoint during production and one timepoint during utilization. Relative transcript levels, with the exception of phaZIb, were unaffected by length of lysozyme treatment, indicating that degradation of the transcripts of interest does not occur during extraction (data not shown). In the case of phaZIb, ~30% degradation was observed at each timepoint.

4.3.3 Reverse transcriptase quantitative PCR (RT-qPCR). RT-qPCR was performed using primers in table 4.1 to determine relative levels of mRNA from eight genes involved in PHB metabolism: phaA, phaB, phaC, phaP, phaR, phaZIa, phaZIb, and phaZIc. 16S rRNA was also quantified. Figure 4.3a shows that the primers amplify specific products in all cases. Since C. necator contains several homologous phasins and depolymerases [16], we performed the control in figure 4.3b. Primers for phaP, phaZIa, phaZIb, and phaZIc were used for colony PCR using the corresponding deletion strains (ΔphaP, ΔphaZIa, ΔphaZIb, and ΔphaZIc, respectively). Primers for phaC were also used as a positive control. The failure to amplify product in the deletion strains show that the primers do not amplify sequences homologous to their intended targets.

Figures 4.4-4.7 present the experimental results as a function of time for the eight gene products in femtograms of cDNA, which was determined by comparison to DNA standards as described in the Materials and methods section. All cDNA quantities are normalized to total extracted RNA. Because there is variation in the efficiency of reverse transcription with different templates, the differences in the y-axis (cDNA) scale among the figures does not correlate with differences in transcript levels between the different genes.

The relative amount of 16S rRNA was determined to vary by less than 6% among all timepoints (data not shown). Since rRNA constitutes the major fraction of total RNA, and the same total RNA was added into each sample, each sample was expected to contain approximately the same quantity of 16S rRNA. This measurement thus serves as a control for RNA concentration determination, loading, and RNA integrity.

The levels of phaA, phaB, phaC, phaR, and phaZIa transcripts relative to total RNA show similar trends during the cell growth and PHB production phases (figure 4.4). This result was expected for phaA, phaB, and phaC since they are thought to be cotranscribed [8]. During the growth phase, the relative levels of all five transcripts decrease, and become stable after nitrogen exhaustion when the production phase begins. In the utilization phase, these five transcripts also share a similar pattern, although the replicate experiments were less similar to each other (data not shown). Relative levels
underwent an initial increase and then gradually decreased over the remainder of the utilization fermentation.

The relative levels of $phaP$ transcripts show a different pattern, increasing six- to seven-fold upon nitrogen limitation (figure 4.5a). As noted above, nitrogen depletion occurred in fermentor 2 between 30 and 60 min after depletion in fermentor 1, and a very similar lag is seen in the rise of $phaP$ transcripts. The $phaP$ transcript is most abundant during the earliest phase of rapid PHB accumulation, when the rate of accumulation is highest. After this initial phase, levels of the $phaP$ transcript decrease to approximately double the starting levels. In the utilization fermentor, levels of the $phaP$ transcript rapidly fall, decreasing six-fold in the first hour and subsequently decreasing a further two-fold (figure 4.5b).

Previous work has demonstrated that accumulation of PhaP depended on the accumulation of PHB [10-12, 27]. In order to demonstrate that this regulation occurs at the transcriptional level, we carried out a fermentation using the strain Re1034, in which the synthase (PhaC) has been deleted and no PHB is synthesized. Fermentation conditions were identical to those in figure 4.1a, except that nitrogen limitation occurred later, at 27.5 h. RNA was isolated from samples taken before and after nitrogen limitation and 1 μg total RNA was used as a template for RT-qPCR. The highest level of $phaP$ cDNA was ~eight-fold lower than the lowest level observed with the wild type strain under similar conditions (0.5 fg vs. 4 fg), despite using ten-fold more RNA template in the RT-qPCR reaction (figure 4.5c). Furthermore, transcript levels did not change with nitrogen limitation. In a recent set of experiments where we performed quantitative Western blotting on samples from cultures grown on complex medium in shake flasks, we noticed a striking correlation between amount of PhaP and PHB [22]. In the current study, we performed quantitative Western blotting and PHB quantitation on samples isolated during cell growth and PHB production. We found a strong correlation ($r=0.99$) between total culture PHB and total culture PhaP (figure 4.6), while no correlation was seen between PHB and $phaP$ transcript levels.

The pattern of $phaZ₁b$ relative transcript levels was similar, but not identical, to the pattern of $phaP$ transcripts. Transcripts of $phaZ₁b$ were below the limit of detection until after 16.5 h and then rapidly increased ten-fold over the next 90 min (figure 4.7a). This increase lagged the observed increase in $phaP$ transcripts by approximately 2 h. As with $phaP$, the mRNA levels decreased after an initial peak. In the utilization fermentor, $phaZ₁b$ mRNA was undetectable (not shown).

This seemed an unusual pattern for a putative depolymerase, so we also carried out quantitative Western blotting for PhaZ₁b during growth, production and utilization (figure 4.8). PhaZ₁b was absent during growth, but was detected 1 h after transcript levels spiked (19 h), and increased throughout the remainder of the production phase. During the utilization phase, PhaZ₁b levels decreased, dropping below the limit of detection by ~6 h in both fermentors.

We also tested whether transcription of $phaZ₁b$ depended on PHB (as seen with $phaP$) by determining transcript levels in Re1034. The levels of the $phaZ₁b$ transcript resembled the levels in the wild type (figure 4.7b), remaining low until nitrogen limitation after which point transcript levels increased sharply, showing that induction of $phaZ₁b$ transcription is independent of PHB production. Note that ten-fold more RNA
was used as template than in figure 4.7a, producing correspondingly higher amounts of cDNA.

*phaZ1c* was assayed for in the wild type strain, but values were below the limit of detection (0.05 fg) at all timepoints (not shown).

**4.4 Discussion.** Three distinct patterns of transcription were observed in the growth and production phases. The first is shared by *phaA, phaB, phaC, phaR*, and *phaZ1a* whose relative transcript levels showed roughly the same trends, decreasing from 12 h through 16 to 18 h and leveling out after 20 h (figure 4.4). The period where a decrease in relative levels is observed is also the period when ammonium is being depleted from the culture. After ammonium is exhausted, the relative transcript levels stabilize for all these genes, during which time the total extracted RNA per cell declines (figure 4.2a). The product of these two figures is proportional to the absolute transcript level per cell for each gene, which undergoes a steady decrease over the course of the fermentation (e.g. 14-fold for *phaA* between 12 h and 25 h).

Kojima et al. recently carried out similar experiments using dot-blots to examine mRNA levels of *phaA*, *phaB*, *phaC*, *phaP*, and *phaR* in *Paracoccus denitrificans* during nitrogen-rich and nitrogen-deficient (PHB producing) conditions [28]. For *phaA* and *phaB*, they saw a large decrease in signal intensity during nitrogen limitation, similar to the decrease in absolute levels we report for *phaA* and *phaB*. However, the authors failed to determine whether their observed decrease is associated with a decrease in total RNA yield, in relative transcript levels, or both. The significance of this omission is clear when considering our *phaA* and *phaB* results. Between 15.5 h and 25 h, the relative level of these two transcripts remains constant (figure 4.4a & b). However, the total RNA yield per cell declines (figure 4.2a) and so the absolute levels of *phaA* and *phaB* (the product of Figures 4.2 & 4.4) decline as well. This same problem is associated with the data from the other transcripts Kojima et al. examined.

Both our data and that of Kojima et al. points out the complexity of interpreting transcriptional data from cells undergoing nutrient limitation. It has been long known that total RNA per cell in prokaryotes varies dramatically with growth rate [29]. Furthermore, the ratio of mRNA to stable RNA (tRNA and rRNA) also changes dramatically based on growth phase and growth rate [30]. Prokaryotic mRNA lacks a polyadenylation signal and is not easily isolable, so normalization to total mRNA is not practical. Comparison to a housekeeping gene is a valid method, but it must be demonstrated that the housekeeping gene is not differentially expressed under the experimental conditions, which is not always the case [31]. Given these complexities, few groups have attempted to examine transcriptional regulation in a changing nutritional environment, except by whole genome array, which is intrinsically normalized to total mRNA [32].

Despite these caveats, we hypothesize that the pattern observed for these five genes represents constitutive transcription. The simplest explanation of our data is that relative levels of *phaA, phaB, phaC, phaR*, and *phaZ1a* change in a similar fashion due to a change in the ratio of mRNA to total RNA rather than being co-regulated, and this is how we interpret our data.

Transcript levels of *phaP* undergo a six-fold increase within 1 h of the shift to PHB production and rapidly decline under utilization conditions. In the absence of PHB
synthase, very little \textit{phaP} transcript is observed and no increase is observed upon nitrogen limitation, demonstrating that PhaP expression is coupled to PHB synthesis at the transcriptional level as several groups have proposed [10, 12, 27]. However, the data in figure 4.6 raise the possibility that PhaP expression may also be regulated at the level of protein synthesis or degradation, as there is a strong positive correlation between levels of PhaP and PHB that is not seen with the \textit{phaP} transcript.

It has been proposed that PhaR in \textit{P. denitrificans} is an autorepressor of the \textit{phaR} locus [33]. Potter et al., propose that \textit{phaR} is also autoregulated in \textit{C. necator}, based on DNase footprinting and on \textit{in vitro} binding assays, although the latter were performed at very high concentrations [12]. It has been well-established that PhaR represses expression of the PhaP protein and acts in some way as a sensor of PHB accumulation, releasing repression at the onset of PHB synthesis [10-12, 27], and the data in figure 4.5 directly show that this occurs at the transcriptional level. If \textit{phaR} were regulated in the same fashion, then the transcript levels of \textit{phaR} should resemble the transcript levels of \textit{phaP}, however that is not the case (compare data in figure 4.4d and 4.5a). There is no indication in this data that levels of the \textit{phaR} transcript depend on the accumulation of PHB. Therefore if PhaR is involved in autoregulation of the \textit{phaR} locus, the mechanism is different from its regulation of the \textit{phaP} locus.

Our results with \textit{phaP} and \textit{phaR} contrast with a recent report by Seo et al. who observed a constant ratio between the levels of \textit{phaP} and \textit{phaR} transcripts when comparing relative transcript levels at different timepoints during PHB production in recombinant \textit{C. necator}. We see a >5-fold change in the first hours of the PHB production phase. The first timepoint that they examined, however, was 12 h after nutrient limitation, pointing out the need to perform analyses during an informative time frame, which in this case is when the rate of PHB accumulation undergoes an abrupt change, signaling a shift to a new metabolic regime.

Levels of the \textit{phaZ1b} transcript in H16 showed a third pattern (figure 4.7a), undergoing a rapid increase of more than ten-fold 1-2 h after the onset of the PHB production phase. After peaking at 18 h, the amount of \textit{phaZ1b} decreased slightly, but still remained high. The \textit{phaZ1b} transcript was completely undetectable in the utilization fermentor and presumably had been cleared during the washing step. \textit{phaZ1b} showed a similar pattern of transcription in the absence of PHB synthase (figure 4.7b), indicating that it is not regulated by PHB synthesis and for the first time demonstrating a regulatory circuit involved in PHB homeostasis in \textit{C. necator} whose regulation is not coupled to PHB. The regulatory protein(s) involved remain to be identified.

The kinetics of PhaZ1b expression do not match our expectation for the expression of an intracellular depolymerase (figures 4.7 & 4.8). Why would the cell need a depolymerase during PHB synthesis and not during degradation? The expression pattern seems to indicate that PhaZ1b is not involved in catabolism of PHB. However, in our previous work, we showed that deleting \textit{phaZ1b} resulted in less complete PHB degradation in the PHB utilization medium [14]. Moreover, in that work we examined PHB degradation in a strain with both \textit{phaZ1a} and \textit{phaZ1c} deleted, and saw substantial degradation over the course of 10 h immediately post-inoculum. In the strain with the additional deletion of \textit{phaZ1b}, no degradation was observed. Despite these observations, we see in the present work that PhaZ1b is no longer present in the cell by 6 h post-inoculum.
Finally, at no time were transcripts of *phaZ1c* observed under these growth conditions. In other recent work we have likewise failed to detect PhaZ1c protein itself [22]. In light of this, it is unsurprising that our previous work failed to detect any effect from deletion of *phaZ1c* [14], despite the fact that PhaZ1c is 46% sequence identical to the major intracellular depolymerase PhaZ1a and 43% sequence identical to PhaZ1b. So far we have only examined a fairly narrow set of fermentation conditions, and may not yet have performed the right experiment to observe *phaZ1c* expression. Given our observation of tightly regulated, transient expression of *phaZ1b*, it seems possible that *phaZ1c* is turned on and off rapidly in response to a specific metabolic condition, and we have not identified that condition. Alternatively, *phaZ1c* may be an unexpressed pseudogene.

**Acknowledgements**

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### 4.5 Tables and Figures

**Table 4.1: Genes and gene products examined in this study, their function, and oligonucleotides used for reverse transcriptase quantitative PCR**

<table>
<thead>
<tr>
<th>Gene/protein name</th>
<th>Protein Function</th>
<th>Oligonucleotides</th>
<th>Product Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PhaA</td>
<td>β-ketothiolase</td>
<td>5'-CAGTACCACATGGGCAATC-3' 5'-CGATCTCTTCAAACTTGCA-3'</td>
<td>139</td>
</tr>
<tr>
<td>PhaB</td>
<td>Acetoacetyl-CoA Reductase</td>
<td>5'-ATCGACACCAACCTGACCTC-3' 5'-TTCAACCCAGAGATGTTGAC-3'</td>
<td>101</td>
</tr>
<tr>
<td>PhaC</td>
<td>PHA Synthase</td>
<td>5'-GCATTCTACCTGCTCAATGC-3' 5'-GATTGGTGGAAGAAGTGG-3'</td>
<td>142</td>
</tr>
<tr>
<td>PhaP</td>
<td>Phasin (granule formation)</td>
<td>5'-CAAGAACGCACAGGAACTG-3' 5'-GTGAACTCAGCTCTGGGTTC-3'</td>
<td>120</td>
</tr>
<tr>
<td>PhaR</td>
<td>Negative regulation of phaP transcription</td>
<td>5'-ACACCCAGACCAGCCTAC-3' 5'-GGGTCAGTTTCGACACCAGAC-3'</td>
<td>105</td>
</tr>
<tr>
<td>PhaZ1a</td>
<td>PHB depolymerase</td>
<td>5'-AGGTCTACGCACGACTGG-3' 5'-GGCATGCCAGATCATGCG-3'</td>
<td>141</td>
</tr>
<tr>
<td>PhaZ1b</td>
<td>Putative PHB depolymerase</td>
<td>5'-ATCTCACCGACTGGCAACA-3' 5'-CAAATGCGCACCAGATGC-3'</td>
<td>130</td>
</tr>
<tr>
<td>PhaZ1c</td>
<td>Putative PHB depolymerase</td>
<td>5'-TGCAATTGCTGCTATGTC-3' 5'-GCACCTCGCTGCTCTTC-3'</td>
<td>141</td>
</tr>
<tr>
<td>16S rRNA</td>
<td>No protein</td>
<td>5'-CGGAAATTACTGGGCGTAAAG-3' 5'-ACGCAATTCACGTACACG-3'</td>
<td>146</td>
</tr>
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</table>
Figure 4.1: Average fermentation results for two parallel fermentations of *R. eutropha* H16. (a) cell growth and PHB production phases; (b) PHB utilization phase. Open squares, fructose; open triangles, ammonium; closed triangles, residual cell mass (RCM); closed squares, PHB. During utilization, ammonium changes were negligible and fructose was not present. Error bars represent the spread of the data. Time scale in (b) is discontinuous to show detail through 1 h.

Figure 4.2: RNA extraction from parallel H16 fermentations. (a), cell growth and PHB production phases; (b) PHB utilization phase.
Figure 4.3: Verification of primer efficacy and specificity. (a) Primers in table 4.1 were used with H16 genomic DNA as template and resolved on 1.2% agarose gel. No non-specific products were observed. Primers for *phaA* (A), *phaB* (B), *phaC* (C), *phaP* (P), *phaR* (R), *phaZ1a* (Z1a), *phaZ1b* (Z1b), *phaZ1c* (Z1c), and 16S rRNA (16S) were used. (b) Primers amplify only the intended gene. Primers for *phaP* (P), *phaZ1a* (Z1a), *phaZ1b* (Z1b), and *phaZ1c* (Z1c) were used with the corresponding deletion strain (1052 Δ*phaP*, 1097Δ*phaZ1a*, 1107 Δ*phaZ1c*, and 1110 Δ*phaZ1b*, respectively) as template. Primers for *phaC* (C) were included as a control for PCR. Markers 766, 500, 300, 150, 50 bp.
Figure 4.4: Relative levels of *phaA*, *phaB*, *phaC*, *phaR*, and *phaZ1a* transcripts share a similar pattern during growth and production. RT-qPCR results from parallel H16 cell growth and PHB production fermentations. Closed symbols are fermentation 1 and open symbols are fermentation 2. Line marks onset of PHB production phase. (a) *phaA*; (b) *phaB*; (c) *phaC*; (d) *phaR*; (e) *phaZ1a*
Figure 4.5: Transcription of phaP is tightly coupled to PHB production. (a) Levels of phaP cDNA from parallel H16 cell growth and PHB production fermentations. Closed symbols are fermentation 1 and open symbols are fermentation 2. Line marking onset of production phase is not averaged to show tight coupling of PHB production and phaP transcription. (b) cDNA from parallel H16 PHB utilization fermentations. Time scale is discontinuous to show detail through 1 h. (c) cDNA from Re1034 (ΔphaC) grown under same conditions as in (a). Ten-fold more RNA was used as template than in (a).
Figure 4.6: PhaP protein levels correlate with PHB. Results from cell growth and PHB production phases in (a) fermentor 1 and (b) fermentor 2. All results are per ml culture. Circles, phaP cDNA from RT-qPCR; squares, PhaP protein; triangles, PHB.
**Figure 4.7: Induction of phaZ1b is not dependent on PHB production.** (a) Levels of phaZ1b cDNA from parallel H16 cell growth and PHB production fermentations. Closed symbols are fermentation 1 and open symbols are fermentation 2. (b) cDNA from Re1034 (ΔphaC) grown under same conditions as in (a). Ten-fold more RNA was used as template than in (a).

**Figure 4.8: PhaZ1b protein levels.** Results from quantitative Western blotting expressed as ng/ml culture. Closed symbols are fermentation 1 and open symbols are fermentation 2. (a) Cell growth and PHB production phases. (b) PHB utilization phase. Cultures were diluted 3-fold from (a) to (b).
4.6 References


Chapter 5: Effects of phaP-related mutations on polyhydroxybutyrate molecular weight and granule size in recombinant Cupriavidus necator

5.1 Introduction. PHB synthesis is an addition polymerization involving sequential addition of monomers through a mechanism of covalent catalysis [1, 2]. In an addition polymerization, growth of individual chains begins from a discrete number of initiator molecules, propagates through the addition of individual monomers to the polymer chains and terminates either through a side reaction, or, in a special case termed a “living polymerization”, when monomer is exhausted. In the case of PHB synthesis, it is well established that many polymer chains are synthesized per enzyme molecule, requiring the existence of chain terminating and re-initiating reactions and ruling out a living polymerization mechanism [3-5]. However, the polydispersity (MW/MN), a measure of molecular weight distribution in a sample, of PHB from bacterial sources is ~2 [6], much less than the values of a typical addition polymerization (>10) and closer to the values of a living polymerization (1-2). This implies that there must be some mechanism to control the timing of the termination reaction, in order to ensure that termination only occurs within a relatively narrow MW range.

As described in section 1.4.2, the presence of phaPp Paracoccus denitrificans in recombinant E. coli harboring the phaCABp operon caused the production of PHB with a reduced MW relative to PHB produced in the absence of phaCp [7]. Our examination of protein levels during PHB production in wildtype C. necator showed that the levels of PhaP are tightly coupled to levels of PHB (figure 4.6), and that the stoichiometry of molecules of PhaP:molecules of PHB is close to one [4, 5]. This suggested that PhaP may have a direct interaction with either the PHA synthase or the PHB chain during elongation, which is responsible for its effect on MW. Alternatively, PhaP may not directly interact with either the synthase or the PHB chain during elongation, but instead become associated with the PHB granule surface at an exposed hydrophobic patch as suggested by the model in the literature [8], producing an indirect affect on PHB MW.

In order to assess these hypotheses regarding the effects of PhaP, PHB MW was characterized in several strains in which PhaP levels had been altered or PhaP had been mutated. Since PhaP is also known to affect granule morphology, MW was compared to granule size. Those strains for which transmission electron microscopy (TEM) images are not available in the literature were imaged using deconvolution fluorescence microscopy in order to obtain information about relative granule size. While the images obtained by this method are significantly lower resolution than those obtained by the more widely used TEM method, the time required to obtain images is much lower, allowing for increased throughput. This was particularly important for the results presented in chapter 6, where several strains had to be constructed and screened for granule size in order to obtain the phenotypes of interest, which would not have been feasible with TEM. The results here show that there is a correlation between MW and granule size when comparing strains containing Class I vs. Class III synthase, PhaP vs. PhaP-GFP, and wildtype vs. ΔphaR (PhaP overexpressed) but not in a strain lacking PhaP (ΔphaP).
5.2 Materials and Methods

5.2.1 Bacterial strains and cultivation conditions. Strains used in this study are listed in table 5.1. Precultures were cultivated aerobically at 30°C in tryptic soy broth-dextrose free (TSB) medium (Becton Dickinson, Sparks, MD). PHB production was examined in minimal medium [9] supplemented with 1% fructose and 0.01% ammonium chloride (PHB-P medium); PHB utilization was examined in minimal medium supplemented with 0.5% ammonium chloride (PHB-U medium). All media contained 10 μg/ml gentamicin.

Each procedure was usually performed in duplicate, with very little variability between samples. Not all experiments were performed in duplicate, however; refer to figure legends. A single colony was inoculated from a TSB plate into 5 ml TSB and cultivated for ~40 h at 30°C. 2 ml of this culture was used to inoculate 100 ml TSB in a 500 ml baffled flask which was grown a further 24 h. An aliquot of cells sufficient to produce 200 ml at OD₆₀₀ = 0.5 was washed and transferred to 200 ml PHB-P medium and grown for 72 h. For PHB utilization experiments, 100 ml of this 72 h production culture was harvested and washed with 0.85% saline before being resuspended in 200 ml PHB-U medium and grown a further 72 h.

5.2.2 Determination of cell dry weight (CDW) and PHB content. At timepoints described in the Results section, 5 ml of each culture was collected for CDW and PHB determination as described in chapter 2.

5.2.3 Extraction of PHB from C. necator for Mₖ analysis. At timepoints described in the Results section, 10 ml of each culture were pelleted in a test tube, washed once with 10 ml cold (4°C) distilled water, pelleted again, and finally resuspended in a small volume of residual supernatant after removing >95% of supernatant. Samples were then frozen at ~80°C where they were stored for 48 hours before lyophilization overnight.

Lyophilized samples were extracted in their original test tubes by refluxing with 5 ml CHCl₃ for 48 hours with stirring. Samples were then brought up to 5 ml, gravity filtered through Whatman #4 qualitative filter paper and tubes and filters were washed with 5 ml fresh CHCl₃. The combined 10 ml was dried under N₂ and then redissolved in 1 ml CHCl₃. PHB was precipitated by addition of 3 ml methanol and the precipitate was recovered by vacuum filtration in a Buchner funnel through Whatman #474 quantitative filter paper. Filters were dried under reduced pressure at 55°C for 24 hours at which time PHB was easily removed as a coherent film on each filter.

5.2.4 Determination of PHB Mₖ. Samples were dissolved in TFE at 1 mg/ml for 24 h after which an aliquot was removed, diluted to 0.25 mg/ml and filtered through a 0.2 μm PTFE filter. Coupled multi-angle light scattering (LS) and GPC were used to determine the Mₖ of PHB as described in section 2.2.5 with a PLgel 10 μm Mixed-B column (Polymer Laboratories, Amherst MA).

5.2.5 Fluorescent Staining of Cells and Deconvolution Microscopy. An aliquot of cells (20 μl) was mixed briefly with dye (20 μl of 50 μg/ml Nile Red (Nile Blue A Oxazone, Sigma Aldrich, St. Louis, MO and 50 μg/ml DiO (3,3'-dioctadecyl-
oxacarbocyanine perchlorate, Invitrogen, Carlsbad, CA) in dimethylsulfoxide (DMSO)) and immediately (<10 s) diluted with 100 μl 75 mM KPi, 66 mM NaCl, pH 7.8 (PBS). Cells were pelleted for 1 min at 13,000 x g, resuspended in 20 μl PBS, and staining was repeated twice more. Control experiments for the staining protocol were performed in which cells were exposed to no more than 1% DMSO in order to confirm that DMSO at high concentration was not causing changes to granule morphology (not shown).

Cells were diluted to OD₆₀₀ = ~ 2.0 in PBS and placed under a cover slip on a polylysine coated slide. Cells were imaged using a DeltaVision optical sectioning microscope on an Olympus IX71 base with a 100x/1.4 N.A. objective, Sedat filter set and a Photometrics Cool Snap HQ camera (Applied Precision, Issaquah, WA). DiO was visualized using setting for FITC (λₑₓ = 490 ± 20 nm, λₑₘ = 528 ± 38 nm). Nile Red was visualized using setting for rhodamine (λₑₓ = 555 ± 25 nm, λₑₘ = 616 ± 73 nm).

Hundreds of cells were examined from each cultivation, and the images presented are judged to be representative of the most commonly occurring phenotype for each cultivation. 20 sections were recorded for each image (Δz = 0.2 μm, total thickness 4.0 μm) and images were processed using Softworx 3.5.0 (Applied Precision, Issaquah, WA) and Adobe Photoshop CS2 (Adobe Systems, San Jose, CA).

5.3 Results

5.3.1 PHB production and Mₛ from Cupriavidus necator H16 (wildtype), Re1052 (ΔphaP) and Re1099 (ΔphaR) during PHB production and PHB utilization. The Mₛ of PHB was examined in three strains, H16 (wildtype), Re1052 (ΔphaP) and Re1099 (ΔphaR, PhaP overproducer). As previously reported, both Re1052 (ΔphaP) and Re1099 (ΔphaR) produced lower amounts of PHB than the wildtype strain [10]. After the switch to PHB-U medium, Re1099 (ΔphaR) exhibited a more rapid degradation of PHB than wildtype, utilizing ~75% of PHB in the first 24 h as opposed to ~40% in H16 (wildtype) and Re1052 (ΔphaP) (table 5.2). Both these deletion strains produced lower Mₛ PHB than the wildtype strain, particularly Re1099 (ΔphaR) whose Mₛ was < 42% of wildtype at 72 h in PHB-P (table 5.2). Note that the difference in Mₛ between H16 (wildtype) and the mutant strains is understated due to degradation of PHB which occurred during HPLC and which only affected the measurements for H16 (wildtype).

5.3.2 Correlation of Mₛ and granule size in four PHB producing strains. It has been previously shown that PHB granules are larger in C. necator lacking PhaP than in wildtype C. necator and smaller when PhaP is overexpressed ([11] and figure 1.4). I was interested in the observation that low Mₛ was correlated with granule size in comparing Re1099 (ΔphaR) to H16 (wildtype) but not in comparing Re1052 (ΔphaP) to H16 (wildtype) because a correlation had been suggested by previous results. Liebergesell et al. observed that PHB produced from heterologous PhaEC₄ᵥ or PhaEC₇ᵥ in a PHB-deficient C. necator strain, PHB-4, was both higher Mₛ and was packaged in larger granules [12]. To expand on these findings, fluorescence microscopy was performed on four C. necator strains, H16 (wildtype), Re1018 (phaP-GFP), Re1031 (phaEC₄ᵥ) and Re1033 (phaEC₄ᵥ phaP-GFP). Re1018 (phaP-GFP) and Re1033 (phaEC₄ᵥ phaP-GFP) contain a precise replacement of the phaP ORF with the phaP-GFP ORF, which encodes a translational fusion of PhaP and GFP [13]. Re1031 (phaEC₄ᵥ) and Re1033 (phaEC₄ᵥ
phaP-GFP) contain a precise replacement of the phaC<sub>Cn</sub> ORF with the co-transcribed ORFs for phaEC<sub>A</sub>. Note that the phaP-GFP strains were used because we had previously noted a change in granule size in these strains (not shown), not because GFP fluorescence was used to image the granules. GFP fluorescence was not imaged because the membrane specific fluorescent dye, DiO, was used and it is much brighter than GFP with similar excitation and emission spectra.

Fluorescence microscopy is unable to resolve clearly delineated granule boundaries in the same manner as TEM. However, in most cases, individual granules appear as brightly stained features bounded by unstained regions, allowing estimation of size relative to other fluorescently stained granules. Even if granules are tightly packed and their size is approaching the limit of resolution, information can be abstracted. TEM and freeze-fracture electron microscopy studies have shown that granules are usually close to spherical, and if not spherical, form convex shapes. Therefore, individual fluorescently stained granules will form no more than one local fluorescence maximum. The distance between individual maxima thus provides a maximum for the diameter of the individual granules, while irregular or concave shapes and a grainy appearance are most likely indicative of tightly packed granules with diameters below the limit of resolution.

Granules were imaged by fluorescence microscopy after 24 h cultivation in PHB-P and PHB was extracted and characterized by GPC/LS at 48 h. Microscopy was initially performed at 48 h, but resolution was poor (not shown), so cultivation was repeated with samples imaged at 24 h. Relative granule sizes were the same at 24 h and 48 h, however. Granules were largest in Re1031 (phaEC<sub>A</sub>) (Figure 5.1 Panel A) as was PHB <i>M</i><sub>W</sub> (2.9 × 10<sup>6</sup> Da, table 5.3). Both H16 (wildtype) and Re1033 (phaEC<sub>A</sub> phaP-GFP) had granules that were smaller than Re1031 (phaEC<sub>A</sub>) but that were difficult to distinguish from each other (figure 5.1, panels B & C). PHB <i>M</i><sub>W</sub> from each was also lower than that of Re1031 (phaEC<sub>A</sub>), at 1.9 × 10<sup>6</sup> and 2.3 × 10<sup>6</sup> Da, respectively (table 5.3). Re1018 (phaP-GFP) had the lowest PHB <i>M</i><sub>W</sub> (1.2 × 10<sup>6</sup> Da) but granules were difficult to resolve in this strain. However, as noted above, it is expected that below a certain size threshold, granules will not resolve into discrete features, but will in aggregate appear as irregular grainy shapes, as is seen in figure 5.1, panel D. This indicates that this strain produces the smallest granules of the four strains examined.

5.4 Discussion. In chapter 4, we saw a strong correlation between levels of PhaP and PHB [14]. Assuming a typical <i>M</i><sub>W</sub> of 1-2 × 10<sup>6</sup>, the ratio of PHB chains to PhaP molecules under these conditions is near 1, a ratio that was also observed in shake flask fermentations at 4 h in TSB and 24 h in PHB-P [4, 5]. This suggests that PhaP may be playing a specific role in the PHB polymerization reaction. For example, it may play a role in termination such as catalyzing a chain transfer reaction. This hypothesis is supported by results in <i>E. coli</i> XL1-Blue harboring the <i>P. denitrificans</i> phaCAB<sub>pn</sub> operon, where it was observed that the presence of the gene encoding the phasin from <i>P. denitrificans</i> caused the production of PHB with a lower <i>M</i><sub>W</sub> than in a strain without the phasin gene [7].

Consistent with the hypothesis that PhaP is controlling PHB <i>M</i><sub>W</sub>, we saw a large decrease in <i>M</i><sub>W</sub> in the ΔphaR strain, in which PhaP was overexpressed approximately 3-fold relative to the wildtype strain [4]. However, in this strain, PhaC<sub>Cn</sub> was also
overexpressed and was present at ~ 5 times the wildtype levels, so it is possible that higher synthase activity is producing lower $M_w$ PHB, as was reported to occur in *E. coli* [15]. Arguing against PhaP control of PHB $M_w$, the ΔphaP strain failed to exhibit a large increase in $M_w$ as would be expected given the ΔphaR results. As a caveat to that point, it should be noted that three other PhaP homologs were recently identified in *C. necator*, one of which, PhaP3, is expressed at high levels in a ΔphaP strain. It may be that PhaP3 is a paralogous replacement for PhaP and that it contributes to $M_w$ control in this strain.

The observation that the phaP-gfp gene replacement strains produce lower $M_w$ PHB than their wildtype phaP counterparts again hints at some role for PhaP in PHB $M_w$ determination. It stands to reason that if PhaP does contribute to PHB $M_w$ determination, that function could be altered by modification of the protein’s primary structure, resulting in the production of lower $M_w$ PHB. While this analysis of recombinant *C. necator* has provided some intriguing observations related to the mechanism of $M_w$ determination, no strong conclusions can be drawn regarding the role of PhaP in PHB $M_w$ determination.

Granule size and PHB $M_w$ was shown to be correlated in some of the strains studied. Such a correlation was also seen by Liebergesell et al. in the work described in the introduction [12]. Although the authors noted that the granules in some of their strains were larger than in the wildtype strain, they did not remark upon the apparent correlation to $M_w$. In particular, the strains which contained the *Thiocystis violacea*, *Allochromatium vinosum*, and *Thiocapsa pfennigii* synthases all produced both higher $M_w$ PHB and larger granules, while the strain harboring the *Lamprocystis roseopericina* synthase produced nearly wildtype $M_w$ PHB and granules similar in size to wildtype granules.

Using deconvolution fluorescence microscopy, it was clear that of the four strains H16 (wildtype), Re1031 (phaEC_A'), Re1033 (phaEC_A' phaP-GFP), and Re1018 (phaP-GFP) granules were largest in Re1031 (phaEC_A'), smaller in Re1033 (phaEC_A' phaP-GFP), similar to Re1033 (phaEC_A' phaP-GFP) in H16 (wildtype), and smallest in Re1018 (phaP-GFP) (i.e Re1031 > Re1033 ≈ H16 > Re1018) (figure 5.1). This was also the order of PHB $M_w$ in these three strains (table 5.3). Elsewhere it has been well characterized that the granules in a PhaP deficient strain are larger than in H16 (wildtype), which in turn has larger granules than a strain overproducing PhaP [11]. However, this was not the order of PHB $M_w$ observed in this study. Instead, H16 (wildtype) produced the highest $M_w$ PHB followed by Re1052 (ΔphaP) then Re1099 (ΔphaR). This observation of larger than wildtype granule size [11] and lower than wildtype $M_w$ PHB in Re1052 (ΔphaP) argues against a simple model in which granules self-organize based on polymer size. In chapter 6, I will revisit the relationship between granule size and $M_w$ in the context of recombinant *Escherichia coli*. 

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5.5 Tables and Figures

**Table 5.1:** *C. necator* strains used in this chapter

<table>
<thead>
<tr>
<th>Strains</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>H16</td>
<td>wildtype, Gm(^R), also termed DSM 428</td>
<td>ATCC 17699</td>
</tr>
<tr>
<td>Re1018</td>
<td><em>phaP-gfp</em> gene replacement strain</td>
<td>[13]</td>
</tr>
<tr>
<td>Re1031</td>
<td><em>phaEC(<em>A)</em></em> gene replacement strain</td>
<td>[16]</td>
</tr>
<tr>
<td>Re1033</td>
<td><em>phaEC(<em>A)</em></em> and <em>phaP-gfp</em> gene replacement strain</td>
<td>[13]</td>
</tr>
<tr>
<td>Re1052</td>
<td>(\Delta)pha(<em>P)</em> strain</td>
<td>[17]</td>
</tr>
<tr>
<td>Re1099</td>
<td>(\Delta)pha(<em>R)</em> strain</td>
<td>[18]</td>
</tr>
</tbody>
</table>

\(^a\)Gm\(^R\), gentamicin resistant

**Table 5.2:** Timecourse of PHB yield and \(M_W\) from batch cultivation of *C. necator*

<table>
<thead>
<tr>
<th>Strain</th>
<th>PHB/CDW (%)</th>
<th>(M_W) (10(^{-6}) × Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PHB-P 24 h</td>
<td>PHB-P 48 h</td>
</tr>
<tr>
<td>H16</td>
<td>51 ± 2</td>
<td>64 ± 0.2</td>
</tr>
<tr>
<td>Re1052</td>
<td>33 ± 0.3</td>
<td>46 ± 2</td>
</tr>
<tr>
<td>Re1099</td>
<td>34 ± 1</td>
<td>50 ± 1</td>
</tr>
</tbody>
</table>

\(^a\)Results from parallel fermentations. \(^b\)Due to instrument error, \(M_W\) measurements are understated for this strain.

**Table 5.3:** PHB yield and \(M_W\) from batch cultivation of *C. necator*\(^a\)

<table>
<thead>
<tr>
<th>Strain</th>
<th>PHB/CDW</th>
<th>(M_W) (Da)</th>
<th>Panel(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H16</td>
<td>70 %</td>
<td>1.9 × 10(^6)</td>
<td>B</td>
</tr>
<tr>
<td>Re1018</td>
<td>71 %</td>
<td>1.2 × 10(^6)</td>
<td>D</td>
</tr>
<tr>
<td>Re1031</td>
<td>63 %</td>
<td>2.9 × 10(^6)</td>
<td>A</td>
</tr>
<tr>
<td>Re1033</td>
<td>64 %</td>
<td>2.3 × 10(^6)</td>
<td>C</td>
</tr>
</tbody>
</table>

\(^a\)Analysis performed at 48 h in PHB-P medium. Results from single fermentation. \(^b\)In Figure 5.1.
Fig 5.1: Granule morphology in 4 strains of *C. necator*. Deconvolved fluorescent optical sections of cells costained with Nile Red and DiO. Each pair of images shows Nile Red (red) and DiO (green) signal overlay on top (granules plus membrane) and Nile Red signal (grayscale) on bottom (granules). Image representative of average morphology in each strain is shown. Scale is identical in every panel (bar = 1 μm). A: Re1031 (*phaEC_Av*); B: H16 (wildtype); C: Re1033 (*phaEC_Av phaP-GFP*); D: Re1018 (*phaP-GFP*).
5.6 References


13. These strains were kindly provided by Dr. Gregory M. York. They were constructed in a manner similar to those described in [23] and [24].


Chapter 6: PHB $M_w$ and granule formation in recombinant *Escherichia coli*

6.1 Introduction. The genome of *Cupriavidus necator* has been reported to encode six potential polyhydroxybutyrate (PHB) depolymerases [1-4], two PHB oligomer hydrolases [5, 6], and four phasins [1, 7], any one of which could have an independent affect on PHB $M_w$. This complexity makes it difficult to interpret the effects of genetic manipulations in *C. necator* on PHB $M_w$, as illustrated in the previous chapter. Both deletion and overexpression of PhaP was shown to reduce PHB $M_w$ (table 5.2), but as described in section 5.4, changes in the expression level of other proteins in the PhaP deletion strain make it impossible to draw conclusions from these observations. This suggests that *E. coli* may be an attractive host for studying the role of the synthase and other proteins in $M_w$ determination, since *E. coli* has no endogenous ability to accumulate high-molecular weight PHB.

Two lines of research in the literature have examined the effects of various gene products on PHB $M_w$ in recombinant *E. coli*. The first, detailed in section 1.4.1, involves the production of PHB with $M_w$ that varies in inverse proportion to synthase activity in strains producing PhaC$_{C_n}$ [8-10]. These results appear to indicate that PHB $M_w$ is at least partially governed by synthase concentration. This is in accord with the observation of Gerngross and Martín that the $M_w$ of PHB synthesized in vitro is inversely correlated to concentration of PhaC$_{C_n}$ [11]. Recently, Kahar et al showed that in *E. coli* producing high $M_w$ PHB, $M_w$ reached a maximum value early in the PHB accumulation stage and remained constant throughout the remainder of the accumulation stage [12]. This shows that the number of PHB chains (total PHB/PHB $M_w$) was increasing, which indicates that a chain termination reaction was most likely occurring. This last point is essential if *E. coli* is to be used as a model system for studying the basis of PHB $M_w$ control.

The second line of research is only represented by one report, in which Machara et al looked at PHB $M_w$ in *E. coli* in the presence and absence of the phasin protein from *Paracoccus denitrificans* [13]. They found that $M_w$ was drastically reduced by the presence of phasin. Seo et al. performed a similar experiment using the phasin from *C. necator*, but looked only at granule morphology and enzyme activity [14]. In this chapter a similar approach is taken to study the relationship between PhaP, PhaR and PHB $M_w$ pointed out by the results described in chapter 5, where a large decrease in PHB $M_w$ in *C. necator* Re1099 ($\Delta$phaR) was seen (~40% of wildtype $M_w$). In addition, the relationship between synthase levels, $M_w$, and granule formation is examined with both the Class I synthase PhaC$_{C_n}$, and the Class III synthase PhaEC$_{AV}$.

6.2 Materials and Methods

6.2.1 Strains, plasmids, and cultivation conditions. Strains and plasmids used in this study are listed in table 6.1. Cultivations were carried out precisely as described in chapter 2, except that *E. coli* MBX1510 precultures were only grown for 4 h. Samples were taken for analysis and microscopy at 24 h because previous work had shown that DH5a strains harboring pAeT41 reached maximum PHB levels at 24 h under the conditions used [15]. Each procedure was usually performed in duplicate, with very little variability between samples. Not all experiments were performed in duplicate, however; refer to figure legends.
Plasmids were constructed as follows. To construct pAGL41, a 5.3 kb fragment containing \textit{phaEC}_{Aw} pha\textit{AC}_{Aw} pha\textit{BC}_{Aw} was isolated by BamHI/EcoRI digestion of pJOE7 [16] and ligated into a 3.1 kb BamHI/EcoRI fragment of pEP2 [17] to yield the plasmid pAGL20. A 2.3 kb BspH1/PstI fragment encoding \textit{phaEC}_{Aw} was isolated from pAGL20 and ligated into a 4.1 kb NcoI/PstI fragment of pMPM-A4Ω [18], producing the plasmid pAGL41. To construct pAGL50, pSP2 was digested with XbaI/HindIII, and the 6.0 kb fragment was isolated; the fragment was treated with T4 DNA ligase to remove 5’ overhangs and a self-ligation reaction was performed to recircularize the plasmid. To construct pAGL51 the 2.2 kb EcoRI/PstI fragment of pAGL41 containing \textit{phaEC}_{Aw} was ligated into the 4.1 kb EcoRI/PstI fragment of pSP2.

6.2.2 Cell dry weight (CDW) measurements, PHB quantitation, PHB extraction, PHB characterization GPC/LS and fluorescence microscopy. Determination of CDW and PHB content was carried out precisely as described in 4.2.2. PHB extraction and characterization was carried out precisely as described in section 2.2.8. Fluorescence microscopy was performed precisely as described in section 5.2.5.

6.2.3 Preparation of cell free extracts. 0.5 ml of culture was pelleted (1 min, 13,000 \times g) and stored at -80°C. Pellet was resuspended in 0.5 ml of cell lysis buffer (50 mM Tris, 1 mM EDTA, 2% SDS, pH=8) at ambient temperature, then sonicated for 30 s (0.8 s on and 0.2 s off) with a Misonix Sonicator (Misonix, Farmingdale, NY) with microtip. Samples were centrifuged for 6 min at 13,000 \times g and the supernatant was stored at -80°C until immunoblotting.

6.2.4. Immunoblotting. Samples were diluted in loading buffer (50 mM Tris, 1 mM EDTA, 2% SDS, 10% glycerol, 0.1% bromophenol blue, pH=8). Standards for PhaC were provided by Dr. Jianmin Tian and were prepared as described [19]. Standards were diluted in loading buffer plus 100 μg/ml BSA to final concentrations of 4, 2, 1, and 0.5 ng/μl. Samples and standards were separated on a 10% Bio-Rad Criterion ready gel (Bio-Rad, Hercules, CA) at 140 V for 70 min. Proteins were blotted onto a PVDF membrane in a Bio-Rad Criterion plate electrophotletter at 80 V for 50 min. Immunoblotting was performed using a Western Light Chemiluminescent immunoblotting system and antibodies to PhaC or PhaEC at a dilution of 1:2000 and 1:1000 respectively. Generation of the antibodies is previously described [20, 21]. Chemiluminescence was detected using a Biochemi System bioimaging system (UVP, Upland, CA).

6.3 Results

6.3.1 Observation of small, regular PHB inclusions (granules) in \textit{E. coli} DH5α. As reported in section 2.3.4 and table 2.2, the strain DH5α pJOE7 (\textit{phaEC}_{Aw}AB_{Cw}) produces lower \textit{M}_W PHB than the strain DH5α pAeT41 (\textit{phaCAB}_{Cw}). These two strains are identical except pJOE7 carries the ORFs of \textit{phaEC}_{Aw} in place of the ORF of \textit{phaC}_{Cw} in pAeT41. Fluorescence microscopy showed that the PHB in these two strains was organized in a dramatically different fashion. In DH5α pAeT41 (\textit{phaCAB}_{Cw}), the PHB formed very large, irregularly shaped inclusions, while in DH5α pJOE7 (\textit{phaEC}_{Aw}AB_{Cw}) the PHB was organized in small round inclusions (figure 6.1). While these inclusions
may or may not have structural dissimilarities from granules in *C. necator*, I will refer to them as granules hereinafter.

In addition, these cells had an elongated phenotype, as did many of the cells in the culture represented by figure 6.2. It has been previously reported that *E. coli* XL1-Blue producing PHB is prone to filamentation due to inactivation of the essential cell division protein, FtsZ [22]. Although no severe filamentation was observed in these cultures, as was observed for XL1-Blue, the elongated phenotype may have been due to suppression of the activity of FtsZ.

6.3.2 Granule formation in *E. coli* depends on concentration of either Class I or Class III synthase. I hypothesized that the differences in $M_w$ and granule phenotype between the *phaEC*$_{Av}$ (pJOE7) and *phaC*$_{Ch}$ (pAeT41) strains may have been due to a higher concentration of the synthase in the *phaEC*$_{Av}$ strain, and that granules would be observed in a strain containing *phaC*$_{Ch}$ if the protein was expressed to high enough levels. The strain DH5α pSP2 (*phaCAB*$_{Ch}$) was cultivated in LB 2% Glucose, 100 μg/ml ampicillin, with 4.0 mM IPTG, conditions which have been shown to produce high levels of synthase activity [10], and fluorescence microscopy was performed after 24 h cultivation. As anticipated, the PHB was organized in granules (figure 6.2).

The plasmids used above contained the synthase (either *phaC*$_{Ch}$ or *phaEC*$_{Av}$) within an operon that also contained *phaA*$_{Ch}$ and *phaB*$_{Ch}$. In order to demonstrate that the synthase itself was sufficient for granule formation, the plasmids pAGL41, pAGL50 and pAGL51 were constructed which contained either *phaC*$_{Ch}$ or *phaEC*$_{Av}$ behind an inducible promoter and were transformed into *E. coli* MBX1510 which contains *phaA*$_{Ch}$ and *phaB*$_{Ch}$ as chromosomal integrations. In order to produce the broadest possible range of synthase concentrations, pAGL41 and pAGL51 were used for expression of the Class III PHA synthase. Both these plasmids contain a high copy ColE1 origin and encode ampicillin resistance. pAGL41, which contains *phaEC*$_{Av}$ linked to the arabinose inducible promoter, P$_{BAD}$ [18], was used without induction in order to produce very low levels of the PhaEC$_{Av}$. pAGL51, in which expression of *phaEC*$_{Av}$ is controlled by P$_{TRC}$ [23], was used without induction or with 0.1 mM IPTG in order to produce intermediate and high levels of PhaEC$_{Av}$, respectively. pAGL50, which was identical to pAGL51 except it encodes *phaC*$_{Ch}$ in the place of *phaEC*$_{Av}$, was used to produce very low levels of PhaC$_{Ch}$ in the absence of inducer, and intermediate levels of PhaC$_{Ch}$ in the presence of 0.25 mM IPTG. A variety of other promoters were tested in order to generate high levels of PhaC$_{Ch}$, but none were identified that produced uniform granule phenotypes in MBX1510 *E. coli*. Consequently, analyses with PhaC$_{Ch}$ were only performed at low and medium levels of enzyme (i.e. 0 and 0.25 mM IPTG).

Fluorescence microscopy on three *E. coli* *phaEC*$_{Av}$ cultures showed that they exhibited the expected phenotypes of an inverse correlation between granule size and synthase concentration (figure 6.3). In the cultures containing MBX1510 pAGL41 (phaEC$_{Av}$), I observed one or a few large granules per cell (figure 6.3, A). MBX1510 pAGL51 (phaEC$_{Av}$) exhibited several intermediate size granules per cell (panels B), while MBX1510 pAGL51 (phaEC$_{Av}$) + 0.1 mM IPTG exhibited many small granules per cell (panel C). Note that as in figure 5.1 panel D, the granules in figure 6.3 panel C are at or below the limit of resolution, so they appear as irregular grainy shapes.
Immunoblotting was performed on cell free extracts (CFE) of these cultures to show that these phenotypes correlated with relative levels of PhaEC_{Av} (figure 6.3 Panel D). Figure 6.4 shows similar results in the MBX1510 phaC_{Cn} strain, except that only phenotypes of large granules (panel A) or intermediate granules (panel B) were observed, and were correlated with protein levels (panel C).

PHB was extracted from these cultures and M_w was analyzed by GPC/LS. The data in table 6.2 show that PHB M_w was inversely correlated with levels of either PhaC_{Cn} or PhaEC_{Av}.

6.3.3 M_w of PHB produced in E. coli is reduced in the presence of PhaP. In section 5.3.1 it was shown that the PHB produced in the Cupriavidus necator strain Re1052 was of a markedly lower M_w than in the wildtype strain (table 5.2). In an attempt to determine whether this was due to the absence of PhaR, the overexpression of PhaP, or to some other factor, E. coli DH5α strains were constructed which contained plasmids encoding either phaCAB_{Cn} alone (pAE41, pSW213), phaCAB_{Cn} and phaR_{Cn} (pAE41, pGY104), or phaCAB_{Cn}, phaP_{Cn} and phaR_{Cn} (pAE41, pGY105) (table 6.1). These strains were grown for 24 h on LB 2% glucose in order to produce PHB which was extracted and characterized by coupled gel permeation chromatography/multiangle light scattering. As shown in table 6.3, DH5α pAE41 pGY105 (phaCAB_{Cn} phaR_{Cn} phaP_{Cn}) produced lower M_w PHB than the other two strains. Although this experiment was not performed in duplicate, a similar experiment was performed using the plasmid pSJ51 [9] as a source of the PHB biosynthetic genes, and the same result, namely dramatically lowered PHB M_w in the presence of phasin, was observed (not shown).

Fluorescence microscopy was also performed in order to confirm the expected phenotypes of the strains in table 6.3, namely, granule formation in the presence of phaP_{Cn} and no granule formation in its absence (figure 6.5, Panels A – C). Finally, to rule out the possibility that expression of PhaP was resulting in accumulation of higher amounts of PhaC_{Cn}, thereby producing the phenotypes of granule formation and M_w change seen in section 6.3.2, immunoblotting with PhaC_{Cn} anti-serum was carried out on CFE from these cultures. The results in figure 6.5, Panel D show that there was no more PhaC_{Cn} in the presence of PhaP (lanes 10, 11 & 16, 17) than in its absence (lanes 6-9 & 12-15), showing that the effect of phaP on PHB M_w was not due to changes in the level of PhaC_{Cn}.

6.4 Discussion. As described in section 1.4.1, the inverse correlation between synthase activity and PHB M_w in E. coli [10] and the insensitivity of PHB M_w to synthase activity in C. necator [24] suggests that there may be an unidentified factor in C. necator that contributes to PHB M_w control. The observation that the strain Re1099 (ΔphaR) produced low M_w PHB implicated either PhaP or PhaR in this process. In the current chapter, I show that the presence of phaP and phaR is sufficient to reduce PHB M_w and induce granule formation, while phaR alone has no effect on M_w or granule formation (table 6.3, figure 6.5). However, although phaP/phaR is sufficient to induce granule formation, it is not required, as shown in figures 6.1, 6.2, 6.3, and 6.4, where granules are observed but the phasin gene is not present. Neither is it required to produce lower M_w PHB as described by Sim et al. for the Class I synthase, PhaC_{Cn} [10], and as shown in table 6.2 for the Class III synthase, PhaEC_{Av}.
6.4.1 Relationship of PHB $M_W$ and granule morphology. The correlation between $M_W$ and granule size in the *C. necator* strains H16, Re1018, Re1031, Re1033, and Re1099 (chapter 5), in the *E. coli* MBX1510 strains in figures 6.3 and 6.4 and table 6.2, and in the *E. coli* DH5a strains in figure 6.5 and table 6.3 would seem to point to a causal relationship between $M_W$ and granule size. One simple model for such a relationship would be that the granules self-organize based on $M_W$ of the polymer produced. Strict causality is ruled out, however, by the results with *C. necator* Re1052 (ΔphaP) which has larger than wildtype granules [7], but produces lower $M_W$ polymer (table 5.2). In addition, the cells in figure 6.3 Panel C have much larger granules than those in figure 6.4 Panel B, although they contain PHB of the same $M_W$ (1.8 × 10^6 Da), providing further evidence against a causal relationship between these phenomena.

6.4.2 Model for production of a narrow $M_W$ distribution in the chain termination reaction. Tian et al. proposed a model for the PHA synthase in which the PHB chain is extruded through an exit channel, within which it is bound tightly to the enzyme [25]. In section 3.4.1, I proposed that the termination reaction takes places via the occasional passage of monomer cofactor in the thiolate form (e.g. S-CoA) through this exit channel, allowing hydrolysis of the PHB chain at an internal ester (figure 3.2). This exit channel functions as a size-based specificity filter, explaining the observation that a smaller cofactor (S-NAC), which would suffer less steric hindrance, is able to carry out termination more frequently than a larger cofactor (S-CoA).

This model is not sufficient to explain the observation in section 2.3.5 that HB-NAC functions as a highly efficient terminator during HB-CoA polymerizations, nor does it explain why PHB has a low polydispersity. Both of these observations suggest that during the polymerization reaction, the synthase switches in some manner from a highly processive state in which termination is seldom or never observed to a less processive state in which termination is more frequent. Furthermore, this switch appears to be a continuous process, so that S-NAC becomes an efficient terminator when PHB has reached ~50 kDa, S-Pant when it is near 300 kDa, and S-CoA not until it is above 1 MDa (table 3.1).

I propose that the mechanism that governs this switch is a change in the tertiary structure of the exit channel on the PHA synthase that changes the size exclusion limit of the specificity filter. In this model, the thiolate form of the cofactor is very efficient at hydrolyzing the PHB chain, as is observed of S-NAC in copolymerizations of HB-CoA and HB-NAC (section 2.4), so it must be excluded from the exit channel early in polymerization for high $M_W$ PHB to be produced. Yet it must enter the exit channel at a higher frequency later during polymerization, or else, at the least, very high polydispersities would be observed and perhaps no termination would be seen at all. A change in the tertiary structure of the exit channel and its binding of the PHB chain during the polymerization reaction would allow the activated cofactor access to the exit channel at a higher rate, causing more frequent termination of the PHB chain. This explains how a given substrate will produce PHB of a $M_W$ with a low polydispersity in a manner that depends on the size of the cofactor.

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6.4.3 Model for $M_W$ determination in the chain termination reaction. The *in vitro* reactions used to study termination in this work contained only purified synthase, substrate, and buffers. The mechanism that causes the change in tertiary structure described above must therefore require only these components. In addition, an accurate description of the mechanism for PHB $M_W$ control must take into account the observations that synthase concentration impacts $M_W$ (tables 3.1 & 6.2 and [10]), as does the presence of phasin (table 5.2 & 6.3 and [13]) and the structure of the phasin (table 5.3).

I propose that the elongating PHB chain exerts a force on the enzyme exit channel and active site in a manner proportional to the chain length, and furthermore that this force acts to destabilize a conformation in which the PHB chain is tightly bound, causing the specificity filter to open up and allowing the cofactor thiolate to enter. In one simple model, the force that the PHB exerts will be proportional to the mass of the polymer in the following manner. During polymerization, monomers of hydroxybutyrate are being added to the polymer chain within the active site of the enzyme and PHB is being extruded from the active site. This is loosely analogous to the actin based mobility system, such as is used by *Listeria monocytogenes* in which actin is polymerized on the cell membrane of that intracellular pathogen, propelling it around the cytoplasm of its host [26]. PHB is thus exerting force on the enzyme active site as it is being synthesized. Assuming the PHB chain is in an extended conformation while bound in the exit channel, that force would cause the flexible PHB chain to buckle within the exit channel, exerting force on the channel walls and destabilizing the exit channel. In the simplest case, that force would be proportional to the mass of the polymer, but as noted, PHB is a flexible polymer, so only a fraction of the force would be translated back to the active site. In addition, the synthase sits on the surface of the granule, so that the newly synthesized PHB is interacting with the bulk polymer of the PHB. Therefore, such a straightforward explanation of the proportionality of force to polymer mass will not suffice.

One working model to explain these observations is presented below, which is presented in a highly simplified schematic in figure 6.6. (I) The PHA synthase (orange) bound to the surface of the PHB granule (pink) as part of a hypothetical complex (represented as two synthases here), shortly after initiating a new PHB chain. The synthase is stabilized by the presence of phasins (green), synthases (red), and other proteins that may be present (not shown). In addition, these proteins serve to constrain the local surface architecture of the PHB granule, resisting any tendency of the granule surface to bulge. In the case of the wildtype organism, the architecture is envisioned as that proposed by Dennis et al and described in detail in section 1.2.2.1 [27], where the synthase is located in the pore complex and bound on the surface by a network of phasin protein fibers. (II) As PHB is synthesized, it fills any available space beneath the synthase complex and is forced into the bulk polymer of the granule. (III) As polymerization continues, the local density of PHB beneath the enzyme steadily increases as the PHB is prevented from freely spreading on the surface due to the presence of other nearby proteins. As a result the force required to extrude the polymer from the enzyme steadily increases, resulting in a continuously greater destabilization of the exit channel.

In this model, the $M_W$ observations related to phasins and synthase concentration are explained as a result of constraining the localization of the PHA synthase on the granule surface and constraining the structure of the granule surface itself. The
combination of these two factors allows for explanations of all the results presented from *in vitro* and *in vivo* experiments, although given the absence of detail with respect to the structure of the granule surface under these conditions, the explanations are necessarily speculative. The inverse correlation between synthase concentrations both during *in vitro* polymerization of HB-CoA by PhaEC\textsubscript{Av} (table 3.1) and in *E. coli* strains expressing PhaC\textsubscript{Ch} or PhaEC\textsubscript{Av} (table 6.2) may be due to interactions between synthase molecules on the surface of the granule, causing a concentration dependent decrease in the mobility of the synthase on the granule surface, i.e. constraining the localization of the PHA synthase. In the ΔphaR *C. necator* strain described in table 5.2, lower than wildtype \(M_w\) is observed because excess PhaP produces additional constraints on the localization of the synthase and on the structure of the granule surface. This also explains the reduction of \(M_w\) in *E. coli* producing PhaP (table 6.3). A similar explanation can be proffered for the strains harboring *phaP*-GFP vs. wildtype *phaP* (table 5.3) wherein the effect of \(M_w\) reduction in the mutant strain is attributable to either stabilization of PhaP, producing higher concentrations on the granule surface, or to the increased size of the GFP-tagged phasin producing a greater constraint on the granule surface. Finally, the most difficult to rationalize observation, that of reduced PHB \(M_w\) in the ΔphaP *C. necator* strain (table 5.2) could be explained as due to the high levels of expression of the PhaP homolog, PhaP3 [1]. It may be that PhaP3 is able to constrain the synthase on the granule surface, promoting termination, but functions as a less efficient emulsifier than PhaP, so that large granules are produced. I reemphasize that these explanations are purely speculative. As further physical details of the granule surface are elucidated, it may become apparent that these explanations are flawed, but at this time the models presented in figures 3.2 and 6.6 and sections 3.4.1, 6.4.2 and 6.4.3 provide a complete working model for the control of \(M_w\) in PHB biosynthesis.

### 6.4.4 Formation of granules in *E. coli*

Phasins have been proposed to function as emulsifiers, preventing granule coalescence during the polymerization of PHB [28]. Three major pieces of evidence support this model: 1) disruption of *phaP\textsubscript{Ch}* causes the accumulation of one large granule per cell [7]; 2) expression of phasin in *E. coli* XL1-Blue harboring *phaCAB\textsubscript{Ch}* or *phaCAB*\textsubscript{pn} (*Paracoccus denitrificans*) [13, 28] or in *E. coli* DH5α harboring *phaCAB\textsubscript{Ch}* (figure 6.5) causes the accumulation of many small granules per cell; and 3) addition of phasin (PhaP\textsubscript{Ch}) to *in vitro* polymerization reactions containing PhaEC\textsubscript{Av} and HB-CoA results in the formation of smaller particles than are observed in the absence of phasin [29]. In the current work, the observation of a strict correlation between PhaP and PHB levels (figure 4.6) led me to hypothesize that the phasin may be performing another, previously unsuspected function, such as catalyzing a chain transfer reaction, as discussed in section 5.4. However, I have found no evidence to support such a hypothesis. Instead my observations are consistent with the proposed role of PhaP as an emulsifier.

Three novel observations in this work are in accord with the emulsifier model. Granules in the *phaP*-GFP strains are notably smaller than granules in the wildtype organism (figure 5.1). This may be because the GFP domain stabilizes the PhaP protein or because the increase in the protein size allows it to cover a larger area of the granule surface. It was also observed that presence of *phaP\textsubscript{Ch}* and *phaR\textsubscript{Ch}* in *E. coli* DH5α harboring *phaCAB\textsubscript{Ch}* induces the formation of granules (figure 6.5). This recapitulates
the observations in XL1-Blue harboring $phaCAB_Cn$ or $phaCAB_Pn$ [13, 28], but is notable because it shows that granule formation is not particular to that strain, and that the ability to form granules is not related to the ability of XL1-Blue to accumulate high levels of PHB [30]. Finally, it was shown that high levels of the PHA synthase are sufficient to induce granule formation in the absence of phasin in *E. coli* (figures 6.2, 6.3 & 6.4). The synthase is an amphipathic molecule, as is PhaP, able to exist in a soluble form in purified preparations [11, 29] and also able to bind to the surface of the granule [31]. As such, I believe that in this recombinant system, the synthase itself is present at such high levels on the surface of nascent granules that it is able to prevent their coalescence in the same manner as the phasin. Differences in the level of PHA synthase may explain the results described in section 1.4.2, where three groups observed different size granules when producing PHB in *E. coli* XL1-Blue [13, 28, 30]. Each of these groups was using a different plasmid vector for expression of the synthase, and it may be that different levels of the synthase in each strain were leading to the different granule phenotypes.
### 6.5 Tables and Figures

**Table 6.1:** Strains and plasmids used in this chapter

<table>
<thead>
<tr>
<th></th>
<th>Descriptiona</th>
<th>Reference or source</th>
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<tr>
<td><strong>E. coli strains</strong></td>
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<td></td>
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<td>DH5α</td>
<td>Lab strain</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>MBX1510</td>
<td><em>phaA</em>&lt;sub&gt;Cn&lt;/sub&gt;, <em>phaB</em>&lt;sub&gt;Cn&lt;/sub&gt; knock-in strain</td>
<td>Metabolix, Inc., Cambridge MA</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<td></td>
</tr>
<tr>
<td>pAeT41</td>
<td><em>phaCAB</em>&lt;sub&gt;Cn&lt;/sub&gt;, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>[32]</td>
</tr>
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<td>pAGL41</td>
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<td>This study</td>
</tr>
<tr>
<td>pAGL50</td>
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</tr>
<tr>
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<td>This study</td>
</tr>
<tr>
<td>pGY104-</td>
<td><em>phaR</em>&lt;sub&gt;Cn&lt;/sub&gt;, Tet&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>[10]</td>
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<tr>
<td>pSW213</td>
<td>Tet&lt;sup&gt;R&lt;/sup&gt;</td>
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aGm<sup>R</sup>, gentamicin resistance; Amp<sup>R</sup>, ampicillin resistance; Tet<sup>R</sup>, tetracycline resistance
### Table 6.2: PHB Yield and $M_W$ in E. coli MBX1510<sup>a</sup>

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>IPTG (mM)</th>
<th>Synthase</th>
<th>PHB (mg/ml)</th>
<th>PHB $M_W$ (Da)</th>
<th>Panel&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td>pAGL50</td>
<td>none</td>
<td>$phaC_{Cn}$</td>
<td>3.3 ± 0.0</td>
<td>3.5 ± 0.02 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
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<td>$phaC_{Cn}$</td>
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<td>1.8 ± 0.03 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>B</td>
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<tr>
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<td>$phaC_{Cn}$</td>
<td>3.2 ± 0.02</td>
<td>1.7 ± 0.08 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>n. s.&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
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<td>$phaE_{Cn}$</td>
<td>1.7 ± 0.0</td>
<td>4.1 ± 0.50 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>C</td>
</tr>
<tr>
<td>pAGL51</td>
<td>none</td>
<td>$phaE_{Cn}$</td>
<td>4.1 ± 0.1</td>
<td>3.5 ± 0.01 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>D</td>
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<tr>
<td>pAGL51</td>
<td>0.1</td>
<td>$phaE_{Cn}$</td>
<td>1.9 ± 0.1</td>
<td>1.8 ± 0.01 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
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</table>

<sup>a</sup>Cultivated in LB 2% glucose with 100 μg/ml ampicillin and 10 μg/ml tetracycline. Results from parallel cultivations. <sup>b</sup>Panel in Figure 6.3. <sup>c</sup>not shown.

### Table 6.3: PHB Yield and $M_W$ in E. coli DH5α pAeT41<sup>a</sup>

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant gene(s)</th>
<th>PHB (mg/ml)</th>
<th>PHB $M_W$ (Da)</th>
<th>Panel&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>pSW213</td>
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<td>6.9</td>
<td>2.9 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
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<td>pGY104-</td>
<td>$phaR$</td>
<td>6.8</td>
<td>2.7 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
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<td>pGY105</td>
<td>$phaP phaR$</td>
<td>7.6</td>
<td>1.2 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
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<sup>a</sup>Cultivated in LB 2% glucose with 100 μg/ml ampicillin and 10 μg/ml tetracycline. pAeT41 encodes $phaCAB_{Cn}$. Results from a single cultivation. <sup>b</sup>Panel in Figure 6.4.
Fig 6.1: Granule morphology in *E. coli* DH5α harboring Class I or Class III synthase. Deconvolved fluorescent optical sections of cells costained with Nile Red and DiO. Each pair of images shows Nile Red (red) and DiO (green) signal overlay on top (granules plus membrane) and Nile Red signal (grayscale) on bottom (granules). Image representative of average morphology in each strain is shown. Scale is identical in every panel (bar = 1 µm). A: *E. coli* DH5α pAeT41 (*phaCABCn*); B: *E. coli* DH5α pJOE7 (*phaEC_AvABCn*).
Figure 6.2: Granule morphology in *E. coli* pSP2 (*phaCAB*<sub>Cn</sub>). Deconvolved fluorescent optical section of cells costained with Nile Red and DiO. Pair of images shows Nile Red (red) and DiO (green) signal overlay on top (granules plus membrane) and Nile Red signal (grayscale) on bottom (granules). Image representative of average morphology is shown. Scale bar = 1 μm.
Figure 6.3: Granule morphology depends on levels of PhaEC_Av in *E. coli* MBX1510 (phaAB_Cu). (A-C) Deconvolved fluorescent optical sections of cells costained with Nile Red and DiO. Each pair of images shows Nile Red (red) and DiO (green) signal overlay on top (granules plus membrane) and Nile Red signal (grayscale) on bottom (granules). Image representative of average morphology in each cultivation is shown. Scale is identical in every panel (bar = 1 μm). A: *E. coli* MBX1510 pAGL41; B: *E. coli* MBX1510 pAGL51; C: *E. coli* MBX1510 pAGL51, 0.1 mM IPTG. D: Anti-PhaEC_Av immunoblot of cell lysates from cultures in panels A-C. Duplicates are from parallel cultures. Lane 1, 2: 5 μl cell free extract (CFE) from A; 3, 4: 5 μl CFE from B; 5, 6: 5 μl CFE from C; 7,8: 1 μl CFE from A; 9, 10: 1 μl CFE from B; 11, 12: 1 μl CFE from C.
**Figure 6.4: Granule morphology in E. coli MBX1510 (pha\textit{AB}_{Cn}) pAGL50 (pha\textit{C}_{Cn}).** (A, B) Deconvolved fluorescent optical sections of cells costained with Nile Red and DiO. Each pair of images shows Nile Red (red) and DiO (green) signal overlay on top (granules plus membrane) and Nile Red signal (grayscale) on bottom (granules). Image representative of average morphology in each cultivation is shown. Scale is identical in every panel (bar = 1 \mu m). A: \textit{E. coli} MBX1510 pAGL50; B: \textit{E. coli} MBX15 pAGL50, 0.25 mM IPTG. C: Anti-Pha\textit{C}_{Cn} immunoblot of cell lysates from cultures in panels A-B and MBX1510 pAGL50, 2.0 mM IPTG (micrograph not shown). Duplicates are from parallel cultures. Lane 1, 2: 5 \mu l cell free extract (CFE) from A; 3, 4: 5 \mu l CFE from B; 5, 6: 5 \mu l CFE from MBX1510 pAGL50, 2.0 mM IPTG; 7,8: 1 \mu l CFE from A; 9, 10: 1 \mu l CFE from B; 11, 12: 1 \mu l CFE from MBX1510 pAGL50, 2.0 mM IPTG.
Figure 6.5: *phaP* induces granule formation in *E. coli* without altering PhaC\textsubscript{Cn} levels. (A-C) Deconvolved fluorescent optical sections of cells costained with Nile Red and DiO. Each pair of images shows Nile Red (red) and DiO (green) signal overlay on top (granules plus membrane) and Nile Red signal (grayscale) on bottom (granules). Image representative of average morphology in each strain is shown. Scale is identical in every panel (bar = 1 µm). A: *E. coli* DH5\textalpha{} pAeT41 pSW213 (*phaCAB\textsubscript{Cn}*); B: *E. coli* DH5\textalpha{} pAeT41 pGY104 (*phaCAB\textsubscript{Cn}phaR\textsubscript{Cn}*); C: *E. coli* DH5\textalpha{} pAeT41 pGY105 (*phaCAB\textsubscript{Cn}phaP\textsubscript{Cn}phaR\textsubscript{Cn}*); D: Anti-PhaC immunoblot of cell lysates from cultures in panel A-C. Duplicates are from parallel cultures. Lane 1: 4 ng PhaC; 2: 2 ng PhaC; 3: 1 ng PhaC; 4: 0.5 ng PhaC; 5: blank; 6, 7: 5 µl cell free extract (CFE) from A; 8, 9: 5 µl CFE from B; 10, 11: 5 µl CFE from C; 12, 13: 1 µl CFE from A; 14, 15: 1 µl CFE from B; 16, 17: 1 µl CFE from C.
Figure 6.6: Model for generation of force at the enzyme active site as a function of polymer size. I: PHA synthase (red) bound to the surface of the PHB granule (pink) after initiating a new PHB chain, surrounded by phasin (green) and other synthases (red) which constrain it and the granule surface. II: Rigid protein network constrains granule surface, so that PHB concentration increases directly under synthase, increasing force required to extrude PHB. III: PHB chain exerts force on exit channel, destabilizing its binding to PHB chain.
6.6 References


Chapter 7: Directions for future research

7.1 Introduction. In the preceding chapters, models are presented for mechanisms governing the $M_W$ of polyhydroxybutyrate during enzymatic polymerization that are amenable to experimental validation. Three specific mechanisms are described. Firstly, the chain termination reaction during the polymerization of polyhydroxybutyrate by the PHA synthase is said to be the result of a transesterification reaction involving the PHB chain and a thiolate released from the substrate in the enzyme active site (section 3.4.1). Secondly, the frequency of this reaction is said to be governed by a size-based selectivity filter which is a function of the tertiary structure of the enzyme (sections 3.4.1 and 6.4.2). Third and finally, the tertiary structure of the enzyme is said to be controlled by a feedback mechanism involving the length of the PHB chain (section 6.4.3). Hopefully we will soon have a crystal structure of a PHA synthase, with which we will be able to evaluate these models, but in the absence of that structure, I describe below potential experiments to test these models. In addition, I discuss potential applications of the research on $M_W$ control and its insights into granule formation that resulted from this work.

7.2 Chain termination reaction. A key piece of evidence in support of the model that the chain termination reaction involves a transesterification by the substrate thiolate is the observation that PHB synthesized from HB-NAC is covalently modified by NAC (section 2.3.2). The native substrate and other substrate analogs should produce similar results, e.g. PHB-CoA should be synthesized from HB-CoA, PHB-Pant should be synthesized from HB-Pant. It is unlikely that NMR will be sufficient for resolving the ends of PHB synthesized from HB-CoA which has a $M_W$ in the vicinity of 3-4 $\times 10^6$ Da. However, it is likely that the structure of the $3 \times 10^5$ Da polymer synthesized from HB-Pant could be resolved by NMR. One important future experiment would then be to synthesize a large amount of HB-Pant, polymerize it using the synthase, and submit the product to NMR. A similar experiment using the native substrate could be performed by using HB-CoA with a radiolabel (e.g. $^{14}$C) in the CoA moiety. Polymer would be readily purified away from free CoA after the reaction, and the radiolabel could be detected. If labeled CoA was not commercially available, it could be synthesized using the method of Nazi, et al [1].

7.3 Frequency of chain termination reaction as a function of tertiary structure. Additional evidence for this model could come from analysis of copolymerizations involving HB-CoA and HB-Pant or HB-Pant and HB-NAC, in a manner similar to the copolymerization experiment with HB-CoA and HB-NAC presented in section 2.3.5. The result from copolymerization of HB-CoA and HB-Pant would be expected to be essentially identical to that seen with HB-CoA and HB-NAC (i.e. bimodal $M_W$ distribution), since the $K_m$ of PhaEC$_{DH}$ for HB-Pant is similar to its $K_m$ for HB-NAC, so HB-Pant monomers would only occasionally get incorporated during the rapid phase of HB-CoA polymerization. This would produce a bimodal distribution of $M_W$, with one population equal in size to polymer produced during an HB-Pant homopolymerization, and the other population with a $M_W$ intermediate to that of a HB-Pant homopolymerization and a HB-CoA homopolymerization. With the HB-NAC HB-Pant
copolymerization, the result would be expected to be quite different, since the $K_m$ of each substrate is similar. Essentially one population would be expected to be seen, with intermediate $M_w$ to that of polymer produced in a homopolymerization of either substrate. Furthermore, NMR endgroup analysis of this polymer should show that it is modified with NAC alone instead of Pant, since the smaller NAC thiolate would be expected to be involved in termination at a much greater frequency than the Pant thiolate. Endgroup analysis could also be performed on the polymer from a HB-CoA HB-NAC copolymerization if the two polymer populations (figure 2.2) could be separated by preparative GPC or if the reaction was quenched during the fast phase of polymerization.

In addition to recapitulating the copolymerization experiments with different substrates, new substrates could be synthesized with different size cofactor moieties. If the enzyme will accept these new substrates, polymer could be made in vitro and its $M_w$ characterized. For example, the model would predict that the substrates hydroxybutyryl-N-(N-Acetyl-$\beta$-alanyl)cysteamine (scheme 7.1) would produce PHB with a $M_w$ between that produced from HB-NAC and HB-Pant.

\[\text{Scheme 7.1}\]

Site directed mutagenesis studies may also prove informative, as point mutations may alter the structure of the exit tunnel, allowing the thiolate greater or lesser access to the PHB chain. In light of that, it is interesting to note that in two recent examinations of PhaC\textsubscript{Re} point mutants, in which a conserved Gly [2] or Ala [3] were mutated, both small residues, nearly all the mutants examined produced higher $M_w$ polymer. However, in the absence of a crystal structure, these results are difficult to interpret.

**7.4 Mechanism for tertiary structure destabilization.** This third aspect of the model is the most difficult to devise an empirical test for. It requires a specific, controlled mechanism to either destabilize the tertiary structure of the exit channel or to change the force that the polymer is exerting on the enzyme. The former may be accomplished during copolymerizations of different acyl-CoA’s. For example, in a HB-CoA, hydroxyhexanoyl-CoA copolymerization, incorporation of the larger monomer into the polymer chain may destabilize the exit channel enough to allow access to $\text{\textsuperscript{S}-CoA}$, resulting in premature termination and production of lower $M_w$ polymer. However, other explanations could fairly be posited if the described result was observed.

A more rigorous, but experimentally challenging, test could be performed if a two phase system for the polymerization reaction could be developed. The synthase would need to be made to dock at the oil/water interface in a manner analogous to its docking on the granule surface as shown in figure 6.6. The PHB chain would then be extruded into the oil phase rather than into the granule, and changing the viscosity of the oil phase would be expected to change the force that the chain is exerting back on the enzyme. More viscous oils would be expected to produce lower $M_w$ PHB. Unfortunately, the technical details of such an experiment would be formidable, not the least of which
would be demonstrating that the synthase was actually localized at the interface and in the correct orientation for the PHB chain to enter the oil phase.

Finally, it may be possible to identify compounds that would be incorporated into the granule during polymerization and change the viscosity of the granule itself. Artificial amorphous granules could be synthesized by the method of Sanders and coworkers [4], incorporating candidate compounds. Atomic force microscopic methods currently under development in our lab would be able to determine the relative viscosity of these granules. PHB could then be synthesized in vitro in the presence of various concentrations of these viscosity changing agents, and both relative viscosity measurements and $M_w$ analysis could be performed on the polymer produced. If several compounds of sufficiently different chemical structure were used and similar results seen, direct effects of interactions of these compounds with the PHA synthase could be ruled out as a cause of the $M_w$ changes, providing evidence for the importance of the granule viscosity in determination of $M_w$.

7.4 Potential applications. If this model proves correct, it will provide a basis for rational engineering of PHA synthesis for desired $M_w$ control. The model implies that protein engineering of the exit channel in the PHA synthase will have dramatic impacts on $M_w$, and that it should be possible to produce enzymes that maintain low polydispersity of product while either increasing or decreasing chain length. In addition, it points the way towards design of fermentation parameters for control of product $M_w$ if additives can be identified to alter the viscosity of granules in vivo.

In addition, the findings on granule formation by the PHA synthase provide another means by which to exploit potential applications of the PHB granule. Recently, the purification of recombinant proteins by translationally fusing them to the phasin protein has been reported [5, 6]. PHA synthase is also bound tightly to the granule surface, and by being able to drive the formation of small granules, could be used to increase the ratio of the protein binding surface to the granule, thereby increasing purification yields. In addition, Potter and Steinbuchel point out in a recent review the need for nanoscale structures in nanomaterials research and applications, a role that PHA granules could potentially fill due to their scale, degradability and the ability to control their size through phasin expression [7]. Here I have described another apparent means to drive granule size which may prove superior to a phasin-based mechanism.

7.5 Concluding remarks. This work provides the first detailed model of the process for $M_w$ control in the enzymatic polymerization of PHB which can account for all known phenomena pertaining to chain termination. It includes both a novel model for the chain termination and a proposal for a mechanism by which that reaction is governed. Hopefully, future work along the lines described above will soon validate this model, or point a way towards the true underlying mechanism of this fascinating phenomenon.
7.6 References


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knockout strains of Ralstonia eutropha H16 for variation in PHB M_W; 3) producing
PHB in Escherichia coli with synthase and phasin genes under inducible promoters
and analyzing effect on M_W
- Studied PHB granule formation using deconvolution fluorescence microscopy in
recombinant E. coli and in R. eutropha during early stages of PHB accumulation
- Analyzed changes in transcription of PHB homeostasis-related genes by real-time
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Advisor: Dr. Anthony J. Sinskey, Department of Biology

Undergraduate Research, SUNY at Stony Brook, 1998-1999
- Performed biophysical measurements of membrane associated diphtheria toxin
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- Developed profitability model used for price setting on auto loans
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