Production of Polyhydroxyalkanoate Copolymers from Plant Oil

by

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ABSTRACT

Polyhydroxyalkanoates (PHAs) are carbon storage polymers produced by a variety of bacteria. The model organism for studying PHA synthesis and accumulation is *Ralstonia eutropha*. This species can be used to convert renewable resources into PHA bioplastics, which can serve as biodegradable alternatives to traditional petrochemical plastics. A promising feedstock for PHA production is palm oil, a major agricultural product in Southeast Asia. Strains of *R. eutropha* were engineered to accumulate high levels of a PHA copolymer containing 3-hydroxybutyrate and 3-hydroxyhexanoate when grown on palm oil and other plant oils. This type of PHA has mechanical properties similar to those of common petrochemical plastics. The engineered strains expressed a PHA synthase gene from the bacterial species *Rhodococcus aetherivorans* I24. The amount of 3-hydroxyhexanoate in the PHA was controlled by modulating the level of acetoacetyl-CoA reductase (PhaB) activity in the engineered *R. eutropha* strains. Whole genome microarray studies were carried out to better understand *R. eutropha* gene expression during growth on plant oils. These results have provided insights that will allow for additional improvements to be made to the engineered strains.

In order to study growth of *R. eutropha* strains on plant oils, fermentation methods were developed to grow the bacteria in oil medium and measure consumption of the carbon source. In one of these methods, the glycoprotein gum arabic was used to emulsify the plant oil. This emulsification reduced the lag phase in oil cultures and allowed representative samples to be taken early in experiments. High density palm oil fermentations were also carried out using unemulsified oil, which is more representative of industrial culture conditions. Techniques were developed for recovery of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) from *R. eutropha* biomass. Methyl isobutyl ketone was used to extract the PHA, and the polymer was precipitated from solution by addition of an alkane. A process model based on this procedure was developed for continuous recovery of PHA. The results described in this thesis include several advancements towards the goal of industrial PHA production from palm oil.

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Finally I thank my parents, Charles and Pamela Budde, for their constant love and support. They instilled in me an appreciation for learning and hard work. None of this would have been possible without them.

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A Note on Polyhydroxyalkanoate Nomenclature

The chemical units that make up polyhydroxyalkanoate (PHA) polymers can exist in three forms:

(i) thioesters in which the hydroxyalkanoic acid (HA) is covalently linked to coenzyme A (CoA)
(ii) monomers within a PHA chain
(iii) free hydroxyalkanoic acids.

Throughout my thesis the CoA thioesters will be described as "HA-CoA," in which HA will vary depending on the particular hydroxyalkanoate being discussed. Monomer units and free acids are both described as "HA." The text will indicate whether monomer units within a polymer chain or free acids are being discussed. PHA copolymers comprised of multiple monomer types are described as "P(HB-*co*-HV)," which in this example is a copolymer containing HB and HV units. The reader should assume that all hydroxyalkanoic acids are of the (*R*)-3-hydroxyalkanoate form, unless otherwise specified.
Abbreviations and terms used in this thesis are listed below.

Abbreviation	Term	
Polymers and monomers		
PHA	Polyhydroxyalkanoate	
SCL-PHA	Short chain length polyhydroxyalkanoate	
MCL-PHA	Medium chain length polyhydroxyalkanoate	
PHB	Polyhydroxybutyrate	
HA	Hydroxyalkanoate	
HB	Hydroxybutyrate	(4 carbon unit)
HV	Hydroxyvalerate	(5 carbon unit)
HHx	Hydroxyhexanoate	(6 carbon unit)
HHp	Hydroxyheptanoate	(7 carbon unit)
HO	Hydroxyoctanoate	(8 carbon unit)
PLA	Polylactic acid	
LA	Lactate	
Lipids		
TAG	Triacylglycerol	
DAG	Diacylglycerol	
MAG	Monoacylglycerol	
FFA	Free fatty acid	
Experimental techniques		
DSC	Differential Scanning Calorimetry	
GC	Gas Chromatography	
GPC	Gel Permeation Chromatography	
HPLC	High Performance Liquid Chromatography	
NMR	Nuclear Magnetic Resonance, used in reference to NMR spectroscopy	
TEM	Transmission Electron Microscopy	
TLC	Thin Layer Chromatography	

Abbreviation	Term
Culture measurements	
CDW	Cell dry weight
RCDW	Residual cell dry weight, defined as CDW minus PHA
Sp act	Specific activity, used in reference to enzymatic assay measurements
SD	Standard deviation
Polymer properties	
M_n	Number average molecular weight
M_w	Weight average molecular weight
PDI	Polydispersity index, defined as M_w/M_n
T_g	Glass transition temperature
T_m	Melting temperature
Organic solvents	
BA	Butyl acetate
MEK	Methyl ethyl ketone
MIBK	Methyl isobutyl ketone

Chapter 1
Introduction to Polyhydroxyalkanoates

Storage of Carbon and Other Nutrients

Organisms throughout nature have evolved diverse systems for storing essential nutrients, such as carbon, nitrogen, and phosphorous. This storage frequently entails the accumulation of polymers, which can be depolymerized when the monomers are needed for synthesis of other metabolites or for energy generation. In many cases the polymers form insoluble inclusions, which are beneficial because they do not influence reactions involving soluble substrates, and because the polymers do not contribute to the osmotic potential of the cell in which they are stored. These storage polymers are synthesized in a nontemplate-dependent manner, as opposed to DNA, RNA, and proteins, whose synthesis is directed by information encoded in other biopolymers (105). Nitrogen can be stored as cyanophycin (73), while phosphorous can be stored in the form of polyphosphate (58). While these compounds are of significant academic interest, carbon storage molecules are more widespread and have greater industrial importance.

Carbon storage is ubiquitous throughout the eukaryotic and prokaryotic worlds. Mammals produce glycogen and triacylglycerols (TAGs) to store carbon and energy. Glycogen is a short term fuel used to buffer the glucose concentration in the blood, while TAGs are used for long term energy storage. Plants store starch and TAGs in their seeds to provide nourishment for growing embryos. Humans have long made use of use of these carbon storage compounds as food, and later developed more technologically advanced applications (e.g. using tallow to make soap and candles). Prokaryotes also store carbon, and microbial carbon storage molecules have the potential to become resources for the production of many industrial goods.

Bacteria have been found to store carbon in the form of glycogen (88), TAGs (2), and polyhydroxyalkanoates (PHAs) (104). PHAs are polyoxoesters synthesized by a wide range of bacteria for carbon and energy storage. (While only prokaryotes have been found to use high molecular weight PHAs for carbon storage, it has been proposed that both prokaryotes and eukaryotes synthesize complexes of low molecular weight PHA and polyphosphate that act as ion channels (17, 95). This chapter will only focus on the high molecular weight polymer.) The first PHA was discovered by

Lemoigne in 1927, when he found that *Bacillus megaterium* accumulated the homopolymer poly(3-hydroxybutyrate) (PHB) (65). PHAs containing monomers other than 3-hydroxybutyrate were first observed by Wallen and Rohwedder (119), and the later finding that *Pseudomonas oleovorans* could store poly(3-hydroxyoctanoate) made it clear that many types of PHA exist in the microbial world (18). Today PHA monomers are generally characterized as either short chain length (C3 – C5, SCL-PHA) or medium chain length (C6 and greater, MCL-PHA) (89). While most PHA monomers are 3-hydroxyalkanoic acids, monomers with the hydroxyl group in other positions can also be polymerized, with the most important example of these being 4-hydroxybutyrate (70).

PHAs play an important role in bacterial growth and survival. It has been shown that in environments with fluctuating carbon levels, PHA producers are able to outcompete rival species (52). In addition to the interest in the roles of PHAs in the environment, there have also been significant efforts to develop PHAs for commercial use. PHAs have the potential to serve as renewable and biodegradable alternatives to traditional petrochemical plastics. In this chapter I will review PHA metabolism, PHA properties, and attempts made to commercialize these polymers. I will pay special attention to PHA synthesis in the bacterium *Ralstonia eutropha*, which I have worked with extensively to achieve my thesis objective of producing useful PHA copolymers from palm oil.

PHA Metabolism

Monomer synthesis

The model organism for studying PHA biosynthesis is *Ralstonia eutropha* H16 (also referred to as *Hydrogenomonas eutropha*, *Alcaligenes eutrophus*, *Wautersia eutropha*, and *Cupriavidus necator* in the literature). *R. eutropha* is a Gram-negative bacterium of the class *Betaproteobacteria* that lives in soil and freshwater environments (85). Its importance as a model organism was established when it was discovered that *R. eutropha* will accumulate high levels of PHB (>75% of cell dry weight) when grown in media that has plentiful carbon but is limited in some other essential nutrient. PHB synthesis is catalyzed by a PHA synthase, whose *in vivo* substrates are CoA thioesters. This polymerization is a stereospecific reaction in which only (*R*)-3-hydroxyacyl-CoA molecules serve as substrates.

The precursor to PHB is (*R*)-3-hydroxybutyryl-CoA (HB-CoA), which can be synthesized from the central metabolite acetyl-CoA. Wild type *R. eutropha* H16 has limited ability to utilize sugars as carbon sources,

with fructose and *N*-acetylglucosamine being the only carbohydrates that have been shown to support growth (85). Fructose is catabolized through the Entner-Doudoroff pathway, and the resulting pyruvate can be converted to acetyl-CoA and used for energy generation in the citric acid cycle, or for production of PHB (85). PHB is synthesized from acetyl-CoA in a three reaction pathway: two molecules of acetyl-CoA are condensed by an acetyl-CoA acetyltransferase (PhaA) to form acetoacetyl-CoA, the acetoacetyl-CoA is reduced to form HB-CoA by a reductase (PhaB), and the HB-CoA is polymerized by the PHA synthase (PhaC), as shown in Fig. 1.1. (Historically PhaA has been referred to as a β-ketothiolase, which is the enzyme's reverse reaction. I will follow this convention throughout my thesis.) Genes encoding the enzymes that carry out each of these reactions were discovered as an operon (*phaC1-phaA-phaB1*) in the *R. eutropha* genome (82, 83, 94). Sequencing and analysis of the *R. eutropha* H16 genome revealed a more complex picture of PHA biosynthesis in this organism (85), as will be discussed later in my thesis (see Chapter 4 for details).

PhaB is a NADPH-dependent reductase (39), so synthesis of PHB requires an adequate supply of this electron donor. One molecule of NADPH is generated by the Entner-Doudoroff pathway, and one molecule is generated by the *R. eutropha* isocitrate dehydrogenase that is part of the citric acid cycle (122). NADH is also generated by the citric acid cycle. Some bacteria have transhydrogenase enzymes that allow for the transfer of reducing equivalents between NAD^+ and $NADP^+$ (13), but it is not known if *R. eutropha* has a gene encoding this activity.

The *R. eutropha* PHA synthase is only able to make SCL-PHA. The copolymer poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) generated considerable interest when it was discovered that *R. eutropha* would accumulate this polymer when odd chain length organic acids were included in the growth medium (22). Metabolism of these acids leads to the formation of propionyl-CoA, which reacts with acetyl-CoA to form ketovaleryl-CoA. Reduction of the ketovaleryl-CoA by PhaB produces (*R*)-3-hydroxyvaleryl-CoA, allowing HV to be incorporated into the polymer chain. It was initially believed that PhaA catalyzed the condensation of acetyl-CoA and propionyl-CoA, but later work demonstrated that another β-ketothiolase (BktB) is largely responsible for this reaction in *R. eutropha* (99). Other organisms, primarily from the *Rhodococcus* and *Nocardia* genera, are able to synthesize P(HB-*co*-HV) with high HV content from unrelated carbon sources. It was discovered that in these organisms propionyl-CoA is produced via the methylmalonyl-CoA pathway (117). Succinyl-CoA from the citric acid cycle is converted to (*R*)-methylmalonyl-CoA by methylmalonyl-CoA mutase. This molecule then undergoes a racemization reaction and is decarboxylated to yield propionyl-CoA.

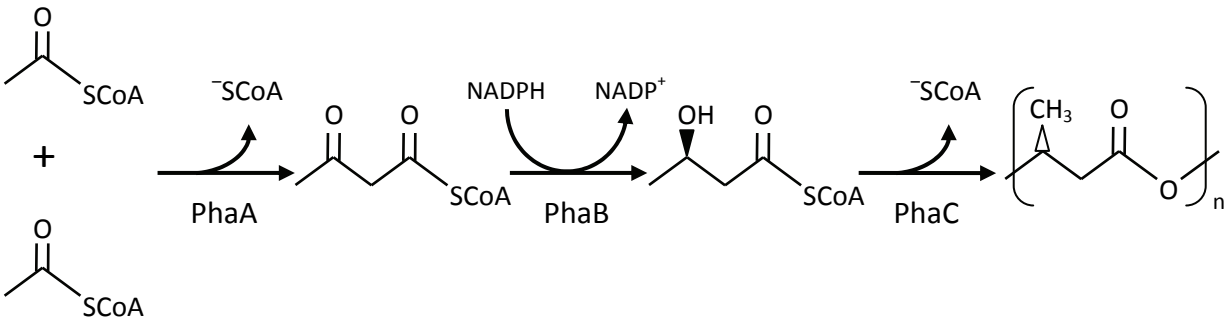

Fig. 1.1: Synthesis of PHB from acetyl-CoA by *R. eutropha*. PhaA is a β-ketothiolase, PhaB is an acetoacetyl-CoA reductase, and PhaC is a PHA synthase.

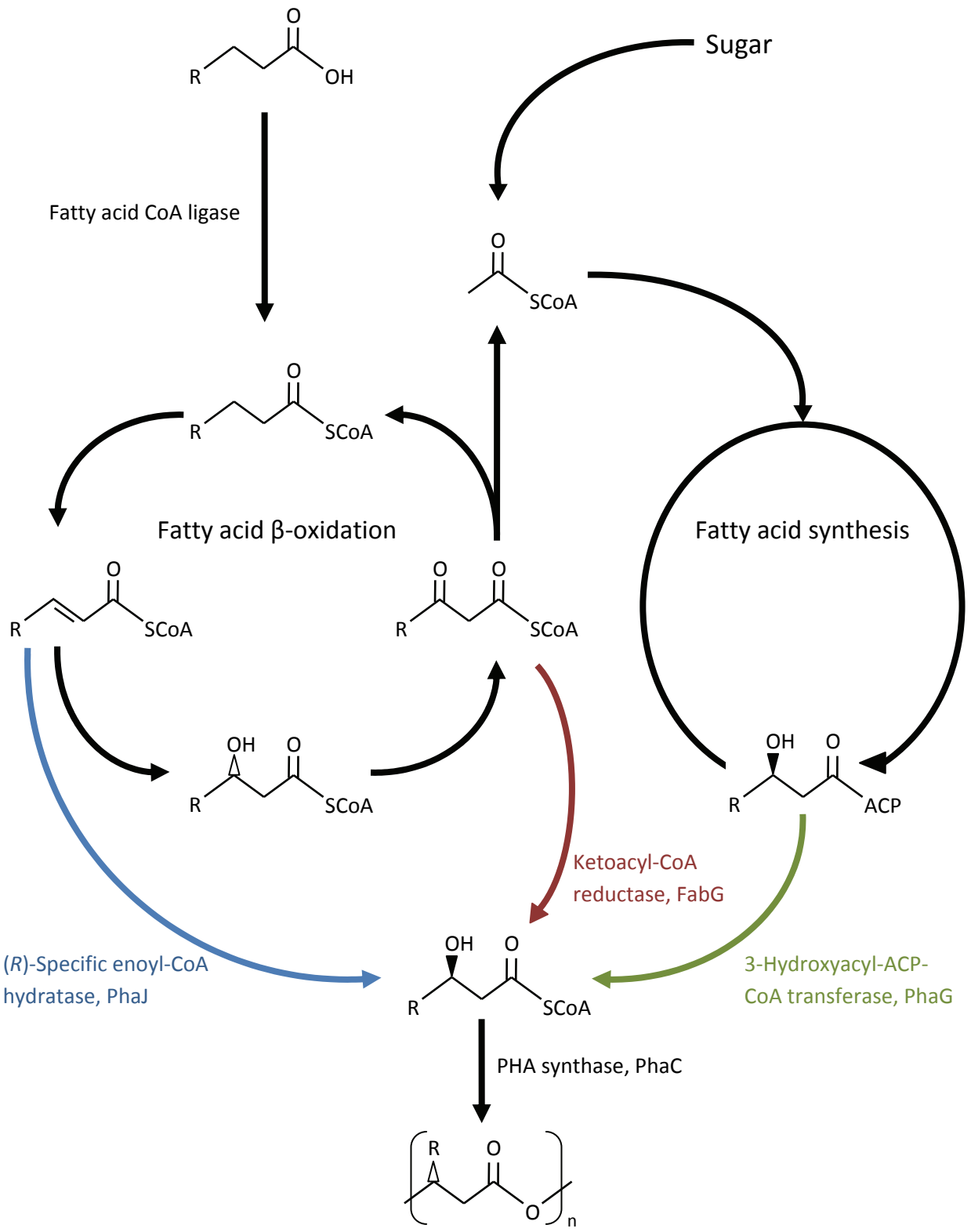

Fig. 1.2: Intermediates from fatty acid β-oxidation and fatty acid synthesis can be converted into monomers for synthesis of MCL-PHA. These enzymatic reactions are shown in blue, red, and green. Note that FabG normally reduces 3-ketoacyl-ACP substrates, but can also reduce 3-ketoacyl-CoA.

Most work involving MCL-PHA synthesis has been conducted using *Pseudomonas* and *Aeromonas* species. Accumulation of MCL-PHA requires both a synthase that can polymerize these monomers, as well as pathways for generation of MCL substrates. There are many potential paths by which intermediates in both fatty acid synthesis and fatty acid degradation can be diverted to form MCL 3-hydroxyacyl-CoA molecules (Fig. 1.2). During *de novo* fatty acid biosynthesis, one of the intermediates is a (*R*)-3-hydroxyacyl thioester, but it is linked to acyl carrier protein (ACP) instead of CoA (19). It was discovered that *Pseudomonas putida* encodes a transferase that allows the (*R*)-3-hydroxyacyl group to be transferred from ACP to CoA (PhaG), thereby creating MCL substrates for the PHA synthase (27, 90).

Fatty acid β-oxidation also provides precursors for monomer synthesis (Fig. 1.2). In the fatty acid degradation pathway, the acids are first activated by ligation to CoA. They are then broken down through a series of reactions, such that every cycle of β-oxidation results in the release of two carbons in the form of acetyl-CoA (19). While some fatty acids in the cell are always turned over and recycled, high flux through this pathway requires that the cells are fed TAGs or fatty acids. One of the intermediates of β-oxidation is 3-hydroxyacyl-CoA, but it is the (*S*) form that cannot be polymerized. The existence of a 3-hydroxyacyl-CoA epimerase has been proposed, but this enzymatic reaction has never been observed. Another intermediate of fatty acid β-oxidation is 3-ketoacyl-CoA. The preferred substrate of the *R. eutropha* PhaB is acetoacetyl-CoA, but it can also reduce other substrates up to C6 in length (39). A related reductase is FabG, which normally functions in fatty acid biosynthesis and reduces 3-ketoacyl-ACP. It has been shown that FabG's from several species are able to reduce 3-ketoacyl-CoA substrates as well, with different enzymes having different activities and substrate specificities (78, 91, 107). Over expression of *fabG* genes in recombinant strains has been shown to increase accumulation of MCL-PHA.

There has been substantial interest in species of the *Aeromonas* genus ever since it was shown that *Aeromonas caviae* stores poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) when grown on plant oil or fatty acids (21). It was later found that *A. caviae* has a gene encoding a (*R*)-specific enoyl-CoA hydratase that allows it to convert fatty acid β-oxidation intermediates into substrates for the PHA synthase (28). The gene encoding this enzyme (*phaJ*) is organized in an operon with the organism's PHA synthase gene. The crystal structure of the *A. caviae* PhaJ has been solved, which provided valuable insights into the mechanism of the enzyme (43). The active enzyme is a homodimer in which each monomer has a "hot dog" fold structure. A histidine residue and an aspartate residue form a catalytic dyad. Residues at the end of the substrate binding tunnel were identified that influence the substrate specificity of the enzyme (43, 115). After *phaJ* from *A. caviae* had been identified, additional *phaJ* genes from other

species were found and characterized (114). Interestingly, an enzyme with (*R*)-specific enoyl-CoA hydratase activity was recently discovered in *Escherichia coli* (81). As wild type *E. coli* does not store PHA, the natural role of this enzyme (MaoC) is unclear.

Bacteria have evolved a number of pathways for synthesis of PHA monomers. In some cases it is clear that the main role of the enzymes is to generate PHA precursors (e.g. PhaA and PhaB), while in others the generation of PHA monomers seems to be a side reaction (e.g. FabG). Metabolic engineers have transferred many of these pathways to new hosts in order to efficiently make desired types of PHA. In some cases the genes can be mutated to change the activity or substrate specificity of the encoded enzymes. In all of these examples, synthesis of a desired PHA requires a PHA synthase that is able to polymerize the monomers produced within the cell.

The PHA synthase

PHA synthases are enzymes that convert soluble 3-hydroxyacyl-CoA monomers into insoluble granules made up of high molecular weight polyester. Genes encoding these synthases have been identified in numerous species of bacteria (89). PHA synthases are divided into four different classes, which are summarized in Table 1.1. For the class I and class II enzymes, the active synthases are homodimers that include a single subunit (PhaC), and the classes are differentiated based on their substrate specificities. Class I synthases produce only SCL-PHA, while class II synthases prefer MCL substrates. Some synthases that make SCL/MCL copolymers, such as the PhaC from *A. caviae*, are therefore difficult to classify with this system. Class III and class IV synthases require two subunits for full activity. In both cases the PhaC subunit shows homology to the class I and class II synthases. While the second subunit (PhaE for class III, PhaR for class IV) is required for full activity, the roles of these subunits are unclear. Significant work has been performed to understand the class I, class II, and class III synthases, while relatively little research has been conducted with the class IV enzymes.

Mechanistic studies of PHA synthases advanced rapidly when it was determined that PHA synthases have a similar structure and mechanism to bacterial lipases (50). Both types of enzymes are part of the α/β hydrolase family, and both act at the interface between an aqueous solution and a hydrophobic surface. The C terminus of class I and class II synthases is highly hydrophobic, and it has been proposed that this domain may interact with the surface of the hydrophobic PHA granule (89). The PhaC subunits of class III and class IV synthases lack this hydrophobic C terminus, but PhaE and PhaR do have

hydrophobic C terminal domains that could serve the same function. Both lipases and PHA synthases contain a lipase box that includes the key catalytic residue (serine for lipases, cysteine for PHA synthases), and whose sequence is typically N-X-X-G-X-C/S-X-G-G. Alignments of PHA synthase amino acid sequences, combined with three dimensional models of synthases, have allowed researchers to determine the roles of several catalytic residues (reviewed in (104)). The best studied of these are a cysteine, a histidine, and an aspartate in the active site (C319, H508, and D480 in *R. eutropha* PhaC). Mutations to any of these residues reduce synthase activity to <0.5% of the wild type enzymes (50, 51).

Table 1.1: A summary of the different classes of PHA synthases (adapted from (89)). Active synthases are dimers made up of two molecules of PhaC (class I and II) or two PhaEC/PhaCR complexes (class III and IV).

Class	Subunit(s)	Example organisms	Protein Size	Substrates
I	PhaC	*Ralstonia eutropha*	60 – 73 kDa	SCL only
II	PhaC	*Pseudomonas aeruginosa* and other pseudomonads	60 – 65 kDa	MCL preferred, some can also use SCL
III	PhaC + PhaE	*Allochromatium vinosum*, *Thiocapsa pfennigii*	PhaC ~40 kDa PhaE ~40 kDa	SCL, some MCL (up to C8)
IV	PhaC + PhaR	*Bacillus megaterium*	PhaC ~40 kDa PhaR ~22 kDa	SCL only

Most experiments designed to elucidate the PHA synthase mechanism have been conducted using *R. eutropha* PhaC and *A. vinosum* PhaEC isolated from recombinant *E. coli*. Enzymatic activity can be measured with a discontinuous assay by monitoring CoA release when the enzyme is incubated with HB-CoA (32). Early efforts to study PHA synthases were complicated by the fact that the enzymes exhibited variable lag phases before maximum activity was observed (32). It was found that this lag phase could be eliminated by priming PHA synthases with short PHB oligomers (50, 125). This advancement allowed researchers to discover that a covalent intermediate is formed at the active cysteine in the synthase (74, 125). It was then determined that the conserved histidine mentioned above activates the cysteine for nucleophilic attack on HB-CoA, while the aspartate serves as a catalyst to activate the hydroxyl group on HB-CoA (51) (see Fig. 1.3).

These findings left one significant question regarding the mechanism of PHA chain elongation: does a noncovalent CoA intermediate form in the active site, or is the growing chain always covalently linked to one of two catalytic cysteines? Both class I and class III synthases are dimers in their active forms (125),

which would allow for a reaction requiring two cysteines. The two proposed mechanisms are illustrated in Fig. 1.3. Differentiating between these two mechanisms was challenging, as high molecular weight polymer is rapidly generated *in vitro*, making it difficult to capture short lived, low molecular weight intermediates. Li and coworkers performed a study using *A. vinosum* PhaEC in which the active cysteine was mutated to a serine (66). The mutated synthase exhibited 0.05% the activity of the wild type enzyme. When the mutant synthase was incubated with radiolabeled HB-CoA, the species $(HB)_2$-CoA and $(HB)_3$-CoA were detected after rapid chemical quench, proving that noncovalent intermediates are formed during polymerization. This finding agrees with the three dimensional model of the PHA synthase, which suggested that the active site was buried within the enzyme, meaning it would be difficult for two PhaC molecules to be oriented such that the active cysteines were in close proximity.

Most mechanistic studies have been conducted using HB-CoA as the substrate for the PHA synthase. Less work has been done to understand the substrate specificity of PHA synthases and the formation of PHA copolymers. An important study of P(HB-*co*-HV) synthesized by *R. eutropha* was conducted by Doi and coworkers (22). Their group used NMR to study the distributions of different diad and triad sequences in the copolymer. They concluded that the reaction proceeds as an ideal random copolymerization, meaning the addition of new monomer units to the polymer chain is independent of the unit at the end of the chain. Multiple groups have used mutagenesis techniques to alter the substrate specificity of class II PHA synthases from pseudomonads (71, 96). While the mutations did have some influence on PHA composition, the roles of individual residues and the mechanism of substrate selectivity is still unclear. The crystal structure of a PHA synthase will have to be solved to gain a better understanding of why different synthases are able to accept different substrates.

An aspect of the PHA synthesis process that is not well understood is how the molecular weight of the polymer is controlled. PHA is a high molecular weight compound, with PHB from *R. eutropha* having been shown to reach weight average molecular weights (M_w) of over 1 MDa (108). It has been determined that PHA synthases produce multiple polymer chains per molecule of enzyme *in vivo* (55), but the mechanism of chain termination and reinitiation is unknown. *In vitro* experiments with purified *R. eutropha* PhaC showed that substrate concentration does not impact PHB molecular weight, but that enzyme concentration does influence the size of the resulting polymer (31). Subsequent *in vivo* experiments conducted using recombinant *E. coli* showed that higher synthase expression resulted in lower PHB molecular weight (98). In these experiments, however, expression of the entire PHB biosynthetic operon was modulated, so different intracellular substrate concentrations were likely

produced in each induction condition. This complication makes it difficult to determine the relative affects of synthase expression and substrate availability on *in vivo* molecular weight. Several compounds have been proposed to act as chain transfer agents that react with the growing polymer at the active site and catalyze chain termination (55). These include water, free CoA, hydroxybutyrate, and 1,3-propanediol (55, 62, 68). While some of these agents have been definitively shown to decrease PHA molecular weight when added to bacterial cultures, it has not been determined whether this is due to interaction of the agents with growing polymer chains, or if the compounds react with stored PHA (62). Tian et al. found that when polymer was released from the synthase, a short PHB oligomer was still bound to the enzyme, suggesting that if the proposed agents are responsible for chain termination, they do not act at the active site (112). *In vitro* studies in which HB-*N*-acetylcysteamine was incubated with synthase resulted in low molecular weight polymer (~700 HB units) being formed that was covalently linked to NAC, indicating that growing polymer could be released from the enzyme (62). The extent to which this happens when HB-CoA is the substrate is unknown, and will depend on the dissociation constant of PHB-CoA. Examination of all the data in the literature suggests that there are multiple routes for PHA chain termination and *in vivo* changes in molecular weight: (i) release of noncovalently bound PHA-CoA from the enzyme (62, 66), (ii) chain transfer of the growing polymer, possibly catalyzed by a nucleophilic residue outside of the active site (112), and (iii) reactions of stored PHA with other intracellular compounds (e.g. propanediol, hydroxybutyrate) that result in decreases of the molecular weight of stored polymer (62, 68). In many studies, it has been observed that PHA copolymers generally have lower molecular weight than the PHB homopolymer (for examples see (54, 59)). This could be explained if the PHA-CoA has a higher K_d than PHB-CoA, resulting in more frequent release of polymer from the synthase.

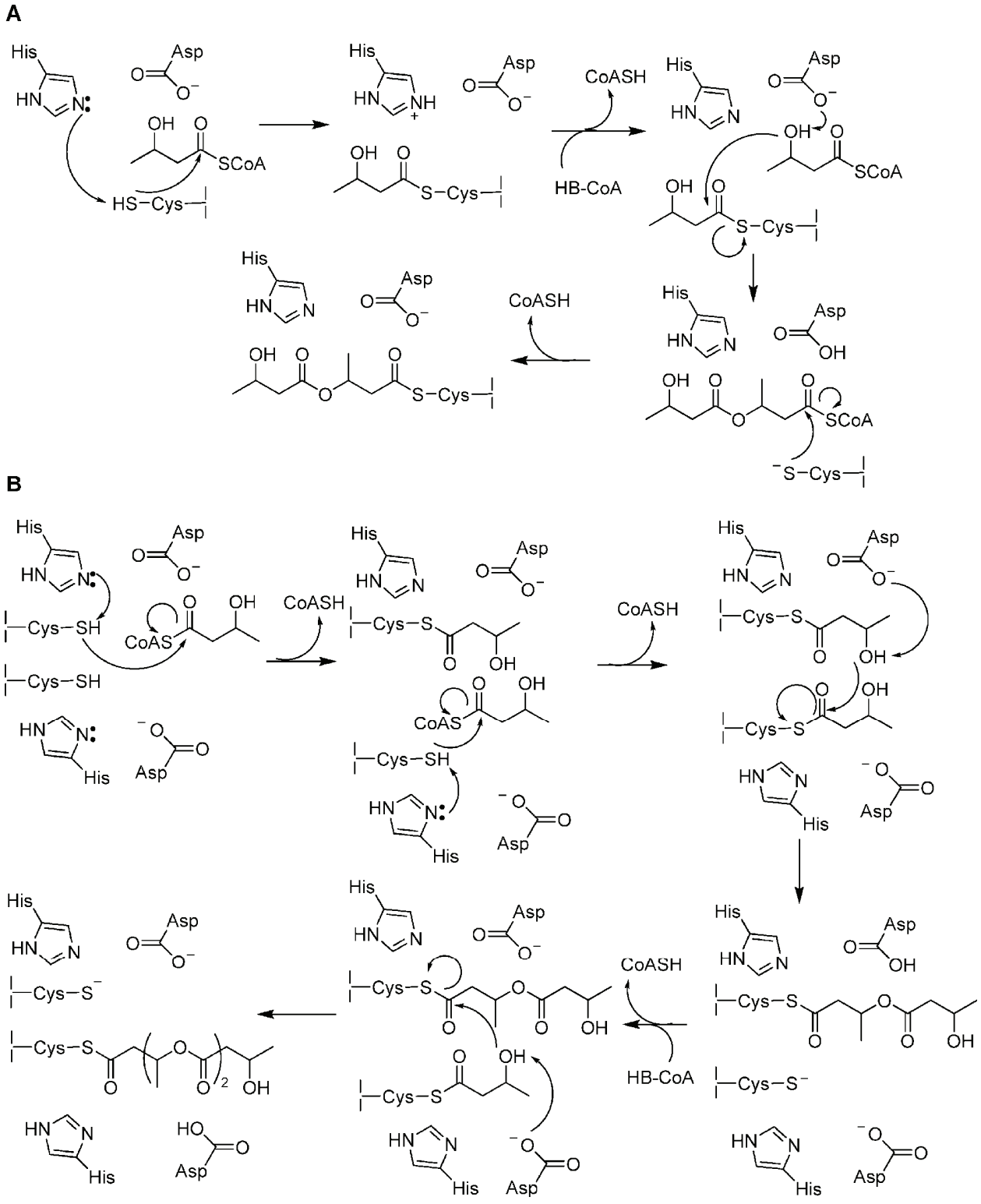

Fig. 1.3: Two mechanisms have been proposed for PHA chain elongation that either do (A) or do not (B) include a noncovalent intermediate. Experimental evidence suggests that (A) is the correct mechanism. The figure is reproduced from (66).

PHA Granule Formation

Intracellular PHA is stored as insoluble inclusion bodies. Transmission electron micrographs revealed that bacterial cells typically contain multiple granules. Even when a high percentage of a *R. eutropha* cell's dry weight consists of PHA, distinct granules can still be seen in TEM micrographs. This observation, along with the early finding that there is lipid associated with purified granules (35), led to the proposal that granule surfaces are coated with lipid and protein, which prevents coalescence. The types and amounts of granule-associated lipids have not been fully quantified, but it has been proposed that the granules are coated with a phospholipid monolayer. The surface coating may also influence the physical state of the polymer. NMR and differential scanning calorimetry experiments have revealed that polymer stored in granules remains in the amorphous state *in vivo* (10, 103). When whole cells are lyophilized or when PHA is isolated from a culture, the polymer rapidly crystallizes. This crystallization depends on the occurrence of nucleation events, and it has been shown that these are rare events given the small volumes of the granules (45). Horowitz and Sanders developed a procedure for synthesis of artificial granules in which crystalline PHB was dissolved and then precipitated in the presence of chemical surfactants (45). The resulting suspension consisted of amorphous granules, showing that the amorphous state of PHA *in vivo* is primarily due to granule size, and not other biological factors.

There are two models described in the literature for how granules are formed *in vivo* (reviewed in (104)). In the micelle model, as multiple synthases make PHA in the cytoplasm, the hydrophobic polymer aggregates, bringing the growing chains together and forming a nascent granule. This model does not provide a ready explanation for how lipid would become associated with the surfaces of granules. In the budding model, PHA synthases are initially localized at the cytoplasmic side of inner membrane. Polymer is synthesized into the lipid bilayer, where it eventually forms a small granule and buds off from the membrane. This model offers an explanation for how granules could become coated with a lipid monolayer. It should be noted, however, that given the large surface area of granules within a *R. eutropha* cell, significant lipid synthesis would be necessary to coat all granules.

A number of microscopy studies have been conducted in attempts to better understand granule biogenesis. Using TEM, Tian et al. observed that early in PHB storage conditions, nascent granules accumulated down the center line of *R. eutropha* cells, and seemed to be associated with dark staining "mediation elements" (113). Although the composition of these elements is unknown, the authors speculated that they could be similar to the scaffolds found in cellulosomes. Fluorescence microscopy experiments conducted using several species of bacteria suggested that granules generally form at the

cytoplasmic membrane, and that in some species they are localized at the cell poles (46, 49). Unfortunately, given the small size of *R. eutropha* cells (widths of ~0.7 μm), it is difficult to resolve distinct PHA granules via fluorescence microscopy in this species, especially early in the granule formation process (113). While the budding model currently has the most support in the literature, more work is necessary to fully understand PHA granule biogenesis in many species. The possibility of PHA scaffolds in *R. eutropha* is an especially intriguing area for future research.

Many proteins are associated with PHA granules *in vivo* (reviewed in (47)). It has been proposed that all enzymes necessary for synthesis of PHB from acetyl-CoA associate with PHA granules in *R. eutropha* (116). In addition to these biosynthetic enzymes, PHA depolymerases also act at the surfaces of PHA granules (47), and may even be present during PHA accumulation (61). The most abundant protein on the surfaces of PHA granules in *R. eutropha* is the phasin PhaP (111, 123). It has been proposed that PhaP is analogous to the oleosins that coat TAG inclusions in plants, and that they stabilize the granules and prevent coalescence (123). Deletion of the *phaP1* gene (the most highly expressed phasin) from the *R. eutropha* genome results in formation of extremely large granules within the cell, while overexpression results in numerous small granules. It is believed that the hydrophobic C terminus of phasins acts as a granule binding domain (123). It is possible that PhaP plays other roles in PHA metabolism as well, such as participating in granule localization.

PHA Depolymerization and Utilization

Two types of PHA utilization occur in nature: intracellular utilization of polymer by PHA-synthesizing organisms, and extracellular utilization of PHA that has been released into the environment from lysed cells. As discussed above, native intracellular granules are composed of amorphous polymer, so intracellular PHA depolymerases use this form of polymer as their substrate. Extracellular PHA rapidly crystallizes, therefore extracellular depolymerases act on semi-crystalline polymer (48). Organisms that feed on extracellular PHA include both bacteria and fungi, and depolymerases from these organisms have been studied extensively (reviewed in (48)). Most extracellular depolymerases show similar domain structures and consist of a signal peptide directing secretion of the enzyme, a catalytic domain, a linker domain, and a substrate binding domain. The catalytic domains contain lipase box motifs with the sequence G-X-S-X-G, in which serine is the key catalytic residue (in contrast to PHA synthases, which contain a cysteine in the lipase box). The amino acid sequences of all extracellular depolymerases

contain strictly conserved serine, aspartate, and histidine residues. The depolymerization reactions catalyzed by these enzymes are stereospecific, with no activity observed when the enzymes are incubated with synthetic (*S*)-PHA. Most extracellular depolymerases are believed to have both exo- and endohydrolytic activity. The products of depolymerization vary depending on the enzyme, with some releasing only HA oligomers, while others degrade polymer to single monomer units. The structures of two extracellular depolymerases have been solved, providing insights into how the enzymes bind to PHA surfaces and how polymer chains enter the active sites (42, 80).

Less in known about intracellular PHA depolymerization and utilization. Most studies in this area have been conducted with the model organism *R. eutropha*. It was established that HB is released from purified *R. eutropha* PHB granules *in vitro*, and that the rate of HB release is increased when the granules are isolated from cells incubated in medium that stimulates polymer utilization (36). A gene encoding an intracellular depolymerase in *R. eutropha* was identified soon thereafter, and it was discovered that the enzyme could only degrade amorphous granules (92). Interestingly, this enzyme (PhaZ1) showed little sequence similarity to extracellular depolymerases and did not contain a lipase box motif. A second depolymerase gene was then discovered (*phaZ2*), but deletion of this gene only resulted in a phenotype in *ΔphaZ1* strains, suggesting that PhaZ2 acts on degradation products generated by PhaZ1 (128). An HB oligomer hydrolase that is not granule bound was also discovered and characterized (PhaY1), which is believed to degrade oligomers that are released from PHB granules by the actions of the depolymerases (37, 57). Analysis of the complete *R. eutropha* genome revealed a total of seven putative depolymerases and two putative oligomer hydrolases, although it is unclear how many of these gene products are actually active within the cell (85).

It is still not known how monomers released from PHB in *R. eutropha* are metabolized. It has been assumed that free HB would be converted to HB-CoA by a HB-CoA ligase (using ATP), activating the molecule for further degradation. HB-CoA could then be converted to acetyl-CoA via oxidation and thiolysis reactions. It has also been proposed based on microarray data that acetoacetyl-CoA is converted to (*S*)-HB-CoA, then crotonyl-CoA, but it is unclear why this pathway (essentially a reverse β-oxidation cycle) would be useful to the organism (84). An intriguing study was recently published, in which Uchino et al. incubated granules isolated from *R. eutropha* with free CoA (116). They observed formation of HB-CoA and acetyl-CoA in suspensions of granules isolated from wild type *R. eutropha* and the Δ*phaZ1* strain. The rate of acetyl-CoA formation increased when NAD^+ was added to the suspensions, but not when $NADP^+$ was added, indicating that different cofactors are used in PHB

synthesis and degradation. Granules isolated from recombinant *E. coli* expressing *phaC1-phaA-phaB1*, *phaZ1*, and *phaP1* also released HB-CoA when incubated with free CoA, leading the authors to speculate that PhaZ1 can catalyze both thiolysis and hydrolysis of PHB. The degradation products that are most prevalent *in vivo* must still be determined.

Regulation

One of the key questions regarding PHB metabolism is how polymer synthesis and degradation are regulated. Enzyme activity measurements suggested that in *R. eutropha* the PHB biosynthetic genes are constitutively expressed (38, 39, 82). This finding was later confirmed by quantifying transcript levels using RT-PCR (61). The RT-PCR study showed that the depolymerase *phaZ1* is constitutively expressed as well, meaning that enzymes for synthesis and degradation of PHB are present in the cell simultaneously. This finding agrees with reports that PHA is continuously turned over, even under PHA storage conditions (23, 108).

Given that transcription and translation of the PHB metabolic enzymes do not change significantly under different growth conditions, there must be other regulatory factors at work. Many of these were first discussed by Oeding and Schlegel in 1973 (79). In this study, free CoA was shown to inhibit the condensation reaction catalyzed by PhaA. In rapidly growing cells, acetyl-CoA enters the citric acid cycle, which results in CoA release and inhibits synthesis of acetoacetyl-CoA. When normal growth is prevented by nutrient limitation, citrate synthase is inhibited, preventing acetyl-CoA from entering the citric acid cycle and allowing for PHB accumulation. Citrate synthase activity is regulated by intracellular nicotinamide concentrations. It has been shown that when normal growth of *R. eutropha* is prevented by nitrogen limitation, the intracellular concentration of NADH increases (63). NADH substantially inhibits the *R. eutropha* citrate synthase, while NADPH (necessary for synthesis of HB-CoA) has a less pronounced inhibitory effect (41, 63). NAD^+ and $NADP^+$ concentrations have no impact on citrate synthase activity. These cofactors may have another regulatory role as well, as it has been shown *in vitro* that high concentrations of NAD^+ and $NADP^+$ inhibit *A. vinosum* PhaEC (53). Concentrations of the oxidized coenzymes in *R. eutropha* were higher during normal growth than in nitrogen starvation conditions, so this could represent another level of regulation. Further studies are necessary to determine the impact of NAD^+ and $NADP^+$ on *R. eutropha* PhaC activity.

Expression of the phasin protein PhaP1 in *R. eutropha* is tightly regulated. Shortly after this protein was discovered, it was determined that PhaP1 production depended on PHB synthesis (123, 127). Later work demonstrated that *phaP1* transcription is triggered by PHB accumulation, and that the concentration of PhaP1 protein is proportional to the amount of stored PHB (61). It was discovered that *phaP1* transcription is controlled by the regulatory protein PhaR, whose gene is located immediately downstream from the *phaCAB* operon on *R. eutropha* chromosome 1 (129). PhaR binds to DNA upstream of *phaP1,* preventing transcription. Deletion of the *phaR* gene leads to constitutive *phaP1* expression. A study of a PhaR protein from *Paracoccus denitrificans* identified both DNA and PHA binding domains (126). The model for regulation of *phaP1* expression is therefore that in the absence of PHA, the constitutively expressed PhaR binds to DNA upstream of *phaP1*, preventing transcription (61, 87). As PHA is synthesized and granules form, the PhaR is sequestered on granule surfaces, allowing transcription of *phaP1*. This model requires efficient sequestration of PhaR, meaning that the protein must either bind PHA more tightly than its recognized DNA sequence, or that the granule surface area is so great that PhaR will encounter PHA surfaces much more frequently than DNA. This regulatory system has the potential to be exploited by metabolic engineers who wish to express certain genes only when PHA is being stored by an organism.

Properties of Polyhydroxyalkanoates

The properties of PHAs determine what applications these plastics are best suited for and how they can be processed. The physical state of the PHA has a major impact on the properties of the material. As mentioned previously, PHA stored in intracellular granules is maintained in an amorphous state. When whole cells are dried or when the PHA is isolated, it is able to crystallize. The crystallinity of PHAs results from the fact that they are isotactic polymers in which the chiral center of each monomer has the (*R*) configuration. The PHB homopolymer crystallizes rapidly and has been studied in great detail. Bulk PHB crystallizes when it is quenched from the melt or cast from solution, and reaches an equilibrium crystallinity of 60 – 70% (11). X-ray diffraction data and molecular modeling revealed that PHB chains take on a 2_1 helical conformation in which the methyl and carbonyl groups point away from the axis of the helix (15). The unit cell is orthorhombic and contains two antiparallel helices. The dimensions of the unit cell are *a* = 5.76 Å, *b* = 13.20 Å, and *c* = 5.96 Å, in which *c* is the direction of the helical axis (15). The

high degree of crystallinity of PHB leads to a plastic that is strong, but very stiff (high Young's modulus) and brittle (low Izod impact strength and elongation to break) (44). This brittleness limits the commercial potential of PHB. The thermal properties of PHB (T_g = 4°C, T_m = 180°C) are also problematic, as the melting temperature is close to the decomposition temperature of the polymer (106). Reductions in PHB molecular weight are observed when the polymer is incubated at temperatures above 170°C and evolution of monomers occurs at temperatures >200°C (64).

It was discovered that many PHA copolymers have more favorable properties than PHB. The first of these PHAs that was studied in detail was P(HB-*co*-HV). Addition of HV units to the polymer chain resulted in material with a lower Young's modulus and a greater extension to break, but the changes induced by incorporation of this monomer were only moderate (44, 106). It was found that the P(HB-*co*-HV) system is isodimorphic, meaning that the HV monomers are able to insert into the PHB crystal structure (12). P(HB-*co*-HV) crystallizes more slowly than PHB, but both polymers eventually reach the same degree of crystallinity at equilibrium (11). 3-hydroxyalkanoate monomers longer than HV (i.e. MCL monomers that are C6 and longer) have a more pronounced effect on polymer properties. The equilibrium degree of crystallinity and T_m of P(HB-*co*-HA_{MCL}) decrease linearly as functions of the mol% of HA_{MCL} in the polymer, and the length of the HA_{MCL} side chains does not influence how these properties change (76). The glass transition temperature of these copolymers also decreases when HA_{MCL} units are added to the polymer, but in this case longer monomers lead to greater decreases in T_g (76). P(HB-*co*-HA_{MCL}) copolymers are weaker than PHB, but also tougher and more flexible. Properties of some of these polymers are summarized in Table 1.2. PHA that consists only of MCL monomer units is a soft, flexible thermoplastic (5). Another PHA copolymer that has received considerable attention is P(3HB-*co*-4HB). The 4HB units have four carbons in the polymer backbone and no side chain. Inclusion of 4HB in the copolymer decreases crystallinity and changes the mechanical properties of the plastic (Table 1.2) (60).

Table 1.2: Thermal and mechanical properties of various PHAs, with polypropylene included as a reference (5, 21, 76, 106, 124). HHx refers to the 3-hydroxyhexanoate monomer. N.D. indicates a value was not determined.

Polymer	T_m (°C)	T_g (°C)	Young's modulus (GPa)	Tensile strength (MPa)	Elongation to break (%)
PHB	180	4	3.5	40	5
P(HB-*co*-20mol% HV)	145	-1	0.8	20	50
P(HB-*co*-10mol% HHx)	127	-1	0.52	21	400
P(HB-*co*-17mol% HHx)	120	-2	N.D.	20	850
P(3HB-*co*-16mol% 4HB)	152	-8	N.D.	26	444
P(4HB)	60	-50	0.15	104	1,000
MCL-PHA[a]	43	-44	0.001	4.7	335
Polypropylene	176	-10	1.7	38	400

[a]The MCL-PHA tested consisted of monomers ranging in size from C6 to C14 (5).

One of the properties that differentiates PHAs from most petrochemical plastics is that they are biodegradable materials. Traditional plastics that are not recycled or sent to landfills persist in the environment, both as large fragments and microscopic particles (110). The impact of this plastic waste on wildlife is not completely understood, but it has been shown that tiny plastic particles and fibers are ingested by many aquatic animals (110). Extracellular PHA serves as a carbon source for many prokaryotic and eukaryotic organisms, which secrete depolymerases to break down the polymer (48). When incubated in water at elevated temperatures, the molecular weight of PHAs decreases due to hydrolysis, which speeds degradation by depolymerases (20). PHAs have been shown to biodegrade in soil, freshwater, and marine environments, both aerobically (72) and anaerobically (1, 14, 25). Degradation of PHAs has been demonstrated to occur more rapidly than degradation of synthetic polyesters (1) and lignocellulose (25). Different types of PHA degrade at different rates, which may be a function of the depolymerases present in a given environment (20, 60).

A number of technologies have been developed for modifying the properties of PHAs, including chemical treatments, processing techniques, and polymer additives. Graft block copolymers can be constructed by linking PHA chains to other polymers (reviewed in (40)), although the biodegradability of the final product will be compromised if all blocks are not biodegradable. Cross-linked polymers are of interest due to their rubber-like properties. Studies of PHA cross-linking have been conducted using PHAs that include either all saturated side chains, or a combination of saturated and unsaturated moieties. Cross-linking can be achieved using peroxides and cross linking agents (29), or through sulfur

vulcanization (30). In both of these studies, gels could be formed with the cross-linked PHA, indicating that successful cross-linking had taken place. Cross-linking led to low crystallinity material with poor tensile strength, but the vulcanized PHA did show reduced tensile set. The molecular weight of the polymer in these studies was very low (<100,000 g/mol), so results could be improved if starting material with higher molecular weight is used. Branched P(HB-*co*-HV) was produced by extruding the polymer in the presence of dicumyl peroxide, and the resulting branched PHA showed improved elasticity (16).

Processing PHAs using certain methods can also lead to improved mechanical properties. Wang et al. found that P(HB-*co*-HV) should be extruded close to its melting point to obtain the highest quality product (121). Drawing PHA fibers and films can change the physical structure of the polymer and alter its mechanical properties. Tanaka and coworkers developed a melt-spinning technique that produced highly crystalline P(HB-*co*-HV) fibers with a tensile strength of over 1 GPa (109). X-ray diffraction studies revealed that the fibers contained both the 2_1 helical crystal structure described earlier, as well as a planar zigzag structure. Gel-spinning techniques have also been developed for preparation of high strength PHA fibers (4, 34). Venkitachalam et al. increased the toughness of P(HB-*co*-HV) films by cross-linking the polymer and drying the films under uniaxial strain (118). PHAs can also be processed in ways that alter their biodegradability. Increasing the porosity and surface area of PHA products allows them to degrade more quickly in the environment (69).

The properties of polymers are routinely altered through the use of additives. These can include fillers, reinforcers, plasticizers, stabilizers, and pigments. As PHA products may be discarded in the environment and decompose, it is important that PHA additives are environmentally friendly. Groups have therefore studied PHA composites in which the bioplastic is reinforced with natural products such as straw and pineapple fibers (7, 67), as well as composites in which wood flour is added as a filler (26). Inorganic nucleating agents can be added to PHA to increase its rate of crystallization, but a study with P(3HB-*co*-4HB) found that nucleating agents did not influence the final extent of crystallization (120). There are several reports in the patent literature of additives that can be used to modulate the properties of PHAs. An example composition for production of bioplastic films includes P(HB-*co*-HV) with acetyl tributyl citrate as a plasticizer and cyclohexylphosphonic acid/zinc stearate as a nucleating agent and thermal stabilizer (6). It is clear that further advances in the control of PHA monomer composition, along with new processing and compounding technologies, will make PHAs more functional and expand the number of applications for which these plastics can be used.

The History of PHA Commercialization

Today the vast majority of commercial plastics are made from fossil resources. It is inevitable that these resources will eventually be depleted, therefore many companies are developing strategies for producing plastics from renewable feedstocks. All polymers made from agricultural resources can be referred to as bioplastics, but this does not necessarily mean that the plastic is biodegradable. One of the first materials that could be considered a bioplastic was rayon, a highly processed form of cellulose used to make fibers and fabrics. Starch can be modified so that it behaves like a thermoplastic, although starch-based materials have very poor resistance to water (http://www.materbi.com). Advances in genetic engineering have allowed firms to manufacture new types of renewable monomers. DuPont, Genencor, and Tate & Lyle have developed a process for making 1,3-propanediol from sugar using an engineered strain of *E. coli* (75). The 1,3-propanediol is polymerized with terephthalic acid to create a new polyester with the trade name Sorona®. New routes are also being explored for synthesis of traditional plastics. Dow and the Brazilian ethanol producer Crystalsev formed a joint venture in 2007 to make polyethylene from sugar cane. Cane sucrose will be fermented to ethanol and then dehydrated to make ethylene, which can be polymerized by normal routes to make polyethylene. The new process is expected to generate less carbon dioxide than traditional polyethylene manufacturing processes.

One of the plastics most often compared to PHA is poly(lactic acid) (PLA). NatureWorks, a subsidiary of Cargill, produces PLA from corn sugar at the industrial scale (24). Corn sugar is fermented to lactic acid, which is then converted to cyclic lactide. The lactide is polymerized to form PLA via a ring opening polymerization reaction. PLA properties can be adjusted by controlling the levels of (*R*)-LA and (*S*)-LA monomers in the polymer (24). This is important, because it makes it possible to produce PLA that is completely amorphous and transparent, allowing the plastic to be used in applications that require clear material (e.g. packaging applications). Clarity represents the one area in which PLA is superior to PHA, which can only be made semi-transparent at this time. In other areas PLA has significant disadvantages relative to PHA, as it biodegrades more slowly than PHA (97) and shows more dramatic decreases in molecular weight than PHA when processed (33). Blends of PHA and PLA have been shown to have attractive properties, and it may be possible to use these alloys for applications in which neither pure PHA nor PLA is suitable (77).

Groups have recognized the commercial potential of PHAs for many years. Researchers at W.R. Grace and Company, a specialty chemicals firm, produced small quantities of PHB for commercial evaluation in

the 1960s, and were granted patents for methods to produce and recover the polymer (8, 9). Their process suffered from low productivity and the material was contaminated with residual bacterial biomass, causing the company to abandon its commercialization efforts (44). In the 1980s Imperial Chemical Industries began working with PHAs, and developed a process for production of P(HB-*co*-HV) in which *R. eutropha* was grown on sugar and propionate (3, 44). The availability of large quantities of this plastic, given the trade name Biopol, allowed many new studies of PHA properties to be conducted. The Biopol process was ultimately determined to be too expensive for commercial viability, so production was halted. The business was sold to Monsanto, who hoped to produce PHA in plants. Much progress was made by Monsanto, including the synthesis of P(HB-*co*-HV) by *Arabidopsis thaliana* and *Brassica napus* (rapeseed) (100). Monsanto eventually decided that PHAs were not part of their long term strategy, and sold their intellectual property assets to Metabolix.

The company Metabolix was founded based on discoveries made at the Massachusetts Institute of Technology by Dr. Oliver Peoples and Professor Anthony Sinskey (82, 83). Metabolix has formed a joint venture with Archer Daniels Midland named Telles, which will make P(3HB-*co*-4HB) from corn sugar using engineered microbes. The Telles production facility uses less non-renewable energy and generates fewer greenhouse gas emissions than traditional plastic manufacturing operations (56). As of June 2010, Telles offers both injection molding grade and sheet extrusion grade plastics. Some Telles grades have also been approved by the FDA for use in food contact applications. PHAs are biocompatible materials that degrade in the human body. Medical devices made from PHAs are being commercialized by Tepha, a company that was spun out of Metabolix in 1998 (70). In 2007, PHA sutures manufactured by Tepha were cleared by the FDA for medical usage. Additional medical devices are under development, including meshes, cardiovascular stents, and drug delivery systems. Procter & Gamble also had a strong PHA research program that resulted in the development of P(HB-*co*-HHx) copolymers that were given the trade name Nodax (76). Procter & Gamble's PHA intellectual property was sold to Meredian Inc. in 2007. Meredian's plans for PHA production are unclear at the time of this writing.

Metabolix is continuing the effort to produce PHAs economically in plants. PHB production in plants was first demonstrated in 1992 by Poirier et al., who worked with *Arabidopsis thaliana* (86). Since that initial publication, various types of PHA have been synthesized using several transgenic crops, including corn, sugar cane, and cotton (reviewed in (101)). Metabolix is focusing on PHA production in switchgrass, an important potential energy crop (93). The company has reported PHB accumulation of 3.7% of dry

weight in switchgrass leaves (102). It is believed that PHA synthesis in plants represents the lowest cost production method, as there is no need to purchase an expensive carbon feedstock. This scheme requires that an efficient process is developed for polymer recovery from plants, which will be challenging given the low PHA content in the plant tissue. Snell and Peoples have laid out a vision for biorefineries in which PHA is recovered from transgenic switchgrass, the cellulose and hemicellulose are converted to fermentable sugars, and the lignin is burned for heat and energy generation (101). In this proposed biorefinery the sugars could be converted to PHA, biofuels, or other industrial chemicals.

The technology for producing PHAs and other biobased chemicals is advancing rapidly, which will one day allow for the replacement of many petrochemical products with renewable alternatives. Large quantities of PHA will soon be commercially available, which will spur many groups to develop new PHA additives, blends, and processing techniques. Many of the initial applications for PHAs will be in sectors that can take special advantage of the biodegradability of these plastics, such as agriculture. As technology improves and production costs decrease, it is expected that PHA adoption will become more widespread. When low cost, cellulosic sugars become available, it will enable large scale, economical production of many additional industrial chemicals. This biobased chemical industry will reduce our dependence on foreign oil and decrease greenhouse gas emissions.

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Chapter 2

Polyhydroxyalkanoate and Triacylglycerol Production by *Rhodococcus aetherivorans* I24

Introduction

The overall goal of my thesis research was to produce useful PHA copolymers from palm oil. The first species studied as a possible production organism was *Rhodococcus aetherivorans* I24. Bacteria of the genus *Rhodococcus* are Gram-positive actinomycetes that grow aerobically and do not form spores. Their genomes have high GC content and many strains harbor linear megaplasmids (20, 29). These bacteria are well known for possessing a variety of unique catabolic pathways that allow them to degrade environmental pollutants and that can be exploited to perform commercially useful bioconversions (9, 18, 29). While genetic tools for modifying *Rhodococcus* strains are not as advanced as in better studied organisms such as *Escherichia coli* and *Bacillus subtilis*, some progress has been made in this area (13, 29).

Recently a number of *Rhodococcus* species have received attention for their ability to store carbon in the form of triacylglycerols (TAGs) and polyhydroxyalkanoates (PHAs). The strain *Rhodococcus opacus* PD630 has been shown to accumulate TAGs in intracellular granules, and it has potential applications in the biofuels industry (5, 31). *Rhodococcus ruber* synthesizes both TAGs and PHAs (4), raising the question of the possible benefits to the organism of making multiple carbon storage molecules. Interestingly, this species produces the copolymer poly(HB-*co*-HV) from sugar (33), while the model organism *Ralstonia eutropha* only makes PHB unless certain HV precursors are included in the growth medium (10). Studies conducted with *R. ruber* and *Nocardia corallina* (a strain from a genus closely related to *Rhodococcus*) have shown that in these species propionyl-CoA is synthesized from succinyl-CoA via the methylmalonyl-CoA pathway (28, 33). Propionyl-CoA can then undergo a condensation reaction with acetyl-CoA via a β-ketothiolase to form the five carbon precursor to 3HV-CoA. These findings suggested that *Rhodococcus* species may be able to make interesting PHA copolymers from palm oil. While poly(HB-*co*-HV) is a more useful bioplastic than PHB, the addition of HV monomers to a PHB chain does not alter the physical properties of the polymer to the same extent as longer chain length monomers (1). This is because HB and HV units can co-crystallize, while monomers longer than

HV disrupt crystallization (6). Therefore production of poly(HB-*co*-HV) alone would not be sufficient to meet the goal of making a bioplastic with wide commercial appeal.

Rhodococci have been studied for their ability to use hydrophobic organic compounds as carbon sources. *Rhodococcus* sp. RHA1 first gained attention when it was demonstrated that this strain could use polychlorinated biphenyls as a sole carbon source (24). Other studies showed that species of *Rhodococcus* could grow on alkanes and diesel fuel, and that biosurfactants were synthesized which may aid in metabolism of these hydrophobic molecules (21). Of special relevance to my research was a report that several species of *Rhodococcus* secrete lipases and are able to grow on plant oils (12). Given that bacteria from the genus *Rhodococcus* grow on insoluble carbon sources, efficiently metabolize fatty acids, and are known to accumulate PHAs, it was decided that these species had potential for converting palm oil to PHA and should be investigated.

A unique advantage of our lab was that we had access to a draft genome sequence of *R. aetherivorans* I24. This strain was originally isolated from soil contaminated with toluene (8). Genome sequencing and assembly were performed at the University of Cambridge, UK (J. Taylor and J. Archer, personal communication) and genome annotation was a collaborative effort between the Archer and Sinskey Labs. Four genes were predicted to encode PHA synthases, based on their amino acid sequence similarity to known PHA synthases, which strengthened our belief that *R. aetherivorans* had potential as a PHA production organism. The putative synthase genes were named C09, D12, H08 Region 1, and H08 Region 2, based on the contigs on which they were discovered. Recombinant expression of these genes in *R. eutropha* revealed that only C09 and D12 encoded active synthases (refer to Chapter 5 for details). An alignment comparing the *R. aetherivorans* C09 and D12 PHA synthases to well characterized synthases from *R. eutropha* and *Aeromonas caviae* is shown in Fig. 2.1. Note that the *R. aetherivorans* C09 enzyme deviates from most other synthases in that in place of the normal lipase box motif at the active site (G-X-C-X-G), this enzyme has the sequence S-X-C-X-G. An alignment of 59 PHA synthases in a recent review showed that only the synthase of *R. ruber* has the same lipase box sequence as the *R. aetherivorans* C09 synthase (22). Note that in Chapter 5 the C09 synthase gene is referred to as $phaC1_{Ra}$ and the D12 synthase gene is referred to as $phaC2_{Ra}$.

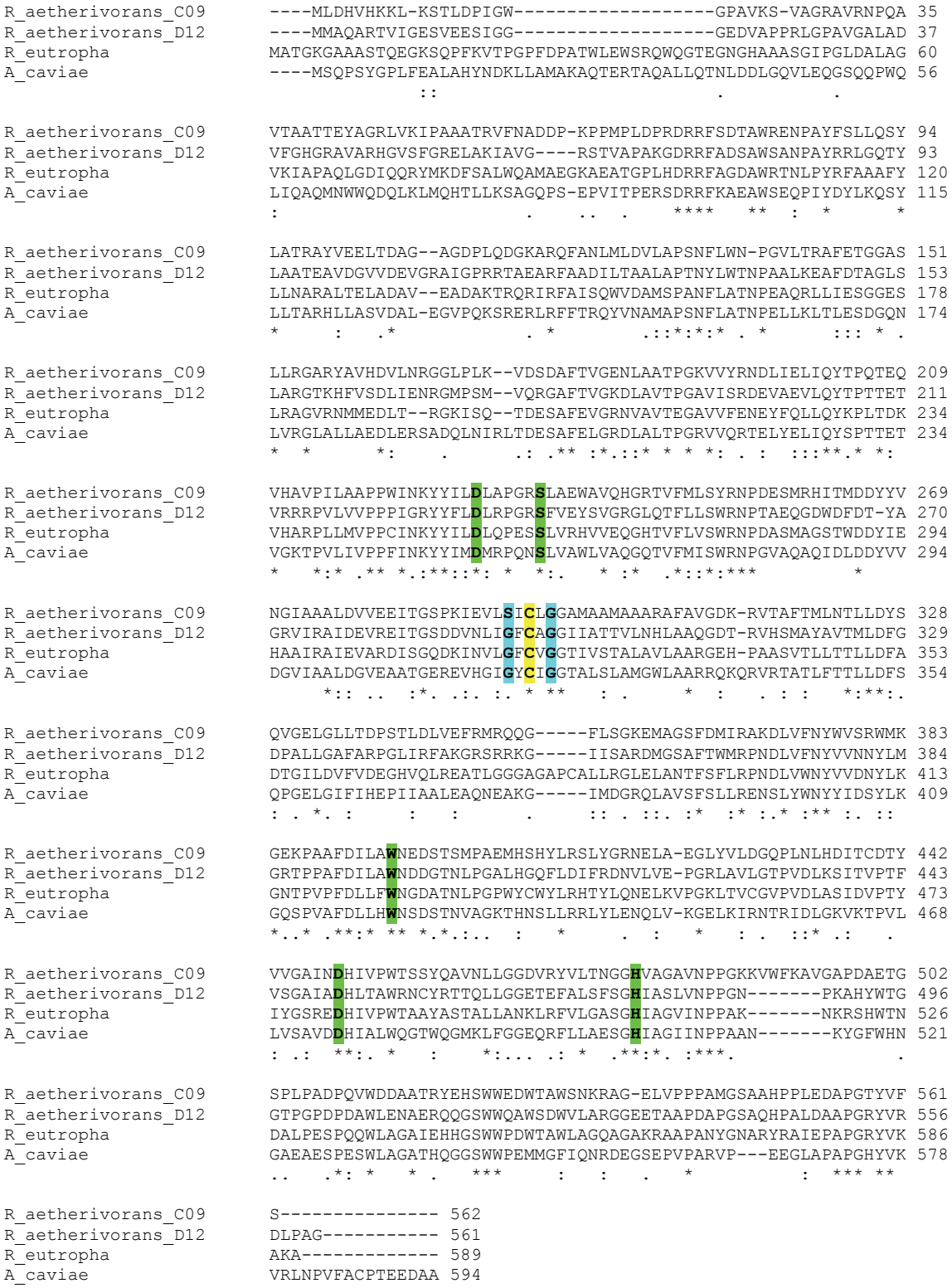

Fig. 2.1: Alignment of the amino acid sequences of four PHA synthases (17). The catalytic cysteine is highlighted yellow, the other lipase box residues are blue, and additional conserved residues shown to influence catalytic activity are green (25).

Materials and methods

Bacterial strain and cultivation conditions

The strain used in these experiments was *Rhodococcus aetherivorans* I24 (8). Solid and liquid lysogeny broth (LB) was routinely used as a rich medium for cell growth (23). The standard minimal medium was used at pH 7 and contained 3.98 g/L K_2HPO_4, 1.65 g/L KH_2PO_4, 1.4 g/L $(NH_4)_2SO_4$, 1.0 g/L $MgSO_4 \cdot 7H_2O$, 15 mg/L $CaCl_2 \cdot 2H_2O$, 1.0 mL/L A9 trace elements solution, and 1.0 mL/L Stock Solution A. The A9 trace elements solution consisted of 0.5 g/L $FeSO_4 \cdot 7H_2O$, 0.4 g/L $ZnSO_4 \cdot 7H_2O$, 0.25 g/L EDTA, 20 mg/L $MnSO_4 \cdot H_2O$, 15 mg/L H_3BO_3, 10 mg/L $NiCl_2 \cdot 6H_2O$, 50 mg/L $CoCl_2 \cdot 6H_2O$, and 5 mg/L $CuCl_2 \cdot 2H_2O$. Stock Solution A consisted of 2.0 g/L $Na_2MoO_4 \cdot 2H_2O$ and 5.0 g/L FeNaEDTA. The carbon source in the minimal medium was either 4% glucose or 2% commercial soybean oil, as indicated in the text. Growth on soybean oil was tested using both oil emulsified with the glycoprotein gum arabic (refer to Chapter 3 for details on emulsified oil cultures) and unemulsified oil. Chemicals were purchased from Sigma Aldrich (St. Louis, MO). In shake flask cultures 1 g/L sterile MOPS buffer (pH 7) was added to the medium to prevent growth inhibition due to acidification of the medium. *R. aetherivorans* was always grown aerobically at 37°C. For flask cultures the orbital shaker was set to 180 RPM.

Batch fermentations were carried out with a 5.5 L fermentor (Lab Fermentor L1523, BioEngineering AG, Wald, Switzerland) using a 3.5 L working volume. The preculture steps consisted of first inoculating 5 mL LB with a single *R. aetherivorans* colony and incubating 24 h on a roller drum. The tube culture was used to inoculate a 50 mL glucose minimal medium flask culture (250 mL flask), which was incubated for 24 h. Cells from this flask culture were used to inoculate a 200 mL glucose minimal medium flask culture (1 L flask), which was incubated until an optical density at 600 nm (OD_{600}) of ~10 was reached. This required 24 to 30 h of growth. The fermentor was inoculated with 10 mL from the 200 mL preculture for an initial OD_{600} of 0.4 – 0.5. All fermentations used glucose minimal medium.

The fermentor was stirred with two six blade impellers and aeration was provided via a ring sparger. The temperature was controlled at 37°C and the pH was maintained at 7.0 ± 0.1 by addition of 2 M phosphoric acid and 2 M sodium hydroxide. The initial stirring rate was 300 RPM and air was provided at 1 VVM. The dissolved oxygen concentration was maintained above 70% using a cascade control scheme that increased the stirring up to 1000 RPM and the air flow up to 2.5 VVM as necessary. A 30% (v/v) polypropylene glycol solution was used as antifoam and was added to the fermentation in

response to an antifoam probe/controller. Deviations from standard fermentation conditions are noted in the text.

Analytical methods

The cell density of cultures was estimated by measuring the OD_{600} with a Spectronic Genesys 20 spectrophotometer (Rochester, NY).

The cell dry weight (CDW) of fermentation samples was measured by taking 5 to 10 mL of culture broth in preweighed plastic test tubes, pelleting at 20,000×*g* for 15 minutes, washing with 0.85% saline, pelleting again, and then drying the cell pellets under vacuum at 80°C. CDW measurements were carried out in duplicate or triplicate and averaged.

Glucose in culture media was quantified using a previously established HPLC method (19). Clarified supernatant from fermentation samples was filtered through 0.2 µm PVDF syringe filters (Pall, Port Washington, NY) and injected onto an Aminex HPX-87H ion exchange column (Bio-Rad, Hercules, CA). The HPLC used was an Agilent 1100 (Santa Clara, CA) and glucose was detected by a change in refractive index. The glucose concentrations were calculated by comparing the peak areas of experimental samples to a standard curve.

Ammonium concentrations in fermentation samples were measured using an ion sensitive electrode for ammonia gas. The Ammonia Gas Sensing Combination Electrode was purchased from Hach (Model 51927-00, Loveland, CO) and was connected to a VWR Scientific ion selective electrode meter (Model 9200, West Chester, PA). 1% (v/v) 10 M sodium hydroxide was added to clarified supernatant samples to convert ammonium ions to ammonia gas and measurements were taken using the electrode. An "air gap assembly" was employed to prevent the electrode from contacting the samples, in order to avoid membrane fouling. The system was calibrated using ammonium chloride standards ranging from 1 mg/L to 1,000 mg/L ammonium.

The amounts of PHA, palmitic acid, and oleic acid in dried cell samples were quantified by the methanolysis protocol reported by Brandl *et al*. (7). Dried cells were incubated with 1 mL $CHCl_3$, 0.85 mL methanol, and 0.15 mL H_2SO_4 for 150 minutes at 100°C, converting PHA monomers and fatty acids into their related methyl esters. The organic phase was analyzed with a Hewlett Packard 6890 gas chromatograph equipped with an Agilent DB-Wax megabore column (30 m × 0.53 mm × 1 µm). A 10 µL

splitless injection was used and methyl esters were detected with a flame ionization detector. The oven temperature was ramped from 70 to 220°C during each run. The concentration of each species was calculated by measuring the appropriate peak area on the chromatogram and comparing the area to a calibration curve generated with standards that had also been subjected to the methanolysis reaction.

Elemental analysis of dried *R. aetherivorans* cells was carried out by Atlantic Microlab, Inc. (Norcross, GA). The C, H, N, O, and S contents of each sample were measured.

Extraction and analysis of storage lipids

At the conclusion of a *R. aetherivorans* glucose fermentation (after 84 h of growth) cells were concentrated and dried at 80°C under vacuum, then ground with a mortar and pestle. 3.7 g of dried cell mass was weighed into a round bottomed flask and 200 mL of chloroform was added. The chloroform was heated to boiling and the extraction was carried out for 96 h in order to dissolve all PHA present. After this time the solution was cooled, and cellular debris was removed with a glass microfiber filter (Whatman International, Cat. No. 1822-055, Maidstone, UK). The filter was washed with additional chloroform, then 600 mL methanol was added to the solution in order to precipitate the PHA (27). The solution was incubated at 4°C for 48 h, after which time a solid white precipitate was visible. This precipitate was collected by passing the mixture through a piece of preweighed filter paper. After drying, the paper was weighed to determine the amount of material recovered. The material was then scraped from the filter paper for further analysis.

3.7 g of dried cell mass prepared in the same manner as above was extracted with hexane in a Soxhlet apparatus for 28 h. Hexane is known to extract neutral lipids from biological samples. After the extraction, the hexane was removed with a rotary evaporator, leaving a viscous orange liquid.

The products of the two extractions were dissolved in deuterated chloroform and analyzed by proton nuclear magnetic resonance (NMR) spectroscopy. NMR spectra were collected using a Varian Mercury 300 MHz spectrometer.

To gain further insight into the hexane extracted lipids from *R. aetherivorans*, thin layer chromatography (TLC) was performed using an established procedure (15). The method used 20 cm × 20 cm glass TLC plates coated with a 250 µm layer of silica gel 60. Two solvent systems were employed: chloroform/methanol/water (65:30:5 by volume, solvent system 1) and hexane/diethyl ether/ acetic

acid (80:20:1.5 by volume, solvent system 2). The plate was first washed by allowing solvent system 1 to travel up the plate. The plate was dried and samples were spotted 1.2 cm from the bottom edge. For each sample, three drops of a 100 mg/mL solution (dissolved in 2:1 chloroform/methanol) were spotted onto the plate with a microcapillary pipette. The plate was then developed twice with solvent system 1 to a distance of 4 cm from the bottom of the plate. The plate was developed once with solvent system 2 to a distance of 14.5 cm from the bottom of the plate. Spots were visualized by spraying the plate with 40% sulfuric acid and then leaving the plate overnight at 80°C, causing organic compounds to char.

Results

Growth of *Rhodococcus aetherivorans* I24 on Plant Oil

R. aetherivorans grew successfully in minimal medium shake flask cultures in which soybean oil was provided as the sole carbon source. Growth occurred when the oil was simply added to the medium and also when the oil was emulsified using gum arabic. Qualitative observations suggested that the growth rate was enhanced and the lag phase was decreased when the oil was emulsified.

The main challenge with the plant oil cultures was quantifying cell growth. When samples from a soybean oil culture were centrifuged in an attempt to measure CDW, some cells pelleted, while others adhered to the layer of unused oil at the top of the test tube. *R. aetherivorans* cells could clearly be identified visually due to their orange color, likely caused by production of carotenoids, which is a common trait among rhodococci (26). The fraction of cells that pelleted and the fraction that were attached to the oil phase could not accurately be determined, but the amount of cell mass associated with the oil appeared to be substantial. Increasing the time and speed of the centrifugation did not separate the *R. aetherivorans* cells from the oil layer.

It was possible to carefully remove the clarified medium between the cell pellet and oil layer with a Pasteur pipette. We hoped that the residual soybean oil could then be removed from the cells by washing the sample with hexane and water. After the hexane wash, however, only a small fraction of cells pelleted, while the majority collected between the organic and aqueous phases. It was impossible to satisfactorily remove the hexane and water while leaving only the cells. When we later concluded that *R. aetherivorans* accumulates TAGs, we identified the additional problem that the hexane wash would extract stored TAGs from the biomass. It was therefore impossible to quantitatively monitor cell growth and lipid production when *R. aetherivorans* was grown on plant oil.

Growth of *Rhodococcus aetherivorans* I24 on Glucose

Given the issues encountered growing *R. aetherivorans* with plant oil as the carbon source, we decided to grow this organism using glucose minimal medium so that we could more easily analyze its growth and PHA production. Results of a typical fermentation are presented in Fig. 2.2. The preculture conditions described in the Methods section were developed to minimize the lag phase of the culture,

and we found that exponential growth had begun by the 8.5 h time point. Ammonium in the medium was depleted by 14 h, so after this time further cell division was impossible. Glucose was still available to the cells, however, allowing for synthesis of carbon storage molecules. CDW continued to increase until the 40 h time point, although surprisingly the observed glucose concentration remained approximately constant after 20 h. The PHA content of the cells reached its maximum early in the experiment, then decreased and remained steady at ~1.4% of CDW after the 14 h time point. No increased storage of PHA was observed in response to nitrogen limitation. Note that because total CDW increased over time, some PHA must have been continually synthesized to maintain a constant percentage of total biomass. As has been observed for other rhodococci, the PHA had high HV content, which stayed constant at ~74mol% throughout the experiment. When analyzing the GC chromatograms of *R. aetherivorans* methanolysis samples, we observed large peaks with late retention times that we believed could correspond to fatty acid methyl esters (refer to the sample chromatogram in Fig. 2.3). Using fatty acid standards, we identified several of these peaks and found that palmitic and oleic acid were the most abundant fatty acids in *R. aetherivorans* samples. Interestingly, the fatty acid profile includes odd chain length fatty acids (as has also been shown for other rhodococci (31)), which are not present in the TAGs in plant oils.

In the next glucose fermentation, we quantified production of PHA, palmitic acid, and oleic acid (Fig. 2.4). In this experiment ammonium was depleted by the 12.5 h time point. We wanted to observe how the *R. aetherivorans* lipid profile responded to resumption of cell growth, so a bolus of ammonium sulfate was added to the culture at 34.5 h. When additional ammonium was available to the cells, the amounts of palmitic and oleic acid rapidly decreased, which is the expected pattern for carbon storage molecules. When nitrogen again became limiting, fatty acid levels increased. As before, the PHA content of the cells remained nearly constant despite the changes in nitrogen availability in the medium. The HV content of the polymer was the same as in the previous fermentation (data not shown). Because of the additional ammonium added to the medium, the total CDW reached in this fermentation was almost three times the CDW achieved in the fermentation shown in Fig. 2.2. In this experiment the glucose concentration consistently decreased as CDW increased, as expected.

We generally observed that the PHA content of *R. aetherivorans* was higher when the bacteria were grown in shake flasks compared to experiments conducted in fermentors. This can be seen in Fig. 2.4, in which the 0 h PHA time point is from a sample taken from the preculture used to inoculate the fermentation. We hypothesized that the reduced oxygen availability in shake flasks relative to the

fermentor could be the reason for the difference in PHA accumulation. We therefore decreased the dissolved oxygen concentration in the fermentor to 30% beginning at 63.5 h. After this point CDW increased slightly then leveled off, but no increase in PHA accumulation was observed. For the final 12 h of the experiment the dissolved oxygen was set to 0%. Total CDW decreased dramatically during this period.

Elemental analysis was performed with dried cells from the fermentation shown in Fig. 2.4. The wt% of C, H, N, O, and S in each sample was determined (Table 2.1). At 34.7 h the cells had accumulated significant lipids. After this point cell growth resumed due to the added ammonium, which is represented by the 39.5 h sample. From 34.7 to 39.5 h, C & H content of the cells decreased, while N content increased. This is consistent with the idea that stored fatty acids were metabolized as additional nitrogen was taken up by the cells. At 45.5 h ammonium in the medium was again depleted and the cells were accumulating fatty acids, leading to increased C & H content in the cells and decreased N content. We believe that phosphorous makes up most of the unaccounted for mass of the dried cells.

Table 2.1: Elemental analysis of dried *R. aetherivorans* cells indicated that C and H content of cells increased during lipid storage and decreased during lipid mobilization.

Element (wt%)	Sample Time (h)		
	34.7	39.5	45.5
C	53.35	47.72	49.79
H	8.22	7.20	7.59
N	4.65	6.89	5.18
O	26.53	26.67	26.88
S	0.13	0.23	0.19
Total	92.88	88.71	89.63

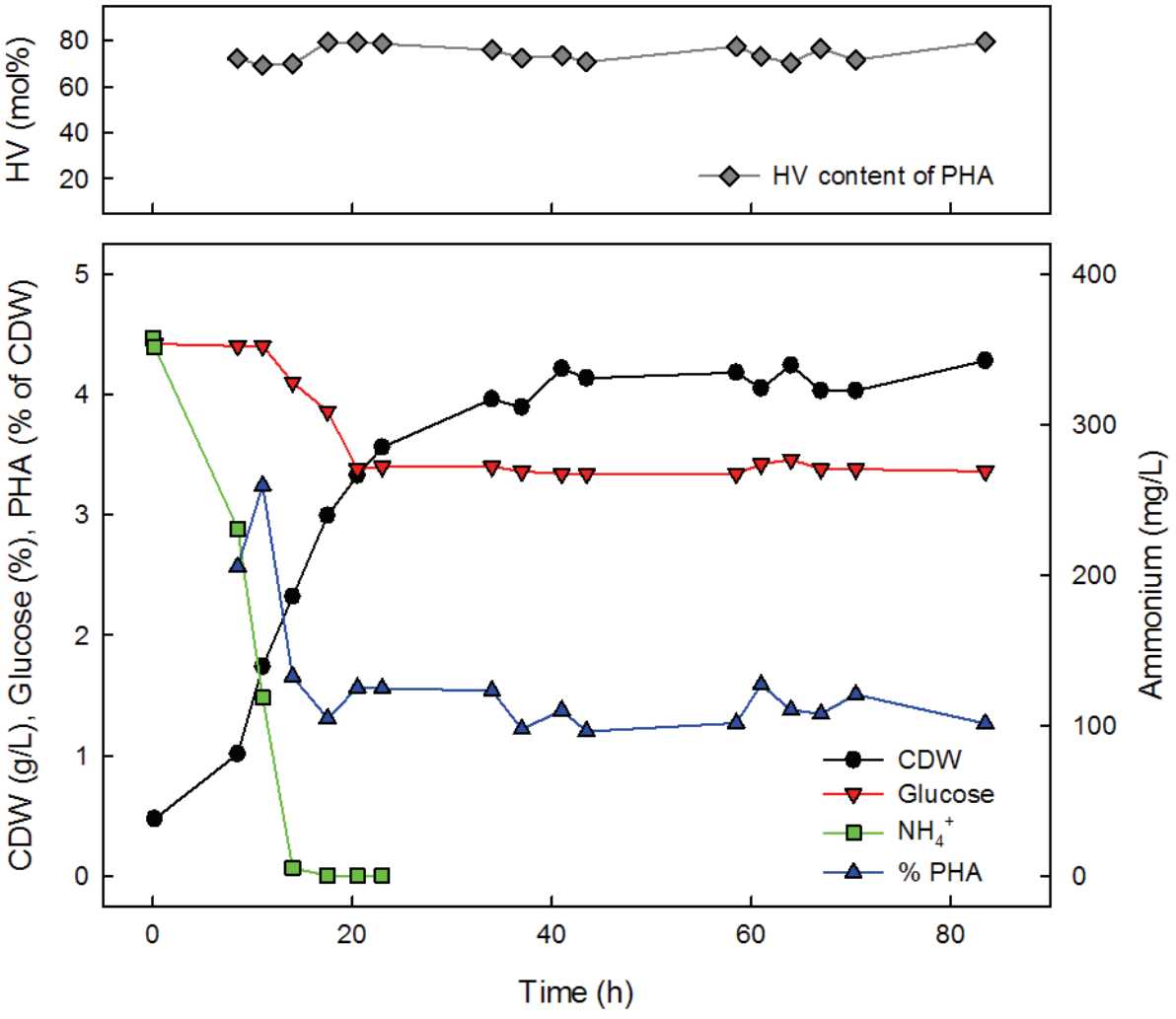

Fig. 2.2: Fermentation I, growth and PHA production of *R. aetherivorans* cultured in glucose minimal medium. The bacteria accumulated a small amount of PHA with high HV content.

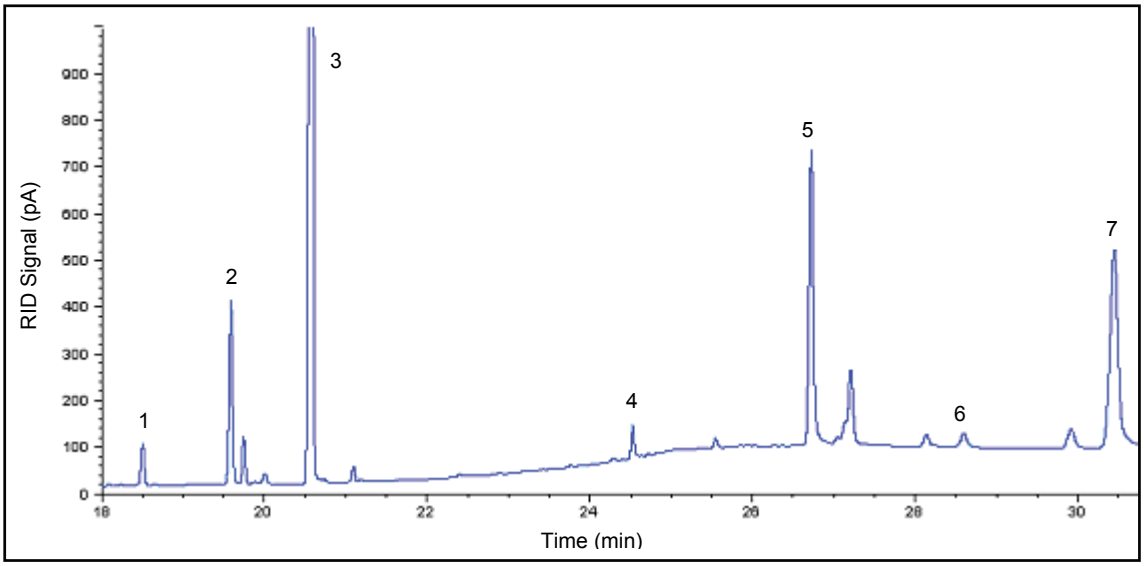

Fig. 2.3: A typical GC chromatogram of a *R. aetherivorans* methanolysis sample. Peaks were identified using standards that were also subjected to the methanolysis reaction. The peaks correspond to the methyl esters of: HB, 1; HV, 2; myristic acid (C14:0), 4; palmitic acid (C16:0), 5; margaric acid (C17:0), 6; oleic acid (C18:1), 7. Peak 3 is methyl benzoate added to the solution as an internal standard.

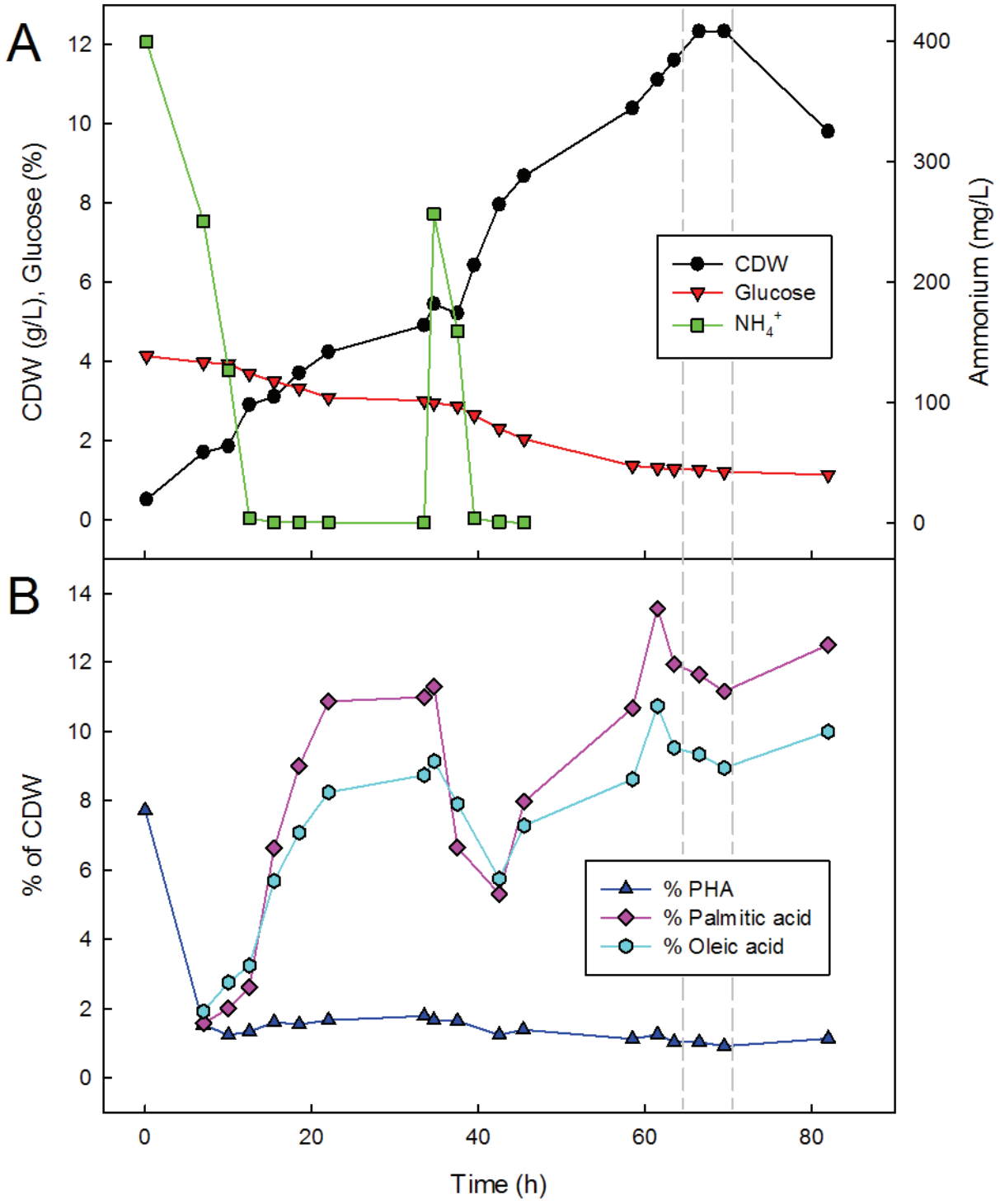

Fig. 2.4: Fermentation II, growth and nutrient consumption of *R. aetherivorans* cultured in glucose minimal medium were measured (A). PHA and fatty acid production were monitored by the methanolysis/GC assay (B). A bolus of ammonium sulfate was provided at 34.5 h. The dashed lines indicate when the dissolved oxygen concentration in the fermentation was reduced, first to 30%, then to 0%.

Analysis of *R. aetherivorans* I24 storage lipids

While the methanolysis/GC assay is useful for quantifying the PHA monomer and fatty acid contents of cells, it has the drawback that the molecules of interest are derivatized during the assay. We therefore performed additional analyses to confirm that *R. aetherivorans* was indeed synthesizing PHA and TAGs. PHA was isolated from dried cell mass using the well established method of dissolving the polymer in chloroform, then precipitating using methanol. The amount of solid recovered by this process was 1.9% of the dry biomass. This value agrees well with the methanolysis data presented in Figs. 2.2 and 2.4. To confirm that the recovered solid was PHA, it was examined using proton NMR, as shown in Fig. 2.5. Comparing the spectrum of the experimental sample to the spectrum of a poly(HB-*co*-HV) standard purchased from Sigma Aldrich, we found that the chemical shifts of all peaks matched, demonstrating that *R. aetherivorans* synthesized the expected copolymer. The HV content of the copolymer was quantified using NMR peak areas, and it was determined that the polymer contained 74mol% HV. Once again, this finding confirms the methanolysis data. Note that peak *f* in Fig. 2.5 cannot be used to quantify HV content, because water present in the $CDCl_3$ generates a peak with the same chemical shift (11), artificially increasing this peak area.

Confirming the presence of TAGs in *R. aetherivorans* cells was especially critical, as fatty acids stored in TAGs cannot be distinguished from cell membrane fatty acids in the methanolysis/GC assay. The hexane extract from *R. aetherivorans* should include all TAGs present, but may also contain other hydrophobic compounds produced by the bacteria. Comparing the proton NMR spectrum of the hexane extract to the spectrum of a known TAG (palm oil) shows the two are extremely similar (Fig. 2.6). Peaks characteristic of the TAG backbone (*a* and *b*) appear in the *R. aetherivorans* extract spectrum, along with a number of peaks resulting from saturated and unsaturated fatty acids. Intriguingly, there are additional peaks in the extract spectrum around 3.5 ppm that do not appear in the palm oil spectrum. The compound(s) responsible for these peaks have not been identified.

TLC was used to further confirm accumulation of TAGs by *R. aetherivorans*. The TLC method was able to clearly separate phospholipids, free fatty acids, and TAGs. Fig. 2.7 shows that the major spot from the *R. aetherivorans* hexane extract matches the TAG standard.

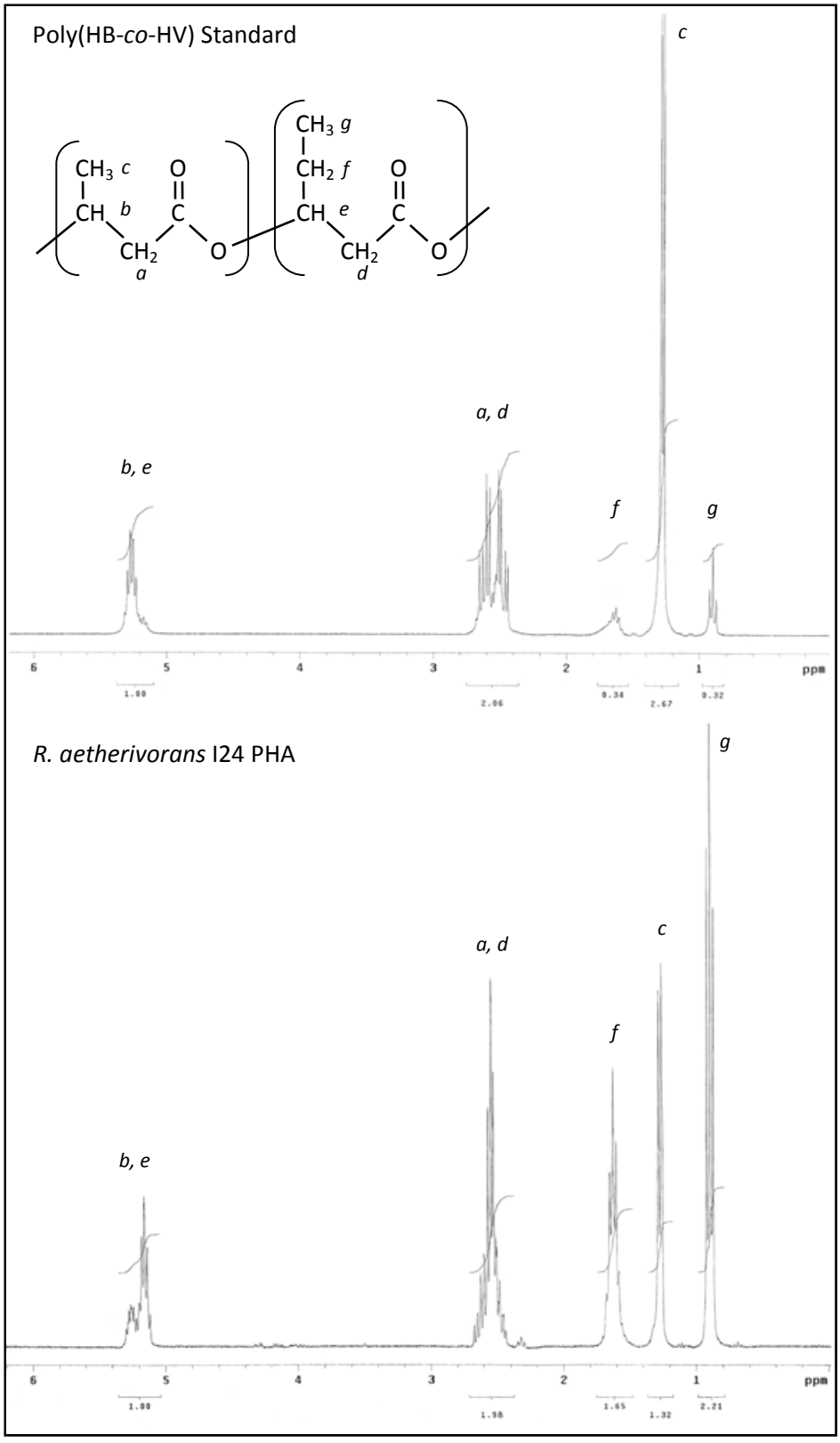

Fig. 2.5: ^{1}H NMR spectra in $CDCl_3$ of a poly(HB-*co*-HV) standard and PHA extracted from *R. aetherivorans* I24. Based on the area of peak *g*, it was determined that the standard contained 10mol% HV and the extracted PHA had 74mol% HV.

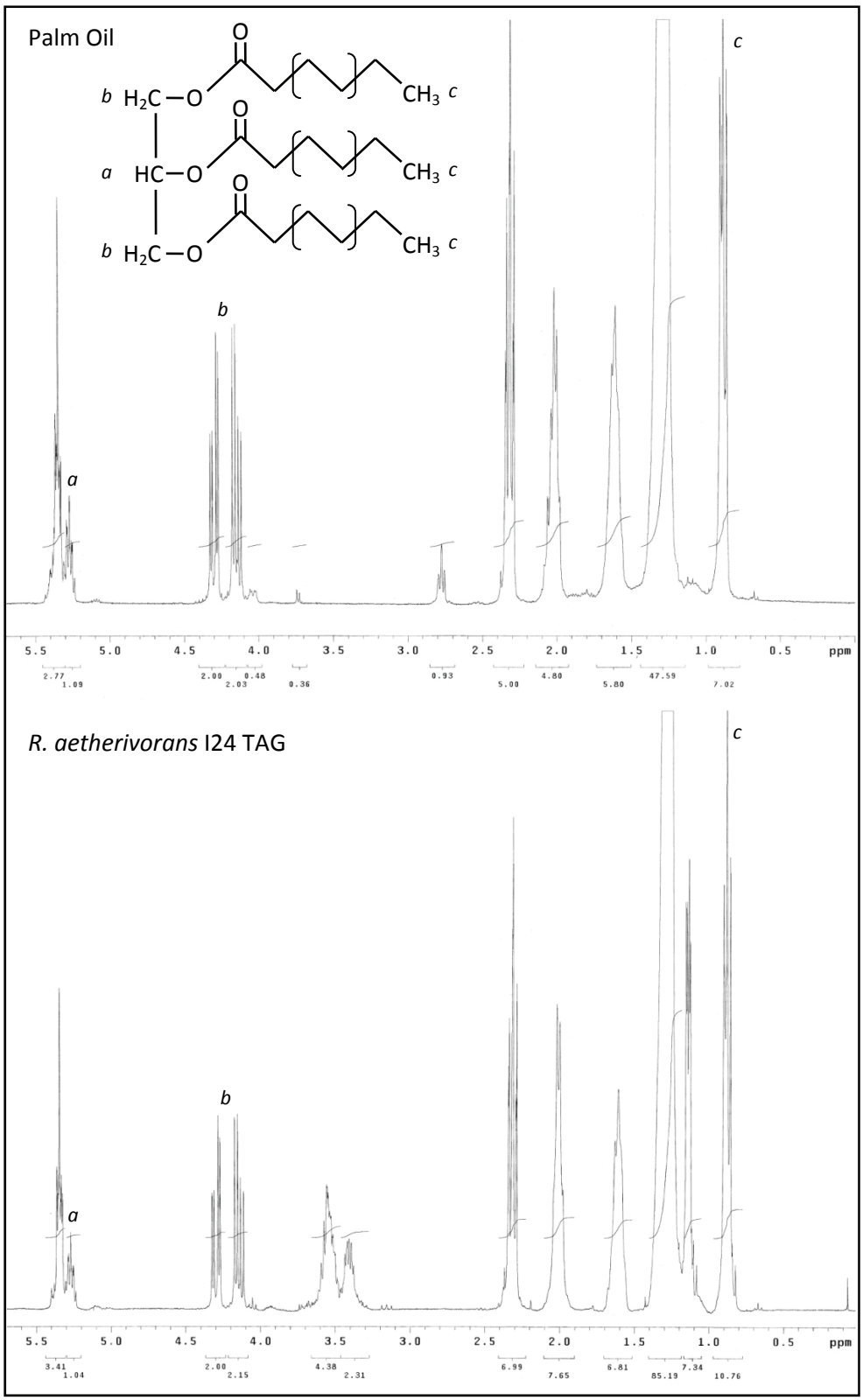

Fig. 2.6: ^{1}H NMR spectra in $CDCl_3$ of palm oil and TAGs extracted from *R. aetherivorans* I24. Peaks characteristic of TAG molecules are labeled *a, b, c*. Peaks from 0.5 to 2.5 ppm resulted from saturated and unsaturated fatty acid protons. The source of the peaks around 3.5 ppm in the *R. aetherivorans* extract is unknown.

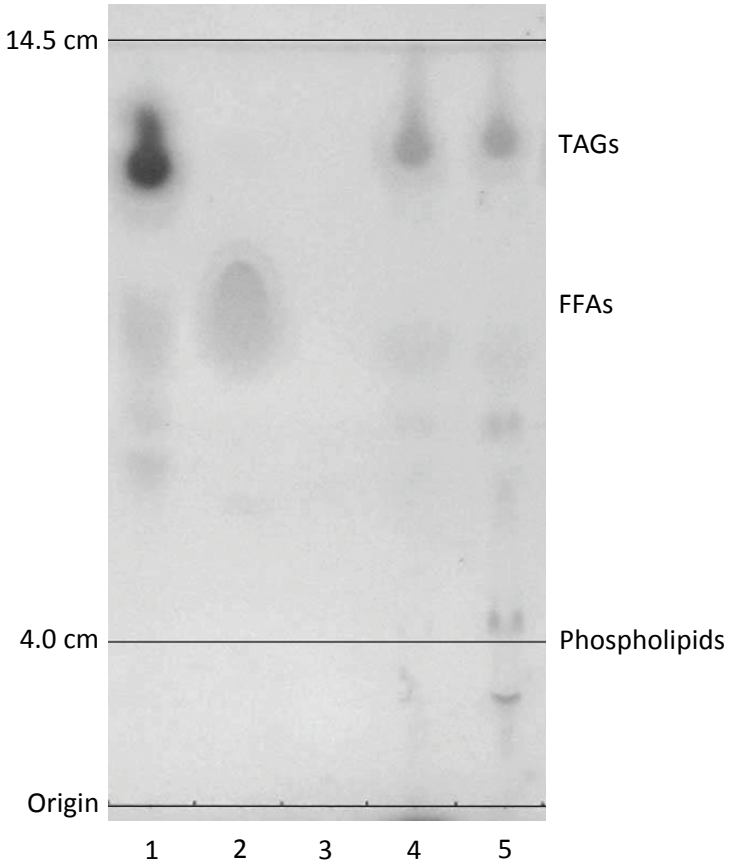

Fig 2.7: TLC analysis revealed that hexane extracts of *R. aetherivorans* grown on glucose primarily consisted of TAGs. The lanes contained: soybean oil, 1; oleic acid, 2; phospholipid standard, 3; fermentation I extract, 4; fermentation II extract, 5. Soybean oil served as a TAG standard, while oleic acid was the free fatty acid (FFA) standard. The phospholipid standard could not be visualized on this plate, but later experiments showed that phospholipids migrated ~4 cm under these conditions. Samples were spotted at the origin, 1.2 cm from the bottom edge of the plate.

Discussion

Studies with *R. aetherivorans* grown in glucose minimal medium showed that this species is able to synthesize both poly(HB-*co*-HV) and TAGs. While the PHA is of some interest due to its high HV content, the fact that the bacteria accumulated <2wt% PHA led us to conclude that *R. aetherivorans* does not have potential as a PHA production organism. The PHA content of the cells remained constant throughout fermentation experiments, regardless as to whether or not nutrient limitation had occurred. This suggests that PHA is not actively used by *R. aetherivorans* for carbon storage. Interestingly, we consistently observed greater PHA accumulation in shake flask cultures than in fermentor cultures. The only difference in the media was that the shake flask medium contained MOPS buffer, although it is unclear whether this difference influenced PHA production. Reducing the dissolved oxygen concentration during a fermentation did not lead to increased PHA production, suggesting that oxygen limitation is not responsible for the higher PHA content observed in flask cultures. Additional work is required to determine the reason for the higher level of stored PHA in flask cultures.

Quantitative RT-PCR was used to analyze RNA extracted from *R. aetherivorans* fermentation samples, in order to determine which of the putative PHA synthase genes were expressed during growth. RNA from the C09, H08 Region 1, and H08 Region 2 genes was detected, suggesting that these potential synthases are expressed, while D12 is not (Tim Ohlrich, Diploma Thesis, unpublished). Given that I discovered only C09 and D12 encode active PHA synthases (see Chapter 5), this suggests that the C09 gene product is responsible for PHA biosynthesis in *R. aetherivorans*.

R. aetherivorans appears to utilize TAGs as its principal carbon storage molecule. The presence of intracellular TAGs was confirmed by NMR and TLC analyses of *R. aetherivorans* hexane extracts. The NMR spectrum of one of these extracts also included peaks that did not appear on the plant oil NMR spectrum (Fig. 2.6). While we cannot definitively identify the source of these peaks, the chemical shifts (~3.5 ppm) are consistent with hydroxylated fatty acids, which are known to be synthesized by *Rhodococcus erythropolis* and other actinomycetes (2, 3, 14). If hydroxyl fatty acids are in fact the source of these peaks, further work is required to determine whether they are incorporated into TAGs or are present in a different hexane-soluble compound.

The maximum fatty acid content measured in the fermentation shown in Fig. 2.4 was 24.3% of CDW. This underestimates total fatty acid content because only palmitic and oleic acid were quantified, and there are certainly other fatty acids present in the cells (Fig. 2.3). The strain *R. opacus* PD630 has been

studied in the most depth for possible industrial TAG production. While *R. aetherivorans* did not reach the fatty acid levels that have been reported for *R. opacus* (16), it is possible that the gap could be closed by optimizing the culture medium and growth conditions for TAG production in *R. aetherivorans*. Genes responsible for PHA production could also be knocked out to prevent flux of acetyl-CoA and propionyl-CoA to PHA. The primary advantages of *R. aetherivorans* over *R. opacus* are that it grows more quickly and is cultured at a higher temperature (16).

The fermentation experiments were conducted using glucose as the carbon source because cell growth in plant oil shake flask cultures proved impossible to accurately quantify. Despite testing several centrifugation procedures, *R. aetherivorans* cells always adhered to the low density organic phase in samples, whether it was residual plant oil or hexane used to wash the cells. It has been shown that other *Rhodococcus* species grew at the interface of the organic and aqueous phases when hydrophobic compounds were used as carbon sources (30), and that adherence to hydrocarbon droplets may be due to an extracellular polymeric substance (32). Additional experiments are required to determine the mechanism by which *R. aetherivorans* adheres to plant oil. We initially hoped to overcome the oil adherence issue by washing the samples with hexane, but this would lead to extraction of TAGs stored by *R. aetherivorans* and make the resulting measurements inaccurate.

We concluded that *R. aetherivorans* was not a suitable organism for additional PHA research because of the low amount of polymer produced, the challenges associated with growing this strain on plant oil, and a lack of well developed genetic tools for strain improvement. Our attention shifted to *R. eutropha*, which grows robustly on plant oil and accumulates high levels of PHA. Although *R. aetherivorans* did not become the main focus of my thesis work, this project still provided important results. The finding that this strain synthesized poly(HB-*co*-HV) encouraged us to characterize the putative synthases from the *R. aetherivorans* genome, and two of these genes have proven valuable for constructing novel recombinant *R. eutropha* strains. We also demonstrated that *R. aetherivorans* accumulated TAGs and found that this strain should be explored as a possible biofuel producer.

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Chapter 3
Growth of *Ralstonia eutropha* on Emulsified Plant Oil

Introduction

Plant oils are important agricultural products that are harvested from a variety of crops worldwide, including soybean, rapeseed, and oil palm. These oils primarily consist of triacylglycerols (TAGs), in which three fatty acids are attached to a glycerol backbone. The types and distribution of fatty acids present in an oil vary depending on the plant species (27). Plant oils have traditionally been used in the food industry, but they can also be chemically processed into other products such as fuels, fine chemicals, and polymers (11, 19, 27). Recent studies have investigated the feasibility of using bacteria to biologically convert plant oils and fatty acids into fuels (15), surfactants (16, 24), and polyhydroxyalkanoate (PHA) bioplastics (1). In order to utilize plant oils, bacteria secrete lipases, which catalyze the release of fatty acids from TAGs (13). The fatty acids are then transported into the cell, where they are catabolized via the β-oxidation cycle (7). Some bacteria also synthesize surfactants that may increase the surface area and bioavailability of hydrophobic carbon sources, allowing for more efficient growth on these compounds (25).

Our group is interested in studying production of PHA from plant oils by the bacterium *Ralstonia eutropha* H16. PHAs are natural polyesters that can be used as biodegradable bioplastics (2), and *R. eutropha* is the model organism for studying PHA synthesis and accumulation (22). Wild type *R. eutropha* is known to make the homopolymer polyhydroxybutyrate (PHB) from plant oils, but the strain can be engineered to make other types of PHA (8). While there are several reports in the literature of PHA production from plant oils with *R. eutropha* (8, 14, 18, 21), when we began our experiments we experienced difficulties using plant oil as a carbon source for bacterial fermentations. Addition of insoluble plant oil to aqueous growth medium creates a heterogeneous mixture, in which the low density oil is concentrated at the top of the vessel. Oil can be dispersed in a fermentor by rapid stirring, but aeration forces the oil back to the top of the fermentor, where it collects on the walls of the vessel. Build up of oil on the vessel walls makes this material unavailable to the cells and leads to errors when attempting to measure oil consumption and product yield. Another issue caused by the heterogeneity of the medium is that taking representative samples early in plant oil cultures is impossible, as there is no way to determine an appropriate depth for the sampling port. *R. eutropha* will eventually grow in a

medium in which plant oil is the sole carbon source, and as the cells grow the oil becomes emulsified over time. We found, however, that lag times in these cultures were long and could vary considerably between experiments. In order to conduct quantitative and reproducible experiments, we therefore developed a method for growing *R. eutropha* cultures in which the plant oil is emulsified using a chemical emulsifying agent. These emulsified oil cultures can be grown in both shake flasks and fermentors.

When developing this method we investigated several surfactants commonly found in biotechnology laboratories: sodium dodecyl sulfate (SDS), Tween 80 (polyoxyethylene (20) sorbitan monooleate), and Triton X-100 (4-(1,1,3,3-Tetramethylbutyl)phenyl-polyethylene glycol). We also tested gum arabic (GA), a natural glycoprotein synthesized by the acacia tree (9, 23). GA is used as a thickener, emulsifier, and stabilizer in the food processing industry, and is classified by the United States Food and Drug Administration as Generally Recognized as Safe (U.S. Code of Federal Regulations, 21CFR184.1330). Surfactants have been shown to interact with microbes in many ways, and can either inhibit or stimulate growth depending on the molecule and the species of bacteria (28). When evaluating our chosen surfactants for use with *R. eutropha*, we therefore examined their toxicity, ability to be metabolized, and influence on PHB production. We found that gum arabic was the most effective emulsifier for growth of *R. eutropha* on plant oil, and we believe this method could also be a useful experimental tool for studying growth of other bacteria on oils.

Materials and methods

Bacterial strain and cultivation conditions

All experiments were performed with *Ralstonia eutropha* H16 (ATCC 17699). The rich medium used in this study was dextrose free tryptic soy broth (TSB) medium (Becton Dickinson, Sparks, MD). We used a phosphate buffered minimal medium (pH 6.8) that has been previously described (6). Various carbon sources, emulsifying agents, and nitrogen sources were added to the minimal medium as described in the text. All media contained 10 μg/mL gentamicin sulfate. Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless noted otherwise. *R. eutropha* was always grown aerobically at 30°C. For shake flask experiments the shaker was set to 200 RPM.

The inhibitory effects of the emulsifiers were studied by preparing TSB using 90% of the normal volume of water. Concentrated stocks of the emulsifying agents were prepared and autoclaved. The emulsifiers were then added to the TSB to the desired concentration, along with the amount of sterile water necessary to bring the TSB to its normal concentration. The abilities of the emulsifying agents to act as sole carbon sources were studied by adding compounds from sterile stocks to minimal medium, along with 0.1% ammonium chloride as the nitrogen source. As gum arabic is a glycoprotein, it contains amino acids and could therefore potentially serve as a nitrogen source. This was studied using minimal medium containing 2% fructose, 0.3% GA, and no NH_4Cl. Optical densities at 600 nm (OD_{600}) were measured using a Spectronic Genesys 20 spectrophotometer (Rochester, NY).

We observed that when GA was autoclaved in the presence of the salts found in the minimal medium, the liquid became slightly cloudy. We believe this was due to some of the protein denaturing and coming out of solution. To determine how this precipitant influenced cell growth and PHB synthesis, minimal medium cultures containing 0.05% NH_4Cl were made. Sets of cultures were then prepared that contained one of the following combinations: (1) 2% fructose and no GA, (2) 2% fructose and 0.3% GA, or (3) no fructose and 0.3% GA. Additionally, flasks were prepared that contained medium with no fructose and 0.3% GA that were not inoculated with *R. eutropha*, in order to determine the amount of material that precipitated over time in the absence of cells. Fructose was used as the carbon source in these experiments instead of plant oil, because this made it easier to examine the effects of GA without the added complication of comparing emulsified versus non-emulsified oil.

Preparation of emulsified oil medium

The oil medium used in this study was designed to stimulate PHB accumulation by *R. eutropha*. A low level of NH_4Cl was supplied to the cells and excess carbon was provided in the form of palm oil (Wilderness Family Naturals, Silver Bay, MN). After nitrogen in the medium was exhausted, cell division ceased and PHB was stored by the cells. We found that in minimal medium with 0.05% NH_4Cl, 1% palm oil provided sufficient carbon, while medium with 0.1% NH_4Cl required 2% palm oil. 1% oil needed 0.3% GA for efficient emulsification, while 2% oil could be emulsified with 0.5% GA. GA is available from Sigma-Aldrich (Cat. No. G9752).

To prepare the medium, a 10× solution of GA was prepared in water. GA dissolves slowly at room temperature, so the solution was stirred rapidly to speed dissolution. The GA solution was then centrifuged (10,500×*g*) to separate out insoluble particles. The sodium phosphate, K_2SO_4, clarified GA, and palm oil were combined, along with an appropriate amount of water, and emulsified by homogenizing with a Sorvall Omni-Mixer for one minute. The amount of water added before emulsification will depend on the particular apparatus used to make the emulsion. We found that concentrated oil emulsions could be made, and then diluted as necessary. We discovered that homogenizers were the most efficient tools for emulsification, but sonicators can also be used. After emulsifying the oil, the medium was autoclaved, cooled, and $MgSO_4$, $CaCl_2$, trace elements, and gentamicin were all added from sterile stocks.

Fermentation Conditions

Emulsified palm oil fermentations were carried out using an Infors Sixfors multiple fermentor system (Bottmingen, Switzerland). *R. eutropha* was grown overnight in TSB, and these cultures were used to inoculate 50 mL minimal medium flask precultures with 2% fructose and 0.1% NH_4Cl. Cells from the minimal medium precultures were used to inoculate each fermentor to an initial OD_{600} of 0.1. Each vessel contained 400 mL of emulsified palm oil medium with 2% palm oil, 0.5% GA, and 0.1% NH_4Cl. The temperature of each fermentor was kept constant at 30°C. The pH of each culture was maintained at 6.8 ± 0.1 through controlled addition of 2 M sodium hydroxide. Stirring was provided by two six-blade Rushton impellers at speeds of 500-900 RPM. Air was supplied at 1 VVM and the dissolved oxygen concentration was maintained above 40% through controlled addition of pure oxygen.

Analytical methods

The cell dry weight (CDW) and PHB content of samples from fructose cultures were measured as described previously (6). Residual cell dry weight (RCDW) was calculated as the total CDW minus the mass of PHB. CDW of samples from oil fermentations was measured by taking 10 mL samples in 15 mL preweighed plastic tubes, centrifuging, washing with 5 mL cold water plus 2 mL cold hexane, centrifuging, resuspending the cells in 2 mL cold water, freezing at -80°C, and lyophilizing. The hexane was included in the washing step to remove unused oil. The PHB content of cells was measured by transferring freeze dried biomass to screw top glass test tubes, and reacting the samples with methanol and sulfuric acid in the presence of chloroform to convert the PHB monomers into methyl 3-hydroxybutyrate (3). The amount of methyl 3-hydroxybutyrate was quantified using an Agilent 6850 gas chromatograph (Santa Clara, CA) equipped with a flame ionization detector and a DB-Wax column (Agilent, 30 m × 0.32 mm × 0.5 µm). The carrier gas was hydrogen (3.0 mL/min) and the temperature program was 80°C for 5 min, ramp to 220°C at 20°C/min, and hold at 220°C for 5 min. A calibration curve for the instrument was generated by treating known amounts of pure PHB as described above and measuring the resulting peak areas on the chromatograms. Fatty acids in lipid samples were quantified using the same methanolysis procedure, with pure fatty acids as standards.

A previous report described a method for measuring plant oil concentrations in which oil was extracted from 2 mL of medium using hexane (14). We tested this method with emulsified palm oil medium and could not achieve quantitative oil recovery. We therefore developed a new method for measuring the concentration of oil and other lipids in emulsified oil medium. For each sample, 10 mL medium was taken in a 15 mL plastic test tube and centrifuged 10 minutes using a swinging bucket rotor. (In this method all centrifugations were performed at room temperature and 7,200×*g*.) The supernatant was transferred to a 50 mL plastic test tube, and the pellet was washed with 5 mL water and centrifuged again to recover oil that had been associated with the cell pellet. This supernatant was then combined with the supernatant from the previous centrifugation (15 mL total). 20 mL of a 2:1 (v/v) mixture of chloroform and methanol were added to the tube, and the sample was vortexed for one minute. The sample was then centrifuged and 5 mL of the organic phase (i.e. the bottom phase) was transferred to a preweighed glass test tube. Solvent was removed by incubating the test tube in a heat block at 40°C, then transferring the test tube to a vacuum oven at 80°C. After drying, samples were weighed to determine the mass of recovered lipid. The lipid concentration in the medium was calculated using the following equation:

$$\text{Lipid concentration} = \text{Mass recovered}\left(\frac{V_{organic}}{V_{transferred}}\right)\left(\frac{1}{V_{sample}}\right)$$

In this equation, $V_{organic}$ is the volume of the organic phase after the extraction. Because the solvent mixture includes methanol, not all of the solvent remains in the organic phase after contact with the aqueous medium. We measured $V_{organic}$ to be 13.33 mL when using chloroform/methanol. $V_{transferred}$ was 5 mL and V_{sample} was 10 mL (the volume of medium taken from the culture).

The ammonium concentration in fermentation samples was determined from cell free supernatants using an enzymatic ammonium assay kit from Sigma Aldrich (Cat. No. AA0100). The assay was carried out following the manufacturer's instructions.

Glycerol was detected using a HPLC assay with an Aminex HPX-87H column (Bio-Rad, Hercules, CA) that was previously used in our lab to measure fructose concentrations (17). A 100 µL injection was used instead of 25 µL in order to detect low concentrations of glycerol. Glycerol had a retention time of 13.6 min.

The lipid species extracted from fermentation samples were examined qualitatively by thin layer chromatography. Lipid samples were dissolved in chloroform and 30 µg were spotted onto a silica gel TLC plate (250 µm thickness). The standard mixture spotted on the plate contained a triacylglycerol (TAG, 1,2-distearoyl-3-oleoyl-*rac*-glycerol, 10 µg), diacylglycerol (DAG, 1,2-dipalmitoyl-*rac*-glycerol, 20 µg), monoacylglycerol (MAG, 1-palmitoyl-*rac*-glycerol, 20 µg), and free fatty acid (FFA, palmitic acid, 10 µg). The plate was first developed with chloroform/methanol/water (60:35:5, by volume) to 5 cm from the origin. It was next developed with hexane/diethyl ether/acetic acid (70:30:1, by volume) to the top of the plate. Spots were visualized by spraying the plate with 3% cupric acetate in an 8% phosphoric acid solution and incubating the plate at 200°C.

Results

Evaluation of potential emulsifying agents

To determine if the potential emulsifying agents inhibited growth of *R. eutropha*, TSB cultures were prepared that included each of the surfactants. The surfactant concentrations used in this experiment were in the ranges needed to generate 1% palm oil emulsions: SDS, 0.05%; Triton X-100, 0.05%; Tween 80, 0.2%; GA, 0.3%. OD_{600} measurements were made over time to monitor cell growth (Fig. 3.1). We found that growth of the GA cultures was nearly indistinguishable from the TSB-only control cultures. The cultures that included Tween 80 grew at the same rate as the controls through 12 h, but exhibited a higher cell density at the 24 h time point. The Triton X-100 cultures grew more slowly than the controls, but did show significant growth by 24 h. Cultures that contained 0.05% SDS did not grow over the course of the experiment. When the SDS concentration was reduced to 0.01% *R. eutropha* was able to grow, but the cultures still grew more slowly than the TSB-only control (data not shown).

Our goal was to study growth of *R. eutropha* on plant oil, therefore it was important that the bacteria could not use the surfactants as an alternate carbon source. To test this, we prepared minimal medium cultures in which each surfactant was the sole carbon source. OD_{600} was monitored and compared to controls that contained no carbon source (Table 3.1). The OD_{600} measurements of the SDS and Triton X-100 cultures actually decreased relative to the controls. We observed aggregation of cells in these cultures, which may have caused the drops in optical density. The GA cultures showed slightly higher OD_{600} values than the controls at both time points, but OD_{600} did not increase over time, indicating that no significant growth was taking place. The Tween 80 cultures showed considerably higher optical densities than the controls, and the OD_{600} readings for these cultures increased over time. This demonstrates that Tween 80 can serve as an effective carbon source for *R. eutropha*.

Because gum arabic is a glycoprotein, it also has the potential to serve as a nitrogen source for *R. eutropha*. This was investigated using minimal medium cultures with 2% fructose and 0.3% GA as the sole potential nitrogen source, along with controls that contained 2% fructose and no GA. Triplicate cultures were inoculated to an initial OD_{600} of 0.1. After 48 h of incubation, the mean OD_{600} of the controls was 0.280 ± 0.008, while the mean OD_{600} of the GA cultures was 0.326 ± 0.006. The increase in OD_{600} of the controls can be attributed to the changes in cell morphology caused by storage of PHB (30). The GA cultures exhibited a slightly greater increase in OD_{600}, but the fact that there was little difference

between the GA cultures and the controls indicates that GA does not serve as an effective nitrogen source for *R. eutropha*.

Table 3.1: Minimal medium cultures with 0.1% NH_4Cl and surfactants as the sole carbon sources were inoculated with *R. eutropha* to an initial OD_{600} of 0.1. The cultures were incubated 24 h and OD_{600} values were measured. Values reported are means from three independent cultures ± SD. The control cultures contained no carbon source. A positive control with 2% fructose reached OD_{600} >10 after 24 h.

Surfactant	Concentration	OD_{600}	
		10 h	24 h
Control	N.A.	0.104 ± 0.001	0.101 ± 0.002
SDS	0.05%	0.071 ± 0.002	0.062 ± 0.002
Triton X-100	0.05%	0.091 ± 0.002	0.078 ± 0.001
Tween 80	0.2%	0.727 ± 0.027	1.229 ± 0.009
GA	0.3%	0.125 ± 0.009	0.123 ± 0.011

Growth of *R. eutropha* in fructose minimal medium with gum arabic

Based on our investigation of the effects of the different surfactants on *R. eutropha*, we determined that GA was the best emulsifying agent to use in plant oil cultures. In order to gain further insight into the influence of GA on cell growth and PHB production, as well as to determine how precipitated GA could influence measurements, we grew *R. eutropha* in fructose minimal medium in the presence or absence of 0.3% GA (Fig. 3.2). The RCDW values with and without GA in the medium were almost identical, showing that this compound does not significantly influence cell growth. GA does slightly reduce PHB accumulation, however, as cells grown in medium with GA made 13% less PHB over 72 h than cells not exposed to GA. Despite this reduction, *R. eutropha* grown in the presence of GA still stored significant polymer (79% of CDW). When GA was the only potential carbon source, the maximum CDW observed was <0.1 g/L, which supports our previous finding that GA cannot serve as a carbon source for *R. eutropha*. The PHB content of cells from cultures lacking fructose decreased steadily from 12 to 72 h (Fig. 3.2B), indicating that the cells mobilized stored PHB in the absence of an exogenous carbon source. The PHB initially present in these cells was accumulated during the precultures.

A set of flasks was also prepared with 0.3% GA medium that was not inoculated with *R. eutropha*, in order to determine how much GA comes out of solution during a typical experiment. This was a concern

because significant precipitation could make CDW and PHB content measurements inaccurate. No mass of precipitated material could be measured at the 2 and 12 h time points. We did detect precipitant after 24 h, which increased in concentration from the 24 to 72 h time points and reached a maximum of 0.046 g/L (data not shown). Comparing this value to the measurements from the *R. eutropha* cultures with fructose and GA, we found that the precipitated GA accounted for approximately 1% of the total CDW and 5% of the RCDW at the 72 h time point.

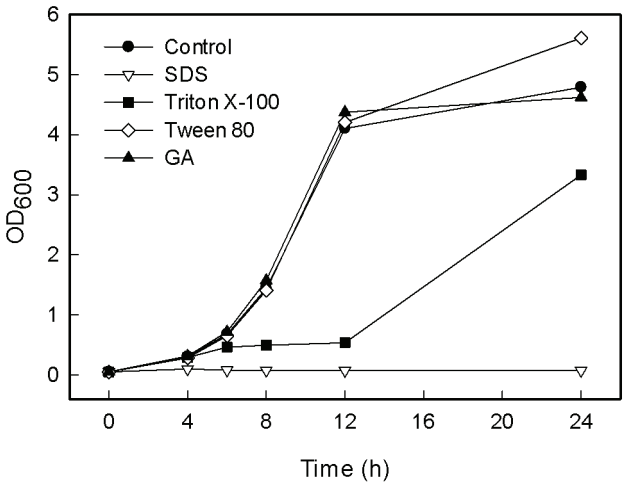

Fig. 3.1: SDS and Triton X-100 inhibited growth of *R. eutropha* in TSB. GA did not inhibit growth in TSB, while addition of Tween 80 to the cultures led to higher cell densities at the 24 h time point. Values reported are means from triplicate cultures.

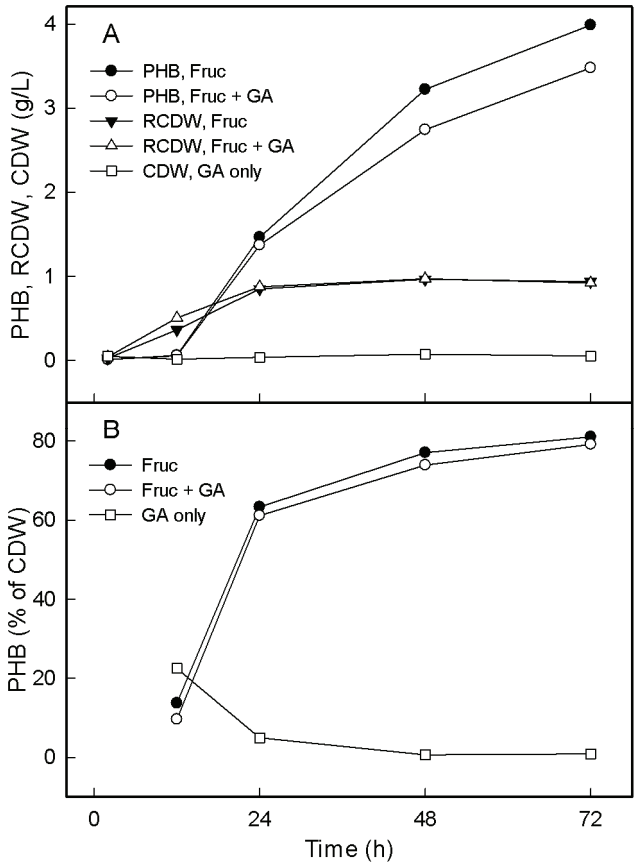

Fig. 3.2: ***R. eutropha*** **was grown in fructose minimal medium with GA (open symbols) and without GA (closed symbols). Growth and PHB production were measured (A), as was PHB content (B). Control cultures with GA and no fructose (open squares) showed that GA could not be used as a carbon source and that cells metabolized stored PHB in these cultures. Values reported are means from triplicate cultures.**

Quantification of oil utilization

We found that a published method for measuring oil concentrations in bacterial growth media that relied on hexane extraction was not quantitative when applied to our emulsified oil medium (14). We therefore tested extraction of palm oil from 10 mL samples using hexane, chloroform, and a 2:1 mixture of chloroform and methanol. (Chloroform/methanol mixtures have long been used to extract lipids from biological samples (12).) As bacteria grow and oil is broken down, other lipids will appear in the culture medium, including monoacylglycerols (MAGs), diacylglycerols (DAGs), and free fatty acids (FFAs). To accurately measure the total amount of lipid present in a medium, an extraction procedure must also recover these polar lipids. To test the extraction of polar lipids, we also measured recovery of oleic acid and a 1:1 mixture of oleic acid and palm oil from GA emulsions. Results of these experiments are shown in Table 3.2.

Table 3.2: Palm oil (PO), oleic acid (OA), and a 1:1 mixture of the two were extracted from minimal medium standards in which the lipids were emulsified with gum arabic (GA). 10 mL samples were taken from each standard and 20 mL of each solvent was used for extraction. All extractions were performed four times, and the reported values represent the means ± SD.

Solvent	Mass of lipid recovered (%)					
	1% Lipid + 0.3% GA			2% Lipid + 0.5% GA		
	PO	OA	PO/OA	PO	OA	PO/OA
Hexane	31.4 ± 1.1	62.1 ± 4.5	50.9 ± 2.7	23.5 ± 0.8	47.6 ± 1.8	42.0 ± 1.7
Chloroform	100.5 ± 1.7	99.8 ± 1.2	101.6 ± 1.6	97.2 ± 4.7	90.6 ± 3.5	96.0 ± 2.1
Chloroform/methanol	102.2 ± 1.2	103.5 ± 2.1	100.2 ± 1.7	98.7 ± 2.7	98.1 ± 0.6	98.0 ± 2.7

Both chloroform and chloroform/methanol recovered greater than 90% of the lipids from each standard. We chose to use chloroform/methanol for measuring lipid concentrations in experimental samples, as it yielded slightly better results with the 2% lipid emulsions. Hexane could not be used to quantitatively recover palm oil or oleic acid. We found that hexane could quantitatively extract emulsified palm oil if sodium hydroxide was added to the samples to break the emulsions (data not shown). Even with this sodium hydroxide treatment, however, oleic acid could not be recovered efficiently. We also tested control standards that contained either 0.3% or 0.5% GA and no lipid. The mass recovered from these samples was negligible, indicating that GA is not extracted by the solvents used in this study (data not shown).

When cells are present in a sample, some of the lipids in the medium are associated with the cell pellet. We attempted to estimate how much lipid was associated with the cells by washing the pellet with hexane after the water washing steps. (Chloroform/methanol could not be used for this step because the chloroform extracts PHB from biomass.) We determined that if the hexane wash is included in the lipid recovery procedure, the observed lipid concentration increases by 1-4% (data not shown). Given the small impact this has on the measurement, we determined that this step does not need to be included in the standard method, but could be added if higher precision is required.

We also discovered that the use of chemical antifoams in a fermentation interferes with the lipid recovery method and should be avoided. Both polypropylene glycol and silicone oil are extracted by the chloroform/methanol method, which will artificially increase the measured lipid concentration in the medium. In our experience, foam formation in emulsified oil cultures was not a significant issue at the cell densities used in this study.

Emulsified oil fermentations

Fermentations were carried out in medium containing 20 g/L palm oil emulsified with 0.5% GA, and cell growth and oil utilization were measured (Fig. 3.3). We observed a measureable increase in CDW by the 9 h time point. This demonstrates that our method leads to cultures with short lag phases, and that we were able to take accurate measurements early in the experiment. By 12 h all the ammonium in the medium was depleted (data not shown), and by 16 h the RCDW reached its maximum value. After this point cell division stopped and further increases in CDW were due to storage of PHB. By 72 h, 79% of CDW consisted of PHB. Six fermentations were carried out in this experiment, and the small error bars in Fig. 3.3 show that this method allows for reproducible growth of *R. eutropha*.

The initial measured concentration of oil in the medium was 17 g/L, which is lower than the 20 g/L added to each vessel. We attribute this discrepancy to the fact that some oil collected on the walls of the vessels and was therefore removed from the medium. Significant oil consumption by the cells was first observed between the 9 and 12 h time points. Oil consumption continued throughout the experiment, although the rate of consumption decreased over time. Measurement of lipid utilization allows for calculation of product yields. We found that over the course of the entire fermentation, polymer was formed at a yield of 0.61 g PHB/g palm oil. If only the PHB production phase of the culture

is considered, the PHB yield is 0.84 g/g palm oil. These values are similar to other yields from plant oils reported in the literature (14).

TLC analysis revealed that over time TAGs in the medium were broken down and the concentrations of FFAs, MAGs, and DAGs increased (Fig. 3.4). Even at the 72 h time point, however, complete breakdown of the TAGs had not occurred. We determined the fatty acid distributions in the lipid extracts from the fermentation medium with a methanolysis assay (Table 3.3). The key observation from this experiment was that the proportion of oleic acid in the medium increases over time, suggesting that *R. eutropha* preferentially uses other fatty acids relative to oleic acid. This could be due to the substrate specificity of the *R. eutropha* lipase, or could result from different rates of transport into the cell for the different fatty acids.

We also developed an HPLC method for detecting glycerol in culture supernatants. Glycerol would be released if all three fatty acids were cleaved from a TAG molecule. We were unable to detect glycerol in the medium at any time point in this experiment, suggesting that any glycerol that appeared in the medium was rapidly consumed by the cells.

Table 3.3: The fatty acid distributions in lipid samples extracted from emulsified palm oil medium at different time points were determined. The values reported are averages from triplicate measurements ± SD.

Fatty acid		Fatty acid content (wt%)		
		2 h	48 h	72 h
Myristic acid	(C14:0)	1.05 ± 0.02	0.91 ± 0.09	1.21 ± 0.2
Palmitic acid	(C16:0)	35.5 ± 0.2	29 ± 2	34 ± 6
Stearic acid	(C18:0)	3.72 ± 0.01	4.9 ± 0.6	5.5 ± 0.7
Oleic acid	(C18:1)	47.3 ± 0.1	55.3 ± 0.5	55 ± 2
Linoleic acid	(C18:2)	12.4 ± 0.1	10 ± 1	4 ± 3

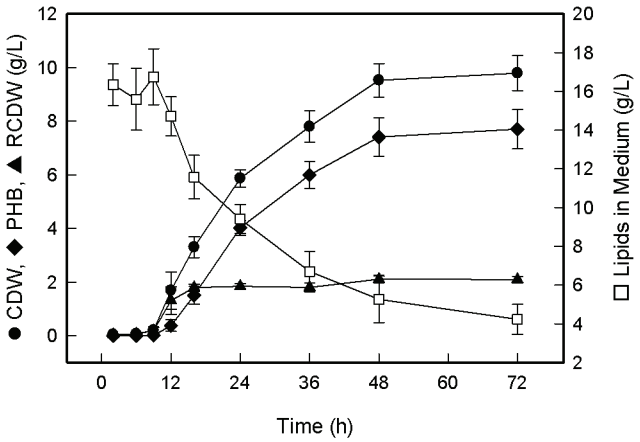

Fig. 3.3: ***R. eutropha*** **was grown in emulsified palm oil fermentations. Cell growth, PHB production, and oil consumption were measured over time. Values reported are means from six fermentations and error bars represent standard deviations.**

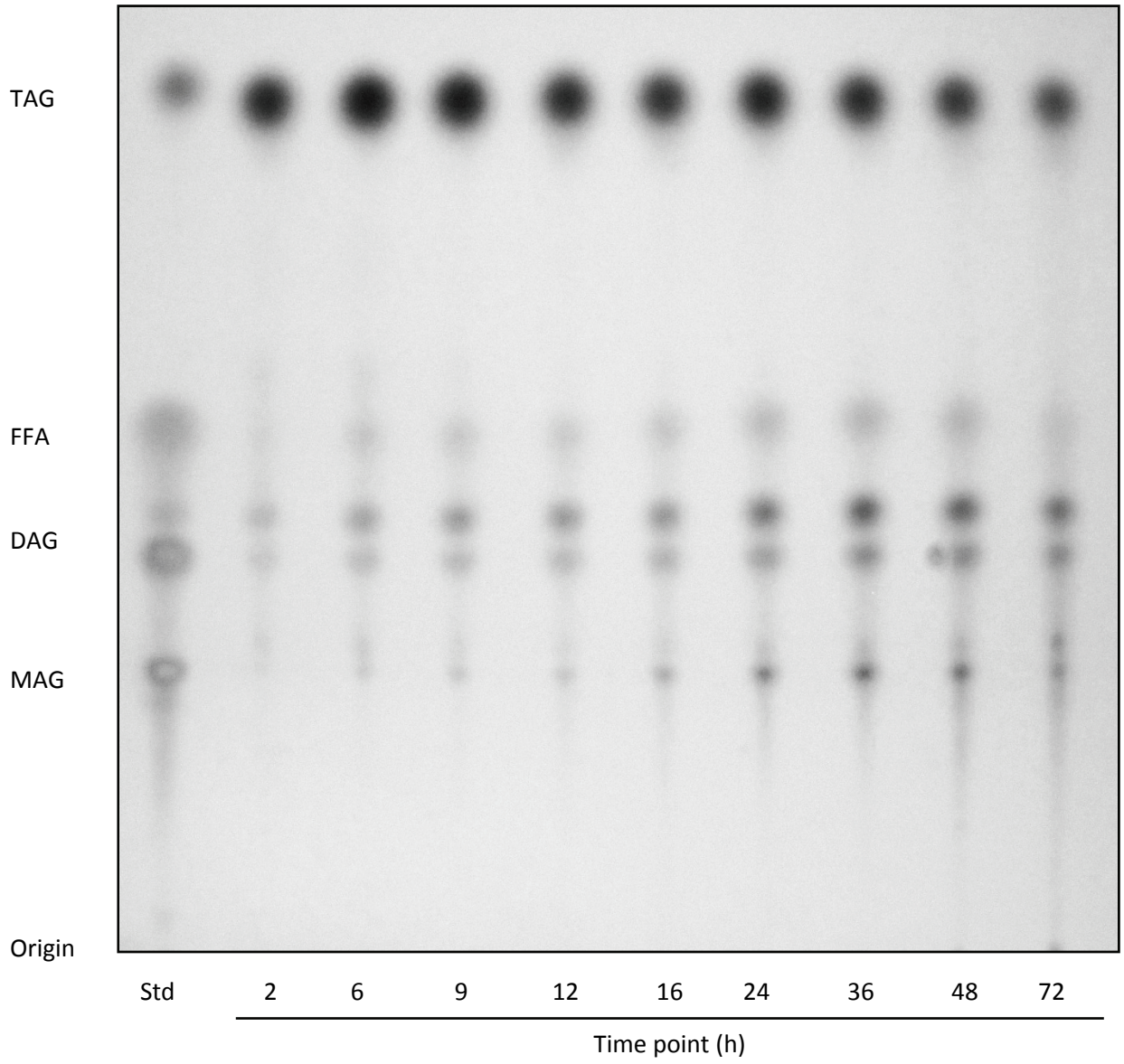

Fig. 3.4: The types of lipids present in the medium of an emulsified palm oil fermentation were analyzed with TLC. Samples were taken at various times during the experiment and lipids were extracted. 30 µg of lipid were loaded for each time point. As the cells grew MAGs, DAGs, and FFAs were generated from the palm oil.

Discussion

Plant oils are excellent potential feedstocks for industrial PHA production due to their high carbon content (1). Our group is most interested in palm oil, a major agricultural product in Southeast Asia with a high oil yield per acre of land (29). While *R. eutropha* is able to grow on non-emulsified palm oil, these cultures exhibit variable lag times and representative samples cannot be taken early in experiments. Oil will eventually become emulsified as the bacteria grow, but the mechanism by which this emulsification occurs is unknown. Some bacteria excrete surfactants (25), but there is no evidence that *R. eutropha* synthesizes these compounds. It is possible that polar lipids released during the breakdown of TAGs (i.e. MAGs, DAGs, and FFAs) could themselves emulsify the oil. Our group recently showed that the putative lipase encoded by gene h16_A1322 (GeneID: 4249488) is essential for robust growth of R. eutropha on non-emulsified plant oil (4).

In order to conduct quantitative, reproducible experiments with plant oil as the carbon source, we developed an emulsified oil culture method for R. eutropha. There are many reports in the literature that use either synthetic or natural surfactants to study growth of bacteria on hydrophobic carbon sources (5, 10, 26, 31). Most of these studies were concerned with the bioremediation of hydrocarbon pollutants. It is known that surfactants can effect bacterial growth in many different ways (28). We therefore evaluated how four different emulsifying agents influenced the growth of *R. eutropha*. Both SDS and Triton X-100 inhibited cell growth at the concentrations tested in this study. The antimicrobial properties of these surfactants had previously been observed in a study of *Bacillus subtilis* (20). Tween 80 did not inhibit growth of *R. eutropha*, but the bacteria were able to use this surfactant as a sole carbon source. Tween molecules contain a fatty acid group. We hypothesize that a *R. eutropha* esterase may cleave the fatty acid from Tween, allowing it to be used as a carbon source by the bacteria. We decided to use GA as the emulsifying agent for our palm oil cultures, because it created stable emulsions and did not significantly affect growth of *R. eutropha*. There were two minor issues with GA: some of the material precipitated when autoclaved, and the presence of GA led to a slight decrease in PHB production (Fig. 3.2). Neither of these issues had a major impact on the results of *R. eutropha* growth and PHB production experiments.

R. eutropha fermentations with emulsified palm oil demonstrated the effectiveness of this method. Cultures had short lag phases and were highly reproducible (Fig 3.3). The cells accumulated high levels of bioplastic, with PHB content of 79% of CDW reached after 72 h. A lipid extraction method was also

developed, which allowed us to monitor oil consumption by the cells and the breakdown of oil in the medium (Fig. 3.4).

We performed emulsified oil fermentations in several bioreactors and learned many lessons on how to best implement this method. Fermentors with fully jacketed vessels work best, because in vessels with partial jackets oil will stick to portions of the vessel wall that are cooler than the bulk medium. We also found it is preferable to use fermentors in which the stirring shaft enters from the top of the reactor and the bottom of the vessel is fully sealed. When the stirring shaft enters through the bottom of the vessel, there is occasionally leakage of culture broth through the mechanical seal. This may be due to the hydrophobic nature of the emulsified oil medium. Chemical antifoams should not be used with emulsified oil cultures because they interfere with the oil extraction method. If foaming is found to be an issue in an experiment, the use of mechanical foam breakers should be investigated.

We have developed a new method for studying growth of *R. eutropha* on plant oils using gum arabic as an emulsifying agent. The emulsified oil medium can be used in both fermentors and shake flasks. While this method is a useful experimental tool, it is unlikely to have industrial applications due to the cost of gum arabic. Our method may also prove useful for studying growth of other bacteria, and could potentially be applied to other hydrophobic carbon sources.

Acknowledgements

I would like to acknowledge three German students from Beuth Hochschule für Technik Berlin who collaborated with me on this project: Stefan Risch, Florian Hübner, and Sebastian Riedel. All three completed diploma theses after doing research in the Sinskey lab. I could not have completed this study without their hard work.

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Chapter 4

The Roles of Multiple Acetoacetyl-CoA Reductases in Polyhydroxybutyrate Biosynthesis in *Ralstonia eutropha* H16

This chapter has been adapted from the paper "The Roles of Multiple Acetoacetyl-CoA Reductases in Polyhydroxybutyrate Biosynthesis in Ralstonia eutropha H16" (4), which has been accepted for publication in the Journal of Bacteriology.

Introduction

Polyhydroxyalkanoates (PHAs) are natural polyesters synthesized by a wide range of bacteria as carbon and energy reserves. PHAs are typically stored when organisms are in an environment in which carbon is plentiful, but the lack of another nutrient limits normal cell growth. It has been found that in environments with fluctuating carbon levels, PHA producers have crucial advantages over rival species (15). In addition to their importance in the microbial world, these polymers have been studied for their potential uses in biodegradable consumer goods (13), medical products (23), and as chemical precursors (5). Although many PHA monomers have been discovered, the most common are 3-hydroxyalkanoates (33). Common PHAs are typically characterized by their constituent monomers as short chain length (SCL-PHA, C4 and C5 monomers) or medium chain length (MCL-PHA, C6 and longer monomers) polymers.

The model organism used to study PHA biosynthesis is the Gram-negative bacterium *Ralstonia eutropha*. This organism accumulates a high percentage of its cell dry weight as SCL-PHA under nutrient limitation. When grown on sugars or plant oils, *R. eutropha* makes almost exclusively poly(3-hydroxybutyrate) (PHB), although the addition of precursors such as propionate to the growth medium can lead to incorporation of 3-hydroxyvalerate into the polymer chain as well (2). An operon of biosynthetic genes from *R. eutropha* encoding enzymes sufficient for synthesis of PHB from acetyl-CoA was discovered in the late 1980s, which consisted of *phaC-phaA-phaB* (26, 27, 37). In this pathway, two molecules of acetyl-CoA are condensed by a β-ketothiolase (PhaA) and the resulting acetoacetyl-CoA is reduced by a reductase (PhaB) to form (*R*)-3-hydroxybutyryl-CoA (HB-CoA), which is the substrate for the PHA synthase (PhaC). Sequencing and analysis of the *R. eutropha* genome revealed the existence of putative

isologs for each of the PHA synthetic genes (30). While the existence of alternate β-ketothiolases was already known (40), most of the potential isologs identified had never been characterized.

Our group wanted to better understand how acetoacetyl-CoA reduction occurs in *R. eutropha*. In addition to the earlier identified *phaB* gene, now referred to as *phaB1* (GeneID: 4249784), the genes *phaB2* (GeneID: 4249785) and *phaB3* (GeneID: 4250155) were discovered on *R. eutropha* chromosome 1. Fifteen other potential isologs were also found to have amino acid sequences that could potentially indicate acetoacetyl-CoA reductase activity (30). The roles of the newly discovered genes in PHB biosynthesis were unclear, especially given the results of an earlier biochemical study that suggested there was a single NADPH-dependent acetoacetyl-CoA reductase in *R. eutropha* (11). In order to determine the roles of the reductase genes in *R. eutropha*, we deleted *phaB1*, *phaB2*, and *phaB3* from the genome both individually and in combination. In addition to characterizing these newly discovered genes, we also hoped to eliminate or diminish formation of HB-CoA by stopping the reduction reaction. Efforts to purify the PHA synthase from *R. eutropha* have been complicated by the high levels of PHB made by this organism (8). Studying formation and growth of PHB granules is difficult because PHB accumulates at a high rate, causing individual granules to coalesce and become indistinct (46). We therefore believed a *R. eutropha* strain with decreased HB-CoA synthesis would be a useful experimental tool, and could also serve as a platform for engineering new PHA synthesis pathways into *R. eutropha*.

Materials and methods

Bacterial strains and cultivation conditions

All experiments were performed with *Ralstonia eutropha* H16 (ATCC 17699) and mutants derived from this strain (Table 4.1). *R. eutropha* strains were grown aerobically at 30°C in both rich and minimal media. The rich medium was dextrose free tryptic soy broth (TSB) medium (Becton Dickinson, Sparks, MD). The minimal medium had an initial pH of 6.8 and was composed of 4.0 g/L NaH_2PO_4, 4.6 g/L Na_2HPO_4, 0.45 g/L K_2SO_4, 0.39 g/L $MgSO_4$, 62 mg/L $CaCl_2$, and 1 mL per 1 L of a trace elements solution. The trace elements solution consisted of 15 g/L $FeSO_4 \cdot 7H_2O$, 2.4 g/L $MnSO_4 \cdot H_2O$, 2.4 g/L $ZnSO_4 \cdot 7H_2O$, and 0.48 g/L $CuSO_4 \cdot 5H_2O$ dissolved in 0.1 M hydrochloric acid. Carbon and nitrogen sources were added to this defined medium as described in the text. All media contained 10 μg/mL gentamicin sulfate. Medium components were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise specified.

Growth in rich medium was carried out by inoculating 6 mL of TSB with a single colony from a TSB agar plate. These cultures were incubated for 24 h on a roller drum, then used to inoculate 100 mL cultures of TSB to an initial optical density at 600 nm (OD_{600}) of 0.05. The 100 mL cultures were grown in 500 mL baffled flasks and shaken at 200 RPM. Samples were taken from the flask cultures at various time points for analysis.

Growth in minimal media was carried out by inoculating 3 mL of TSB with a single colony from a TSB agar plate. These cultures were incubated 24 h on a roller drum, then used to inoculate 5 mL of minimal medium containing 2% fructose and 0.1% NH_4Cl. Aliquots from the minimal medium precultures were used to inoculate minimal medium flask cultures to an initial OD_{600} of 0.05. The minimal medium generally contained 2% fructose and 0.05% NH_4Cl. This fructose concentration was sufficiently high that carbon limitation never occurred in these cultures. In one set of experiments 1% emulsified palm oil (Wilderness Family Naturals, Silver Bay, MN) was used as the sole carbon source in place of fructose. 0.3% gum arabic (Sigma-Aldrich) was included in the palm oil medium as the emulsifying agent. Gum arabic is a natural glycoprotein (9) that is not metabolized by *R. eutropha*. The oil was emulsified by mixing the medium with a Sorvall Omni-Mixer for one minute. Flask cultures had volumes of either 50 mL (in 250 mL baffled flasks) or 100 mL (in 500 mL baffled flasks), and were shaken at 200 RPM. Samples were taken from the flask cultures at various time points for analysis.

Plasmid and strain construction

The method for deletion and insertion of genes in the *R. eutropha* genome follows the procedure described by York et al. (48). Standard techniques were used to amplify, manipulate, and prepare DNA (36). DNA was routinely amplified using high fidelity Taq polymerase (Qiagen, Valencia, CA) and digested using restriction enzymes from New England BioLabs (Ipswich, MA). All oligonucleotide sequences used in this study are provided in Appendix 4.1.

Plasmids used to make markerless deletions in the *R. eutropha* chromosome were created by first constructing stretches of DNA in which the regions upstream and downstream of a given gene were connected. This was done by first amplifying ~500 bp of sequence upstream and downstream of the gene. Primers were designed such that the two fragments had identical 16 bp sequences at the ends that were to be connected. The 16 bp sequence included a SwaI restriction site for future cloning applications. A single, connected DNA fragment was created by overlap extension PCR. Primers used in the overlap PCR were designed so that the final product had BamHI restriction sites at each end. The product of the overlap PCR was cloned into a TOPO vector (Invitrogen, Carlsbad, CA) and sequenced. The TOPO vector was digested with BamHI, the fragment for making the deletion was isolated and then ligated into the backbone of pGY46 digested with the same enzyme. The plasmid pGY46 has previously been used to delete the *R. eutropha phaC1* gene (48). Gene deletion plasmids were transformed into *E. coli* S17-1 and introduced into *R. eutropha* via conjugative transfer. *R. eutropha* strains with potential deletions were assessed by diagnostic PCR.

Complementation experiments were carried out by integrating potential reductase genes into the genome of strain Re2115. The genes *phaB1*, *phaB2*, *phaB3*, and *fabG* (GeneID: 4246984) were amplified from the *R. eutropha* genome by PCR, cloned into TOPO vectors, and sequenced. The gene *phaB3* was cloned twice, once using the sequence as annotated in the published genome (30), and once using an alternate start codon 30 bp upstream of the annotated start. Primers were designed such that all genes had an AscI restriction site upstream of the gene and a PacI site downstream of the gene. Additionally, the primers that hybridized to the 5' end of each gene were designed so that the 11 bp immediately upstream of the start codon of each gene were AGGAGATCTCC, which ensured that each gene had an identical ribosome binding site (RBS) in the complemented strains. The TOPO vectors containing each gene were digested with AscI and PacI, then the DNA fragment with the gene was isolated and blunted using the New England BioLabs Quick Blunting kit. Finally each gene was cloned into the SwaI site of pCB42 (the plasmid used to delete *phaB1*), creating plasmids for integrating each gene into the Re2115

genome at the Δ*phaB1* locus. Gene integration was carried out as previously described (48). All strains and plasmids used in this study are described in Table 4.1.

Polymer analysis

PHB and cell dry weight (CDW) were measured by transferring 4-9 mL of culture to preweighed glass test tubes at various time points. Cells were pelleted, washed with 5 mL cold water, pelleted again, and dried under vacuum at 80°C. Samples from palm oil cultures were prepared using the same protocol, except the washing was performed with a mixture of 4 mL cold water and 2 mL cold hexane. The hexane was added to remove unused oil from the samples. PHB content and CDW were determined from the dried samples using established methods (17, 49). The residual cell dry weight (RCDW) was calculated as the CDW minus the mass of PHB.

PHB molecular weight was measured for polymer extracted from strains grown in fructose minimal medium. After 48 and 72 h of growth, 40 mL of culture was harvested, pelleted, washed with 25 mL cold water, pelleted again, resuspended in 5 mL water, frozen at -80°C, and lyophilized. At each time point samples were also taken for quantification of PHB content. Freeze dried cells were weighed into glass test tubes and sufficient chloroform was added so that the dissolved PHB would have a concentration of 3 mg/mL. The test tubes were incubated in a Reacti-Therm heating/stirring module (Pierce, Rockford, IL) at 50-55°C with refluxing for 48 h, with stirring provided by magnetic stir bars. Chloroform lost due to evaporation was replaced over the course of the extraction. At the end of the extractions, the samples were cooled and cellular debris was removed by filtering the PHB solutions using 0.2 µm PVDF syringe filters (Pall, Port Washington, NY).

The molecular weight of the extracted PHB was measured using gel permeation chromatography (GPC). Molecular weights were determined relative to a series of low polydispersity polystyrene standards with peak molecular weights ranging from 1.1 kDa to 13.2 MDa (Polymer Laboratories, Part No. PL2010-0104). All molecular weight standards and experimental samples contained isopropanol as an internal standard to normalize retention times. Samples were run on an Agilent 1100 HPLC (Santa Clara, CA) connected to a computer running Chemstation software. Polymers were separated using a PLgel Olexis guard column and two PLgel Olexis analytical columns, all connected in series and purchased from Polymer Laboratories (Part No. PL1110-6400 and PL1110-1400). Chloroform was used as the mobile phase at a flow rate of 1 mL/minute with the columns held at 30°C. 100 µL of each sample was injected

onto the columns and the eluted polymer was detected with a refractive index detector. Calibration of the system and analysis of the experimental samples was performed using the Agilent GPC Data Analysis software package.

Enzymatic assays

Acetoacetyl-CoA reductase activity was measured for the soluble fraction from cellular lysates of *R. eutropha* strains. Cultures were grown in fructose minimal medium, harvested after 24 h of growth, pelleted, and stored at -80°C. Lysates were prepared by thawing the pellets on ice and resuspending them in 50 mM potassium phosphate buffer (pH 6) using 5 mL buffer per gram of wet cell mass. One mL of suspended cells was transferred to a 2 mL screw top plastic vial containing 0.6 g of 0.1 mm zirconia/silica beads (BioSpec Products, Bartlesville, OK). Vials were loaded onto a FastPrep-24 (MP Biomedicals, Solon, OH) and treated twice at 6.0 m/s for 40 s, with a 5 minute break between treatments. After lysis, the samples were centrifuged for 15 minutes at 4°C. Remaining insoluble debris was removed from the supernatants by filtering through 0.45 µm low protein binding Supor syringe filters (Pall), yielding the soluble lysate fractions that were used for enzymatic assays. Lysates were stored on ice while conducting the experiments. Reductase activity using either NADPH or NADH as the cofactor was measured at pH 6 and 25°C using the published protocol (11). The assay reactions contained 50 mM potassium phosphate buffer, 0.1 mM NADPH or NADH, and 32 µM acetoacetyl-CoA. When acetoacetyl-CoA was not included in the assay, addition of *R. eutropha* lysate did not result in increased NADPH or NADH oxidation relative to the no lysate control.

The β-ketothiolase activity in the soluble fraction of cellular lysates of *R. eutropha* strains was measured in the thiolysis direction using acetoacetyl-CoA as the substrate. Lysates were prepared as described above, except cells were harvested after 48 h of growth and the cell pellets were resuspended in 150 mM 4-(2-Hydroxyethyl)-1-piperazinepropanesulfonic acid (EPPS) buffer, pH 8. The enzymatic activity assays were carried out at 25°C as described by Slater et al. (40). The assay reactions contained 150 mM EPPS (pH 8), 50 mM $MgCl_2$, 40 µM acetoacetyl-CoA, and 0.1 mM CoA. In both the reductase and β-ketothiolase assays, acetoacetyl-CoA sodium salt was purchased from MP Biomedicals, while all other chemicals were from Sigma-Aldrich. Protein concentrations necessary for specific activity calculations were measured using a modified Bradford assay (50). For both assays, specific activities for pairs of strains were compared using the one-tailed Student's *t*-test, with relevant results given in the text.

Purification of His-Tagged Proteins

Reductase enzymes from *R. eutropha* were expressed in *E. coli* and purified. The expression and purification methods were adapted from a protocol previously used for purification of *E. coli* FabG (12). The *R. eutropha* genes *phaB1*, *phaB2*, *phaB3*, and *fabG* were amplified via PCR with primers that added a BamHI site to the 5' end of each gene and a BlpI site to the 3' end of each gene. The PCR fragments were digested with these restriction enzymes and cloned into pET-15b cut with BamHI and BlpI, resulting in genes encoding N-terminal His-tagged versions of each enzyme. Plasmids were transformed into *E. coli* strain Tuner(DE3), which allowed for inducible protein expression. The *R. eutropha* genes contain codons that are rare in *E. coli*, therefore each Tuner(DE3) strain was also transformed with pRARE2. All strains were grown at 30°C in lysogeny broth (LB) containing 100 µg/mL ampicillin and 34 µg/mL chloramphenicol. When an OD_{600} of 0.5 was reached, protein expression was induced by addition of 0.5 mM IPTG. Cells were harvested 4 h after induction and pelleted. Cell pellets were resuspended in sodium phosphate buffer (20 mM, pH 7.4) and lysed with a French press. Cell debris was removed by centrifuging the lysates and filtering the supernatants through 0.2 µm low protein binding filters. Clarified lysate was loaded onto a Ni Sepharose Fast Flow column (GE Healthcare, Piscataway, NJ) and fractions were collected with a FPLC. The elution buffer contained 20 mM sodium phosphate and 0.5 M NaCl (pH 7.4), and was run at a flow rate of 5 mL/min. The imidazole concentration in the buffer was increased from 40 mM to 500 mM over the course of each purification. Fractions in which only the protein of interest was visible on a SDS-PAGE gel were collected, concentrated, and dialyzed against storage buffer (20 mM sodium phosphate, 0.2 M NaCl, pH 7.4). The specific activities of the purified enzymes were measured using the reductase assay described above.

Table 4.1: Strains and plasmids used in this study.

Strain or plasmid	Description[a]	Reference or source
R. eutropha strains		
H16	Wild type strain, Gm resistant	ATCC 17699
Re2106	Δ*phaB2*, made from H16 with pCB28	This study
Re2107	Δ*phaB3*, made from H16 with pCB29	This study
Re2111	Δ*phaB1*, made from H16 with pCB42	This study
Re2112	Δ*phaB1* Δ*phaB2*, made from Re2106 with pCB42	This study
Re2113	Δ*phaB1* Δ*phaB3*, made from Re2107 with pCB42	This study
Re2114	Δ*phaB2* Δ*phaB3*, made from Re2106 with pCB29	This study
Re2115	Δ*phaB1* Δ*phaB2* Δ*phaB3*, made from Re2112 with pCB29	This study
Re2139	*phaB1* inserted into Re2115 genome, made with pCB65	This study
Re2140	*phaB2* inserted into Re2115 genome, made with pCB66	This study
Re2141	*phaB3* inserted into Re2115 genome, made with pCB67	This study
Re2142	*fabG* inserted into Re2115 genome, made with pCB68	This study
Re2143	*phaB3* inserted into Re2115 genome, made with pCB76	This study
E. coli strains		
DH5α	General cloning strain	Invitrogen
S17-1	Strain for conjugative transfer of plasmids to *R. eutropha*	(39)
Tuner(DE3)	Strain for inducible protein expression	Novagen
Plasmids		
pCR2.1-TOPO	Vector for cloning and sequencing PCR products, confers Km resistance	Invitrogen
pGY46	Plasmid for deletion of *R. eutropha phaC1*; encodes *sacB, oriV, oriT, traJ*; confers Km resistance; based on pJQ200mp18; all other deletion plasmids use backbone from this plasmid	(32, 48)
pCB28	Plasmid for deletion of *R. eutropha phaB2*	This study
pCB29	Plasmid for deletion of *R. eutropha phaB3*	This study
pCB42	Plasmid for deletion of *R. eutropha phaB1*	This study
pCB65	Plasmid for insertion of *phaB1* into *R. eutropha* genome at *phaB1* locus, based on pCB42	This study
pCB66	Plasmid for insertion of *phaB2* into *R. eutropha* genome at *phaB1* locus, based on pCB42	This study
pCB67	Plasmid for insertion of *phaB3* (annotated start codon) into *R. eutropha* genome at *phaB1* locus, based on pCB42	This study
pCB68	Plasmid for insertion of *fabG* into *R. eutropha* genome at *phaB1* locus, based on pCB42	This study
pCB76	Plasmid for insertion of *phaB3* (upstream start codon) into *R. eutropha* genome at *phaB1* locus, based on pCB42	This study
pET-15b	Plasmid expression of His-tagged proteins in *E. coli*, confers Ap resistance	Novagen
pRARE2	Plasmid supplying tRNAs for seven rare *E. coli* codons, confers Cm resistance	Novagen
pET-15b-*phaB1*	Plasmid for expression of PhaB1 with N-terminal His-tag	This study
pET-15b-*phaB2*	Plasmid for expression of PhaB2 with N-terminal His-tag	This study
pET-15b-*phaB3*	Plasmid for expression of PhaB3 with N-terminal His-tag	This study
pET-15b-*fabG*	Plasmid for expression of FabG with N-terminal His-tag	This study

[a]Abbreviations: Gm, gentamicin; Km, kanamycin; Ap, ampicillin; Cm, chloramphenicol

Results

Growth and PHB production of *R. eutropha* reductase mutants in different media

All of the different *phaB* deletion strains were initially grown in fructose minimal medium and TSB rich medium to determine the influence of the mutations on cell growth and PHB accumulation, as shown in Fig. 4.1. In minimal medium, the RCDW of all strains increased until nitrogen in the medium was exhausted, sometime between 12 and 24 h. After nitrogen limitation occurred, RCDW remained constant and all strains reached approximately the same RCDW (Fig. 4.1B). Similarly, the RCDW for all strains was nearly identical in TSB (Fig. 4.1D). This indicates that deletion of the reductase genes did not influence cell growth. In fructose minimal medium, all strains in which *phaB1* was present accumulated similar amounts of PHB (Fig. 4.1A). Strains in which *phaB1* was deleted, but *phaB3* was present showed similar PHB production to H16 from 12 to 48 h, but at the 72 h time point there was clearly less PHB in these strains than the wild type strain. Strains in which both *phaB1* and *phaB3* were deleted produced markedly less PHB than the other strains in the study. After 72 h in fructose minimal medium, strains Re2113 (Δ*phaB1,3*) and Re2115 (Δ*phaB1,2,3*) contained <20wt% PHB, compared to 75wt% for H16. Deletion of *phaB2* did not produce an observable phenotype in this medium, or any of the other conditions tested.

It has been shown that when *R. eutropha* is grown in rich medium, it typically accumulates a relatively low level of PHB early in the culture, which is then metabolized as the cells continue to grow (35). All strains with the *phaB1* gene exhibited this pattern of PHB production in TSB, while strains in which *phaB1* had been deleted made almost no PHB at any point during the experiment (Fig. 4.1C). There is some variation in the amount of polymer produced by the strains that make PHB in TSB (H16, Re2106, Re2107, Re2114), which is most evident at the 9 and 12 h time points. We believe the observed variations between these strains were due to difficulties in measuring the low levels of PHB, and not phenotypic differences between strains.

After observing different PHB production by some reductase mutant strains in fructose minimal medium and TSB, we decided to test another minimal medium in which a different carbon source was provided. Plant oils have been proposed as potential feedstocks for industrial PHA production (1). We therefore grew several reductase mutants in minimal medium with palm oil as the sole carbon source and measured PHB production (Fig. 4.2). The strain with only *phaB1* (Re2114) produced the same amount of PHB as H16, as was the case in the fructose medium. The strain with only *phaB3* (Re2112), however,

accumulated almost no PHB in the palm oil medium, in stark contrast to its behavior in fructose medium.

Expression of reductase genes

Our group recently reported whole cell gene expression microarray data for *R. eutropha* H16 grown in minimal media with fructose or trioleate as the sole carbon source (3). Trioleate served as a model for plant oils. Transcript levels were measured with each carbon source during the growth phase and PHB storage (i.e. nitrogen limited) phase of the cultures. We examined this data to determine expression of the reductase genes under different conditions (Table 4.2). Gene expression is reported relative to *fnr3* expression (GeneID: 4248836), a FNR-like transcriptional regulator. We found that expression of this gene varied <20% across all conditions studied (3), making *fnr3* a suitable gene to use for normalization. By this analysis, *phaB1* was the most highly expressed reductase. Expression of this gene was approximately constant in fructose cultures, but increased in trioleate cultures when nitrogen limitation was reached. Expression levels of *phaB2* were very low under all conditions. Expression of *phaB3* was relatively high during the growth phase of fructose cultures, but expression decreased over tenfold after nitrogen in the medium was depleted. There was little *phaB3* expression at any point in the trioleate cultures. The gene *fabG* was expressed in the presence of both carbon sources, and in both cases gene expression was lower under nitrogen limited conditions.

Table 4.2: Expression of reductase genes was measured in *R. eutropha* H16 fructose and trioleate minimal media cultures during growth and PHB storage conditions (3). Gene expression is reported relative to *fnr3* expression (see Results section for details).

Gene	Relative gene expression			
	Fructose, growth	Fructose, PHB storage	Trioleate, growth	Trioleate, PHB storage
phaB1	7.48	7.32	1.31	6.55
phaB2	0.02	0.05	0.03	0.08
phaB3	0.79	0.06	0.03	0.02
fabG	1.55	0.30	0.85	0.29

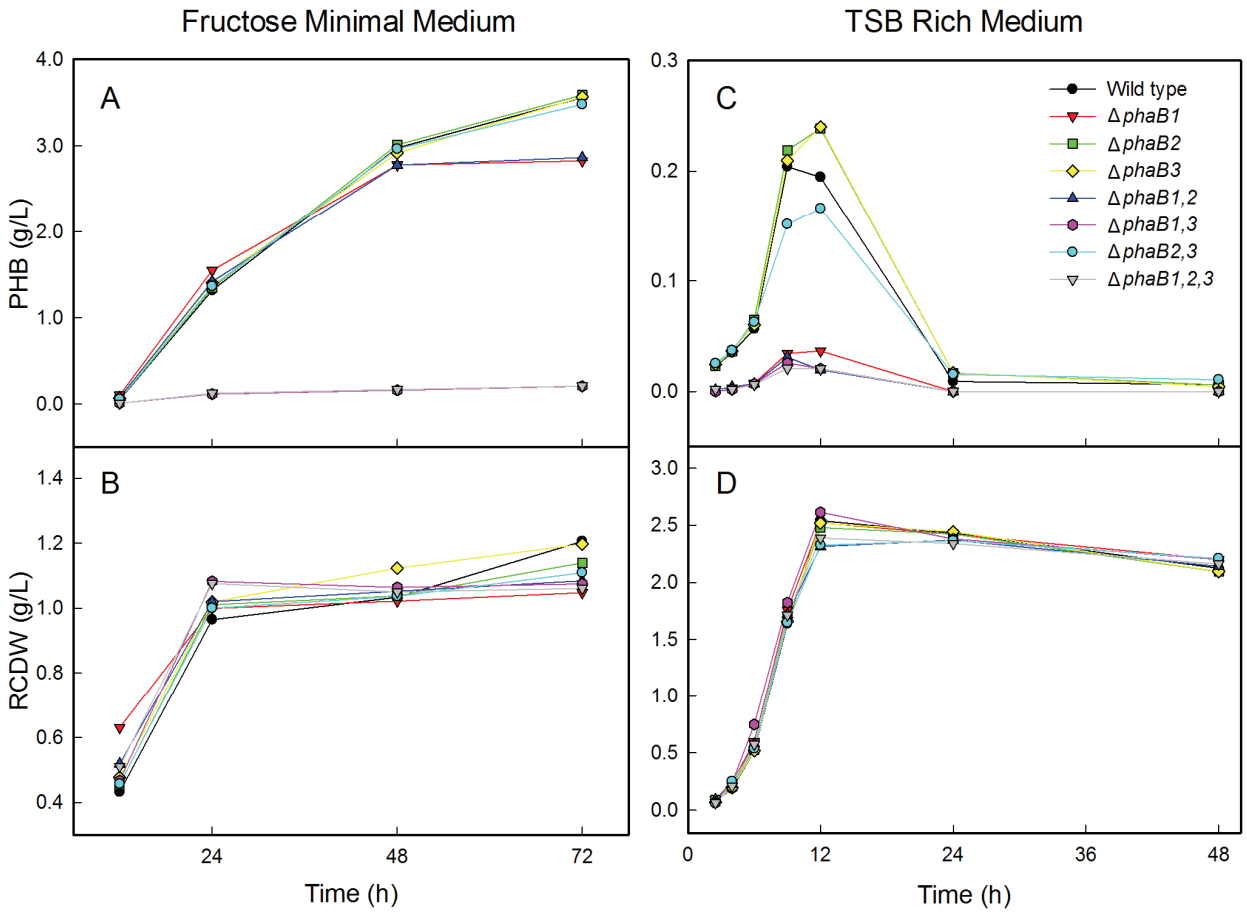

Fig. 4.1: ***R. eutropha*** **reductase mutants exhibited different levels of PHB accumulation in both minimal and rich media. Strains were grown in fructose minimal medium, and PHB production (A) and RCDW (B) were measured. In plot (A) note that the data points for the low PHB producing strains Δ*phaB1,3*** **(pink hexagons) and Δ*phaB1,2,3*** **(grey triangles) overlap. Strains were also grown in TSB, and PHB production (C) and RCDW (D) were measured. All data points are averages from duplicate cultures.**

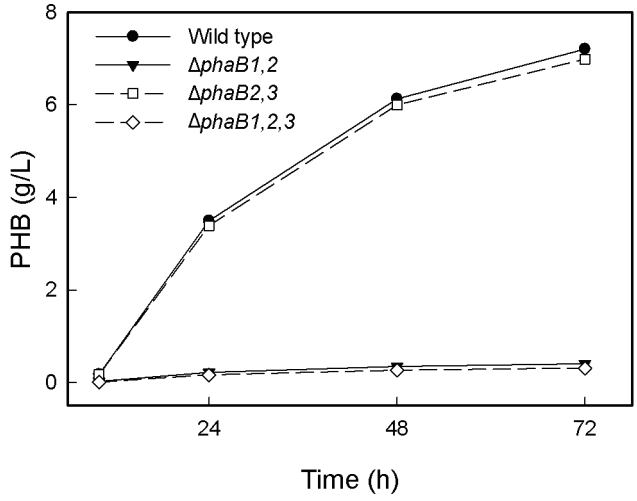

Fig. 4.2: PHB accumulation of Re2112 (Δ*phaB1,2*) changed when palm oil was used as the sole carbon source instead of fructose. Select strains were grown in palm oil minimal medium and PHB production was measured. All data points are averages from duplicate cultures.

Complementation of reductase mutations

In order to confirm that the deletion of the *phaB* genes was responsible for the observed decreases in PHB accumulation, we introduced reductase genes into Re2115. Because of reported issues with plasmid stability in *R. eutropha* (41), we chose to integrate the reductase genes into the Re2115 genome at the original *phaB1* locus, which also meant all reductase genes would then be expressed from the promoter of the *phaCAB* operon. In addition to the *phaB* genes, we investigated the ability of *R. eutropha fabG* to restore PHB production. FabG is a 3-ketoacyl reductase that is part of the fatty acid synthesis pathway (31). This enzyme's normal function is to reduce 3-ketoacyl-[acyl carrier protein] molecules, as opposed to the 3-ketoacyl-CoA substrates used by PhaB1.

The complemented strains were grown in fructose minimal medium and PHB production was measured (Fig. 4.3). We found that the strain in which *phaB1* was reintroduced to the Re2115 genome (Re2139) stored slightly more PHB than H16. The strain in which *phaB2* was added to Re2115 (Re2140) made more PHB than Re2115, but substantially less than H16. When we initially integrated *phaB3* into the Re2115 genome (Re2141) PHB accumulation was the same as Re2115 (data not shown). This was an unexpected result, as Re2112 made significantly more PHB than Re2115 in fructose cultures. When cloning *phaB3* to make Re2141, we used the start codon shown in the annotated genome. After examining the genome sequence, we determined that there was an alternate start codon 30 bp upstream of the annotated start. Addition of these bases to *phaB3* results in an open reading frame the same length as *phaB1*. We cloned the *phaB3* gene again using the upstream start codon and created Re2143. Surprisingly, PHB production was not only restored, but Re2143 actually made significantly more polymer than H16. It is unclear why the addition of 10 amino acids (MKKIALVTGG) to the N-terminus of PhaB3 was necessary to restore PHB production in this experiment, but protein alignments show that many of these residues are well conserved in both the PhaB sequences and FabG sequences from several species (6). We therefore concluded that the start codon of *phaB3* in the published genome is misannotated. The addition of *fabG* to the *phaB1* locus of Re2115 had no observable impact on PHB production.

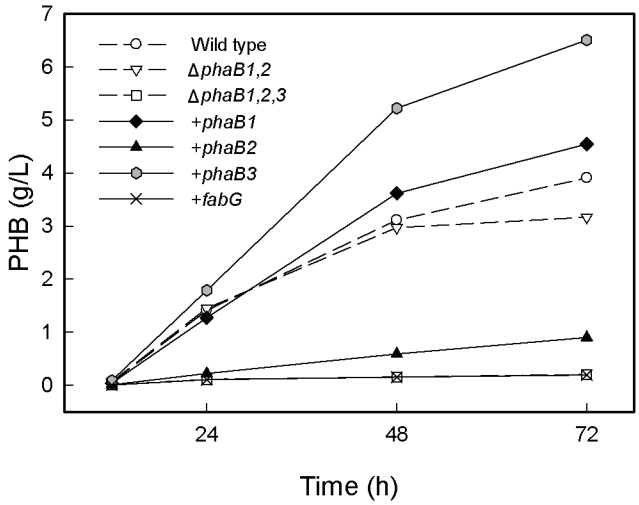

Fig. 4.3: Addition of reductase genes to the Re2115 genome restored PHB production. H16, Re2112, and Re2115 (dashed lines) were grown at the same time as the complemented strains (solid lines, genes added to each strain are indicated in the figure legend) in fructose minimal medium and PHB production was measured. The points labeled *+phaB3* are from strain Re2143. Note that the *ΔphaB1,2,3* and *+fabG* points overlap. All data points are averages from duplicate cultures.

PhaB and PhaA activities in mutant strains

The NADPH-dependent acetoacetyl-CoA reductase specific activity measurements for the various strains correspond well to the PHB production data (Table 4.3). Deletion of *phaB3* from H16 resulted in a slight decrease in activity, while deletion of *phaB1* led to a dramatic decrease. Both the *phaB1* and *phaB3* single mutants have significantly higher reductase activity when compared to Re2115 ($p < 0.01$ for Re2107, $p < 0.05$ for Re2111). Deletion of *phaB2* had no significant effect on reductase activity, which agrees with the earlier finding that deletion of this gene did not impact PHB accumulation. Addition of *phaB1* and *phaB3* to the Re2115 genome led to increases in reductase activity, but neither Re2139 nor Re2143 reached the activity of Re2114. At the 24 h time point at which cells were harvested from fructose medium to make these measurements, all strains except Re2113 and Re2115 had approximately the same PHB content, despite their differences in reductase activity. These data suggest there is a threshold value for reductase activity that allows for wild type levels of PHB production, and that Re2113 and Re2115 fall below this threshold. When NADH was provided as the cofactor, there was no significant difference in the specific activities of the *phaB* mutants (single factor ANOVA test, $\alpha = 0.15$, $F < F_{crit}$), indicating that these reductases chiefly use NADPH as the electron donor.

After finding that Re2139 and Re2143 made more PHB than H16, we measured β-ketothiolase activity in some of the mutant strains to determine if mutations at the *phaB1* locus influenced thiolase activity, as *phaA* is located immediately upstream of *phaB1* in the genome (Table 4.4). Re2112 and Re2115 had greater thiolase specific activity than H16 ($p < 0.01$), suggesting that deletion of *phaB1* increased *phaA* expression. Addition of genes to the Re2115 genome again altered thiolase activity, with each of the complemented strains showing different results. Although Re2139 made more PHB than H16, there was not a statistically significant difference in the thiolase activities of the two strains. Re2143, which exhibited the highest PHB accumulation, had significantly greater thiolase specific activity than H16 ($p < 0.05$).

It has been shown *in vitro* that copies of all enzymes necessary for PHB synthesis from acetyl-CoA are present on the surfaces of PHB granules isolated from *R. eutropha* (47). As all enzyme assays in this study were performed using only the soluble fraction of cell lysates, it is possible that some reductase and thiolase activity was not accounted for in our experiments. However, as significant differences in both PhaA and PhaB activities were found in strains making similar amounts of PHB (e.g. H16 and Re2112), we are confident that differences in soluble specific activities reflect actual differences in enzyme levels and are not due to differences in PHB content.

Table 4.3: Mutant strains of *R. eutropha* showed different levels of acetoacetyl-CoA reductase specific activity when NADPH was used as the cofactor, but similar levels when NADH was the cofactor. All strains were grown in duplicate and each sample was measured twice (n = 4 for each strain). Measurements were made with soluble lysate fractions. Values are reported as means ± SD. One unit of activity is defined as the amount of enzyme needed to convert 1 µmol acetoacetyl-CoA to product per minute at 25°C.

Strain	Genotype	Sp act, NADPH (U/mg)			Sp act, NADH (U/mg)		
H16	Wild type	4.2	±	0.3	1.12	±	0.08
Re2106	Δ*phaB2*	4.0	±	0.4	1.08	±	0.05
Re2107	Δ*phaB3*	3.4	±	0.2	1.03	±	0.03
Re2111	Δ*phaB1*	0.14	±	0.03	0.97	±	0.09
Re2112	Δ*phaB1,2*	0.15	±	0.04	1.1	±	0.2
Re2113	Δ*phaB1,3*	0.09	±	0.01	0.95	±	0.07
Re2114	Δ*phaB2,3*	3.3	±	0.4	1.2	±	0.2
Re2115	Δ*phaB1,2,3*	0.11	±	0.01	1.2	±	0.3
Re2139	Re2115::*phaB1*	0.5	±	0.1	1.0	±	0.1
Re2143	Re2115::*phaB3*	0.2	±	0.1	1.1	±	0.2

Table 4.4: The β-ketothiolase specific activity of *R. eutropha* strains was altered by changes at the *phaB1* locus. All strains were grown in duplicate and each sample was measured twice (n = 4 for each strain). Measurements were made with soluble lysate fractions. Values are reported as means ± SD. One unit of activity is defined as the amount of enzyme needed to convert 1 µmol acetoacetyl-CoA to product per minute at 25°C.

Strain	Genotype	Sp act (U/mg)		
H16	Wild type	0.7	±	0.1
Re2112	Δ*phaB1,2*	2.7	±	0.2
Re2115	Δ*phaB1,2,3*	2.9	±	0.3
Re2139	Re2115::*phaB1*	0.7	±	0.2
Re2140	Re2115::*phaB2*	2.3	±	0.2
Re2143	Re2115::*phaB3*	1.0	±	0.1

Activities of purified reductases

His-tagged versions of PhaB1, PhaB2, PhaB3, and FabG were expressed in *E. coli* and purified to homogeneity. The upstream start codon was used when PCR amplifying *phaB3* for this experiment. The specific activity of each reductase was measured using acetoacetyl-CoA as the substrate and NADPH as the cofactor. Assays were carried out in triplicate and the average specific activities in U/mg were as follows: PhaB1, 1,110 ± 50; PhaB2, 6.3 ± 0.1; PhaB3, 44 ± 7; FabG, 0.64 ± 0.03. The negative control (crude lysate from Tuner(DE3) harboring empty pET-15b) had a specific activity of <0.05 U/mg. It is notable that PhaB1 and PhaB3, the reductases shown to contribute to PHB biosynthesis in our genetic studies, had the highest specific activities.

Molecular weight of PHB from mutant strains

We next wanted to determine how the size of the PHB chains synthesized by some of the mutant strains compared to polymer from H16. PHB from H16, Re2112, Re2115, and Re2143 was extracted and the molecular weights were measured via GPC relative to polystyrene standards (Table 4.5). We report the number average (M_n) and weight average (M_w) molecular weights for each sample. We observed that for H16, Re2112, and Re2115 PHB molecular weight decreased with the amount of polymer stored by a given strain. Re2143 deviated from this trend, as PHB from this strain had a lower molecular weight than polymer from H16, despite the fact that Re2143 was more productive. For all strains, polymer extracted from the 72 h samples had lower molecular weight than polymer from the 48 h samples.

Table 4.5: PHB extracted from different *R. eutropha* strains had different molecular weights. All strains were grown in triplicate and samples were taken 48 and 72 h post inoculation. M_n and M_w values are reported as means ± SD.

Strain	Genotype	M_n (MDa)		M_w (MDa)	
		48 h	72 h	48 h	72 h
H16	Wild type	0.69 ± 0.05	0.64 ± 0.06	3.5 ± 0.5	2.9 ± 0.7
Re2112	Δ*phaB1,2*	0.40 ± 0.02	0.29 ± 0.03	1.70 ± 0.09	1.19 ± 0.02
Re2115	Δ*phaB1,2,3*	0.208 ± 0.009	0.179 ± 0.008	0.528 ± 0.006	0.461 ± 0.005
Re2143	Re2115::*phaB3*	0.53 ± 0.01	0.421 ± 0.009	1.7 ± 0.2	1.2 ± 0.1

Discussion

The number of different acetoacetyl-CoA reductases encoded in the *R. eutropha* genome and their roles in PHB biosynthesis were previously unclear. Biochemical studies initially suggested that there was one NADPH- and one NADH-dependent reductase expressed in *R. eutropha*, with only the NADPH-dependent enzyme producing the (*R*)-HB-CoA necessary for polymerization (11). The gene *phaB1* was subsequently discovered using a genetic screen, and the existence of additional *phaB* genes was proposed (27). Analysis of the *R. eutropha* genome revealed *phaB2* and *phaB3*, as well as 15 other *phaB* isologs (30), although it is unclear what cutoff was used in predicting these additional isologs. Phylogenetic analysis of the nucleotide and encoded amino acid sequences of *phaB1*, *phaB2*, and *phaB3* suggests that these genes are paralogs that resulted from gene duplication events (see Appendix 4.2).

The results of this study indicate that in some growth conditions both PhaB1 and PhaB3 contribute to PHB biosynthesis, while in others only PhaB1 provides the reductase activity necessary for HB-CoA formation. Interestingly, even strains with the least reductase activity (Re2113 and Re2115) showed a low level of PHB accumulation in minimal media, suggesting that either some reductase activity is provided by other enzymes (see Appendix 4.2), or that there is a secondary route for synthesis of HB-CoA. Our findings are analogous to work done with *R. eutropha* β-ketothiolases, in which multiple enzymes are present, but PhaA provides the majority of the activity for PHB synthesis (40). Although in the case of the thiolases, the different enzymes exhibit different substrate preferences.

Efforts to purify acetoacetyl-CoA reductase enzymes from a glucose utilizing *R. eutropha* mutant, prior to the discovery of the PHB biosynthetic genes, resulted in the suggestion that there was a single NADPH-dependent reductase (11). We were unable to replicate the growth conditions from this study, as wild type H16 cannot use glucose as a sole carbon source (30). Assuming that both *phaB1* and *phaB3* were expressed by the mutant during growth on glucose, it is possible that PhaB1 and PhaB3 co-purified, as the enzymes have nearly identical molecular weights (PhaB1, 26.4 kDa; PhaB3, 26.0 kDa, assuming upstream start codon) and similar peptide sequences (52% identity, ClustalW2 alignment (20)). Given that *phaB1* is expressed at a much higher level than *phaB3* (Table 4.2), it is also possible that the low level of PhaB3 in the cellular lysate escaped detection.

Interesting questions exist concerning the regulation of *phaB3* expression. In fructose minimal medium, mutants with only *phaB3* remaining in the genome showed similar PHB production to wild type through 48 h of growth (Fig. 4.1). In the final 24 h of the experiment little additional PHB was made in these

strains, while polymer continued to accumulate in strains containing *phaB1*. Expression data shows that *phaB3* is expressed during growth on fructose, but that expression decreases dramatically when nitrogen limitation is reached (Table 4.2). Without continued formation of PhaB3 protein, breakdown of PhaB3 in Re2112 would eventually lead to insufficient reductase activity for normal PHB synthesis. We also found that *phaB3* was expressed in fructose cultures but not trioleate cultures, which explains the lack of PHB accumulation by Re2112 in Fig. 4.2. While there are many examples of genes whose expression is regulated by the presence of certain carbon sources (10), it is unclear why it might be advantageous for *phaB3* to be regulated in this manner. Given that *phaB1* is constitutively expressed at a high level, it is possible that there was little pressure driving the evolution of *phaB3* regulation, so there may not be a satisfying explanation for the expression pattern of this gene.

We found that deletion of *phaB2* did not lead to an observable phenotype in any of the conditions examined in this study. While the specific activity of purified PhaB2 protein was lower than the activities of PhaB1 and PhaB3, it is clear that *phaB2* does encode an active acetoacetyl-CoA reductase. This observation, combined with the low levels of *phaB2* expression measured in fructose and trioleate cultures (Table 4.2), suggests that this gene is not expressed in normal laboratory conditions. Another group similarly concluded that the *phaC2* gene, which is immediately downstream of *phaB2* in the *R. eutropha* genome, is unexpressed (28).

Complementation experiments showed that all three *phaB* genes could restore some level of PHB production. Despite the fact that native *phaB2* expression was never observed, insertion of this gene at the *phaB1* locus led to increased PHB storage in Re2115, although not to the level observed in the wild type. Insertion of *fabG* at the *phaB1* locus had no significant impact on PHB accumulation. We found that while purified FabG was able to reduce acetoacetyl-CoA, its specific activity was significantly lower than the purified PhaB enzymes. While other groups have successfully used *fabG* genes from *E. coli* and pseudomonads to synthesize PHA precursors, plasmids were used for heterologous gene expression, supplying multiple gene copies per cell (25, 34, 43). It is therefore possible that higher levels of *fabG* expression in *R. eutropha* could increase PHB accumulation in Re2115. In addition, the previously studied FabG's showed preferences for substrates longer than C4 (25), so the *R. eutropha fabG* may be useful for synthesis of MCL-PHA rather than PHB. The contribution of natively expressed *fabG* to PHB biosynthesis in Re2115 requires further study, but it is clear that FabG does not play a major role in PHB production in wild type H16.

The data collected in this study allows us to determine which step limits PHB production during nitrogen limited growth on fructose. Previous work in which PHA biosynthetic genes were over-expressed in *R. eutropha* indicated that increases in synthase activity do not affect the rate of PHB accumulation (16, 18, 24). These results imply that the β-ketothiolase and/or reductase reactions limit flux through the PHB pathway. The fact that reductase activity can be decreased from the wild type level to the level of Re2111 and Re2112 with little change in PHB production suggests that the reduction of acetoacetyl-CoA is not the limiting step in polymer formation. Thiolase activities differed between H16 and Re2111/Re2112, which presents a possible complication in the analysis. We found, however, that Re2139 had similar thiolase activity to H16 and lower reductase activity while still making significant PHB, which confirms that the reductase step does not limit PHB production. Only when reductase activity drops below the level of Re2111/Re2112 does reduction of acetoacetyl-CoA become limiting in the pathway, as is observed with Re2115. The PHB hyper-producing strain Re2143 had reductase activity above the limiting level and greater thiolase activity than H16, allowing for increased flux through the pathway and greater PHB accumulation than the wild type organism.

The results of our study illustrate the challenges inherent in using genetics to study different genes in a co-transcribed operon. It has been established that a single nonsense mutation in *phaC1* can dramatically alter PhaA and PhaB activities (24). In our study, deletion of *phaB1* increased thiolase activity, likely by increasing expression of *phaA*. As *phaA* and *phaC1* are co-transcribed (21), deletion of *phaB1* could similarly increase PHA synthase expression. Strains in which *phaB1* or *phaB3* was inserted into the Re2115 genome had lower reductase activities than when the native *phaB1* was present (Re2112). The gene insertion procedure leaves several additional base pairs at both ends of the open reading frame not normally present in the operon. It is known that modifications to the intergenic regions of an operon can influence gene expression by changing post-transcriptional processes (29), which may have been the case here. It is also possible that the new RBS used with the inserted genes could be less favorable than the native RBS of each gene. Inserting different reductase genes into the PHB operon will alter the secondary structure of the resulting polycistronic mRNA, potentially effecting translation of all genes in the operon differently in each complemented strain (19), which could explain why different levels of thiolase activity were observed in the complemented strains.

Analysis of the PHB molecular weight data is complicated by the fact that the strains examined likely have different rates of HB-CoA synthesis, and may also have different levels of PHA synthase activity. It has been shown with purified *R. eutropha* synthase *in vitro* and with recombinant *E. coli in vivo* that

higher levels of synthase activity lead to shorter polymer chains (7, 38). The influence of substrate concentration on PHB molecular weight is less clear. A study with the *R. eutropha* synthase *in vitro* using HB-CoA as the substrate found that substrate concentration does not influence molecular weight (7). Later experiments using the PHA synthase from *Allochromatium* v*inosum* with (*R*)-3-hydroxybutyryl-*N*-acetylcysteamine as the substrate showed that low molecular weight polymer was made at the lowest substrate concentration tested (22). As the substrate was increased, however, PHB molecular weight reached a plateau and did not increase significantly with further increases in substrate concentration. We have shown that changes to the *phaB1* locus alter thiolase activity, so it is probable that *phaC1* expression is also changed by these mutations, thereby influencing PHB molecular weight. Despite this issue, valuable insights on the influence of *in vivo* HB-CoA availability on PHB molecular weight can still be gained from the data presented in this study. Comparing the molecular weights of PHB from H16, Re2112, and Re2115 it is found that molecular weight decreases with the amount of PHB accumulated. This suggests that lower intracellular HB-CoA concentrations could lead to shorter polymer chains. Comparing Re2112 and Re2115 is especially valuable, as these strains have the same *phaB1* deletion, which should have the same influence on synthase expression. Restoring PHB production in Re2115 by the addition of *phaB3* increases PHB molecular weight, although not to the level observed in H16. All together, the molecular weight results gathered here suggest that diminished HB-CoA synthesis *in vivo* corresponds to lower PHB molecular weight. This presents a potential conflict with the *in vitro* results showing no effect of HB-CoA concentration on polymer chain length. Clearly the *in vitro* system, which is a batch reaction, varies from the *in vivo* system, in which substrate is continuously synthesized. The substrate concentrations in the *in vitro* studies may not match the effective intracellular HB-CoA concentrations generated by the strains in this study, and therefore miss the influence of substrate concentration on polymer length. Additionally, PHB made by *R. eutropha in vivo* is stored within granules, in which there are many proteins present on the granule surface (14). *In vitro* experiments are unlikely to capture the complexity of these granules and the effects interacting proteins may have on the length of PHB chains.

We also observed that PHB molecular weight decreased as culture time increased from 48 to 72 h. Previous work has demonstrated turnover of PHB in *R. eutropha*, with a concurrent decrease in the molecular weight of the stored polymer (44). The reason for this decrease is not well understood, but it may be related to the finding that at least two PHA depolymerases are expressed in *R. eutropha* as PHB is being produced (21). The mechanisms by which PHB molecular weight is controlled *in vivo* is clearly an area for further investigation.

We have shown that in fructose minimal medium the enzymes encoded by *phaB1* and *phaB3* provide the reductase activity for normal PHB biosynthesis in *R. eutropha*. In other media only the product of *phaB1* is important. Deletion of the *phaB* genes resulted in *R. eutropha* strains with significantly reduced PHB accumulation. In most prior work, decreases in PHB synthesis in *R. eutropha* were achieved by altering the PHA synthase (42). The Δ*phaB1,2,3* mutant has diminished PHB accumulation, while still expressing the wild type synthase. As previous efforts to study PhaC from the native host grown under PHB production conditions have been complicated by the presence of large quantities of stored PHB (8), our strains may represent useful experimental tools for future studies. The impact of decreased acetoacetyl-CoA reductase activity on metabolite pools in *R. eutropha* was not explored in this study, but is an intriguing opportunity for future work. Finally, the high level of HB-CoA synthesis in wild type *R. eutropha* has led to difficulties in engineering this species to make PHA copolymers with substantial fractions of monomers other than HB. Efforts are underway in our lab to construct strains based on Re2115 that are able to produce useful PHA copolymers from a variety of carbon sources.

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Appendix 4.1: Oligonucleotide primer sequences

Sequences of oligonucleotide primers used in this study. Primers 1-6 were used in the deletion of *phaB2*, primers 7-12 were used in the deletion of *phaB3*, and primers 13-18 were used in the deletion of *phaB1*. Primers 19-28 were used to clone the indicated genes so that they could be integrated into the *R. eutropha* genome. Note that *phaB3* was cloned twice for genome integration, once using the annotated start codon, and once using a start codon 30 bp upstream of the annotated start. Primers 29-36 were used to clone the indicated genes so that they could be inserted into pET-15b and expressed in *E. coli*. Overlap regions for overlap extension PCR are underlined, as are restriction sites (BamHI, AscI, PacI, and BlpI). RBS sequences are italicized.

Primer	Name	Sequence (5' to 3')
1	*phaB2* upstream F	CGGATCCGGAGTTCCTCCATGCCAGACCA
2	*phaB2* upstream R	GCCGATTTAAATGCCGCCATCTCGTCAGCTCCGTGA
3	*phaB2* downstream F	CGGCATTTAAATCGGCGCAATGAGGATCGACGACAGGA
4	*phaB2* downstream R	CGGATCCCCTTTGACAACAGCTCACTGG
5	*phaB2* diagnostic F	CGTCAACGATGCGCCAGACT
6	*phaB2* diagnostic R	GCAAAGCGCTGATCGCTGAGGT
7	*phaB3* upstream F	CGGATCCGCAATGTGCAAGCCTTCGTTGA
8	*phaB3* upstream R	GCCGATTTAAATGCCGCCATACCACCTGTTACCAGTGCA
9	*phaB3* downstream F	CGGCATTTAAATCGGCGCACATGCAGTAAGGGTGCT
10	*phaB3* downstream R	CGGATCCCTGACGAGGAGCTTCTGAAGCT
11	*phaB3* diagnostic F	CCAAGGCCAACTTTGCCAAGA
12	*phaB3* diagnostic R	GCGTACTGCTGTCAAAGCCG
13	*phaB1* upstream F	CGGATCCCCACGATCAAGAGCTATGCCA
14	*phaB1* upstream R	CGCTCATTTAAATGCCGCGTCCACTCCTTGATTGGCTTCGT
15	*phaB1* downstream F	GCGGCATTTAAATGAGCGCCTGCCGGCCTGGTTCAACCA
16	*phaB1* downstream R	CGGATCCGGTCTGGGTGTCGTAGAGCCT
17	*phaB1* diagnostic F	GGACCTGATGGAGATCAACGA
18	*phaB1* diagnostic R	CATCTGCTTGTTGTCGTGCC
19	*phaB1* clone F	GATGGCGCGCC*AGGAGATCTCC*ATGACTCAGCGCATTG
20	*phaB1* clone R	GCGTTAATTAACAGGTCAGCCCATATGCAGGCCGCCG
21	*phaB2* clone F	GATGGCGCGCC*AGGAGATCTCC*ATGGCCGGACAACGCATT
22	*phaB2* clone R	GCGTTAATTAAGGCTGGGCGGTGACGTCCTGTCGTCGATCCTCATTGCAGG
23	*phaB3* clone F (annotated)	GATGGCGCGCC*AGGAGATCTCC*ATGGGTGGGCTGGGAGAA
24	*phaB3* clone R (annotated)	GCGTTAATTAACCTGGCCCAGCACCCTTACTGCATGTGCTG
25	*phaB3* clone F (longer ORF)	GGCGCGCCA*AGGAGATCTCC*ATGAAGAAAATTGCACTGGT
26	*phaB3* clone R (longer ORF)	GGTTAATTAACCTTACTGCATGTGCTGCCCGCCATTGATGGCGA
27	*fabG* clone F	GATGGCGCGCC*AGGAGATCTCC*ATGACCAAACTTCTCGACAAC
28	*fabG* clone R	GCGTTAATTAACGCGCCCGTTTGGTTCGGAAAACTACTACTTCAGGCG

Primer	Name	Sequence (5’ to 3’)
29	*phaB1* purify F	GAATGGATCCGATGACTCAGCGCATT
30	*phaB1* purify R	GAATGCTCAGCTCAGCCCATATGCAG
31	*phaB2* purify F	GAATGGATCCGATGGCCGGACAACG
32	*phaB2* purify R	GAATGCTCAGCTCATTGCAGGTGTTG
33	*phaB3* purify F	GCCGGGATCCGATGATGAAGAAAATT
34	*phaB3* purify R	GAAGGCTCAGCTTACTGCATGTGCTG
35	*fabG* purify F	GCATGGATCCGATGACCAAACTTCTC
36	*fabG* purify R	GCCGGCTCAGCTTAATTCATGTACAT

Appendix 4.2: Phylogenetic analysis of putative *R. eutropha* reductases

The amino acid sequence of PhaB1 was used as the query to BLAST against the *R. eutropha* genome. The resulting hits with scores ≥ 100 bits are shown in the table below. We constructed phylograms based on the amino acid (Fig. 4.4) and nucleotide (Fig. 4.5) sequences of all genes with scores ≥ 125 bits (11 genes). The sequences were first aligned using the ClustalW algorithm (20) and then phylograms were assembled using the MEGA version 4 software package (45). The PhaB1, PhaB2, and PhaB3 amino acid sequences are grouped together and are distinct from FabG. The nucleotide sequences of the *phaB* genes also form a distinct group. This suggests that these genes are paralogs resulting from gene duplication events. We also note that it is possible one or more of the genes in the table below encode enzymes that provide the residual acetoacetyl-CoA reductase activity that allows for the low level of PHB accumulation observed in Re2115 (Δ*phaB1,2,3*).

Rank	GeneID	Locus Tag	Name	Annotated description	Score (bits)
1	4249784	H16_A1439	PhaB1	acetoacetyl-CoA reductase	503
2	4249785	H16_A2002	PhaB2	acetoacetyl-CoA reductase	265
3	4250155	H16_A2171	PhaB3	acetoacetyl-CoA reductase	261
4	4246984	H16_A2567	FabG	3-ketoacyl-[acyl-carrier-protein] reductase	169
5	4249992	H16_A3164	H16_A3164	short chain dehydrogenase	167
6	4455589	H16_B1904	H16_B1904	3-oxoacyl-[acyl-carrier-protein] reductase	164
7	4457279	H16_B2510	H16_B2510	3-ketoacyl-[acyl-carrier-protein] reductase	159
8	4457116	H16_B0361	H16_B0361	3-oxoacyl-[acyl-carrier-protein] reductase	146
9	4455013	H16_B0385	H16_B0385	3-oxoacyl-[acyl-carrier-protein] reductase	133
10	4247211	H16_A2460	H16_A2460	putative beta-hydroxyacyl-CoA dehydrogenase	125
11	4456093	H16_B1334	H16_B1334	short chain dehydrogenase	125
12	4247373	H16_A0931	H16_A0931	dehydrogenase with different specificities	124
13	4249498	H16_A1287	H16_A1287	3-oxoacyl-[acyl-carrier-protein] reductase	123
14	4455625	H16_B0201	H16_B0201	short chain dehydrogenase	119
15	4455935	H16_B0062	H16_B0062	gluconate 5-dehydrogenase	118
16	4455545	H16_B0101	H16_B0101	short chain dehydrogenase	117
17	4249460	H16_A1334	H16_A1334	3-hydroxybutyrate dehydrogenase	113
18	4250059	H16_A1814	H16_A1814	D-beta-hydroxybutyrate dehydrogenase	112
19	4249518	H16_A1267	H16_A1267	short chain dehydrogenase	111
20	4456026	H16_B1696	H16_B1696	short chain CoA dehydrogenase	108
21	4456782	H16_B1075	H16_B1075	3-oxoacyl-[acyl-carrier-protein] reductase	107
22	4249490	H16_A1325	H16_A1325	short chain dehydrogenase	106
23	4248116	H16_A2152	H16_A2152	short chain dehydrogenase	105
24	4455773	H16_B1834	H16_B1834	3-ketoacyl-[acyl-carrier-protein] reductase	103
25	4456835	H16_B1442	H16_B1442	short chain dehydrogenase	103
26	4249320	H16_A1531	H16_A1531	gluconate 5-dehydrogenase	100
27	4247572	H16_A2473	H16_A2473	short chain dehydrogenase	100

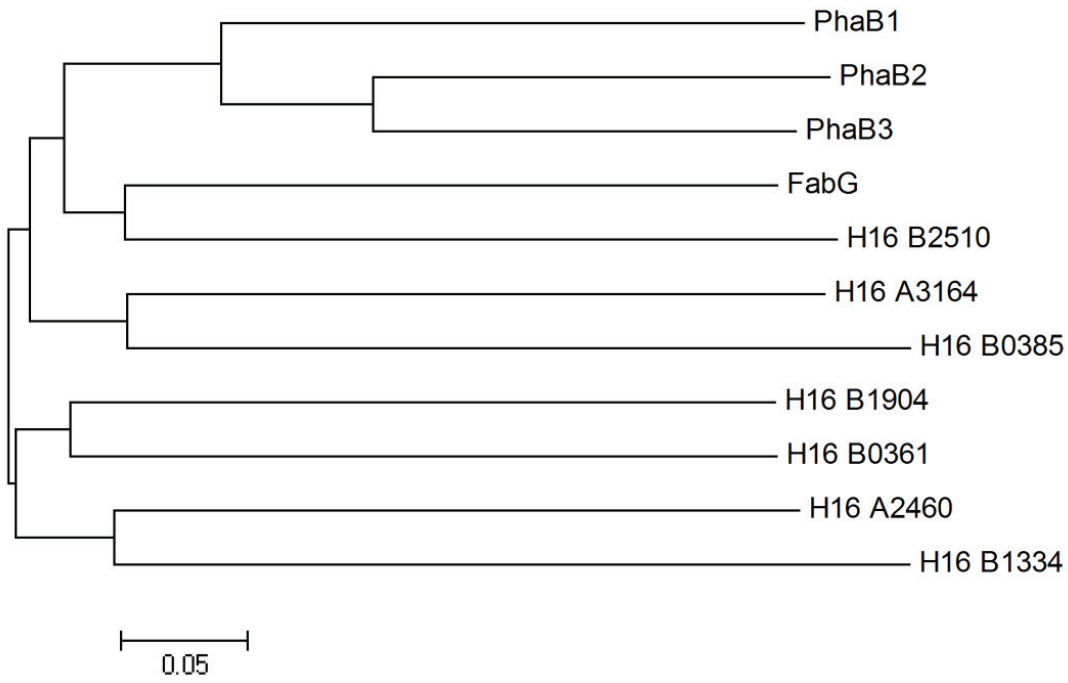

Fig. 4.4: A phylogram based on the amino acid sequences of predicted proteins from the *R. eutropha* genome with significant homology to PhaB1. Phylogenetic relationships were established using the Neighbor-Joining method with the p-distance model. The optimal tree with the sum of branch lengths = 3.112 is shown. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option).

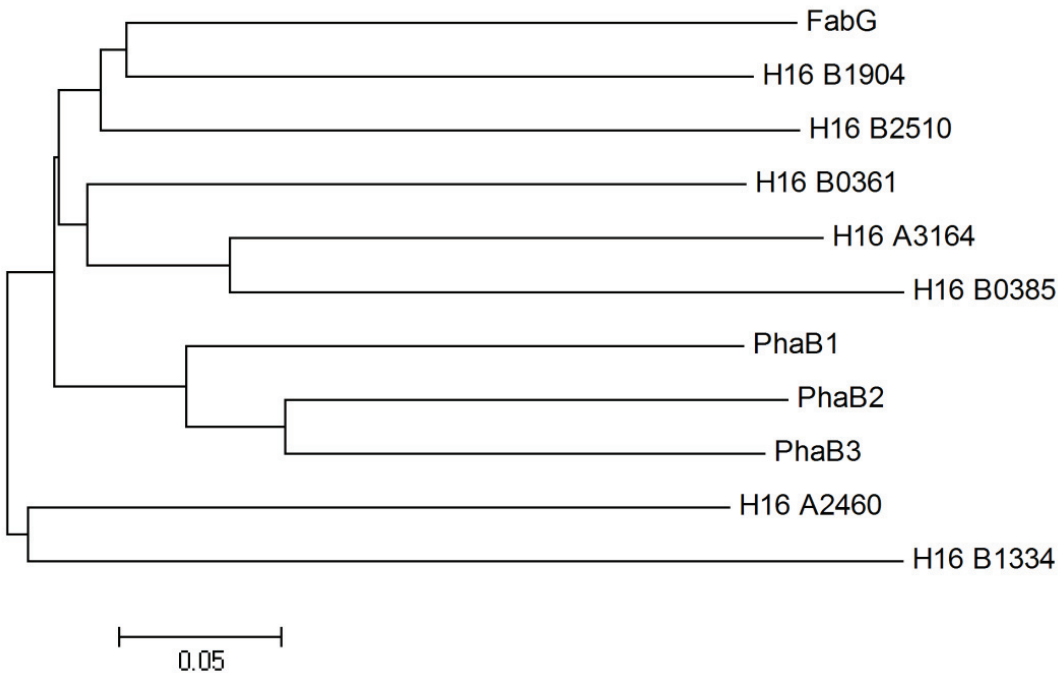

Fig. 4.5: A phylogram based on the nucleotide sequences of predicted genes from the *R. eutropha* genome encoding proteins with significant homology to PhaB1. Phylogenetic relationships were established using the Neighbor-Joining method with the p-distance model. The optimal tree with the sum of branch lengths = 2.335 is shown. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option).

Chapter 5

Production of Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) with Engineered *Ralstonia eutropha* Strains

Introduction

Polyhydroxyalkanoates (PHAs) are polyesters synthesized by bacteria as carbon and energy storage compounds (2). The first PHA discovered was the homopolymer poly(3-hydroxybutyrate) (PHB) (21). It was established that other types of PHAs also exist in nature when Wallen and Rohwedder extracted PHA copolymers from sewer sludge (39), and when de Smet et al. observed that *Pseudomonas oleovorans* can synthesize poly(3-hydroxyoctanoate) (9). Today PHAs are characterized as containing short chain length (SCL, C3-C5) and/or medium chain length (MCL, C6 and greater) monomers (29).

There has long been interest in using PHAs as biodegradable bioplastics that could serve as alternatives to petrochemical plastics. The commercial potential of PHB was first investigated by W.R. Grace and Company (3), who determined that this polymer has several issues that limit its value. PHB is a highly crystalline polymer that lacks toughness and begins to decompose near its melting temperature, making it difficult to process (20). Many studies were later conducted investigating poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (P(HB-*co*-HV)), but it was found that introduction of HV units into the polymer had limited impact on the material properties (16). This is due to the fact that HB and HV units are able to co-crystallize (4). Insertion of MCL monomer units into PHB chains leads to more dramatic changes to the properties of the plastic (26). The best studied member of this class of PHA is poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (P(HB-*co*-HHx)). When compared to PHB, P(HB-*co*-HHx) has a lower melting temperature, lower Young's modulus, and longer elongation to break (10, 26). This means that P(HB-*co*-HHx) is a tougher, more flexible plastic than PHB.

The model organism for studying PHA synthesis and accumulation is *Ralstonia eutropha* H16, because it accumulates large quantities of polymer when grown in nutrient limited conditions (28). Wild type *R. eutropha* will only produce SCL-PHA, and thus is limited as an industrial PHA production organism. One of the first organisms identified that synthesizes P(HB-*co*-HHx) was *Aeromonas caviae* (33). This bacterium and related species store P(HB-*co*-HHx) when grown on plant oils and fatty acids, but exhibit a low level of PHA accumulation (10). Investigations of *A. caviae* have revealed how this organism is able

to synthesize P(HB-*co*-HHx). The PHA synthase from *A. caviae* ($PhaC_{Ac}$) efficiently polymerizes both HB-CoA and HHx-CoA (11). *A. caviae* also has a gene encoding a (*R*)-specific enoyl-CoA hydratase (*phaJ*), which allows for conversion of fatty acid β-oxidation intermediates into PHA precursors (13, 15).

Plant oils and fatty acids are appealing feedstocks for industrial PHA production because of their high carbon content, and because metabolism of these compounds can influence the monomer composition of the resulting PHA (1). Several groups have produced P(HB-*co*-HHx) using *Aeromonas* strains or recombinant *R. eutropha* expressing $phaC_{Ac}$ (8, 17, 22, 25). In these cases, however, production of PHA with high HHx content (>5mol%) required feeding the cells short chain length fatty acids, which is undesirable because these are more costly than raw plant oil. Mifune and coworkers recently reported engineered *R. eutropha* strains that expressed an evolved $phaC_{Ac}$ and $phaJ_{Ac}$ (24). Strains from this study were able to accumulate high levels of PHA (>75wt%) with up to 9.9mol% HHx content when grown on soybean oil.

Our group hypothesized that the high levels of HB-CoA produced by *R. eutropha* when it is grown on plant oil could limit incorporation of other monomers into the PHA, even if the strain expressed a PHA synthase that could polymerize both HB-CoA and HHx-CoA. We recently reported a *R. eutropha* strain in which genes encoding acetoacetyl-CoA reductases were deleted, and this strain makes significantly less PHB than wild type (Chapter 4). We hoped to engineer this strain to produce P(HB-*co*-HHx). We integrated the *A. caviae* PHA synthase gene into the genome of this strain, as well as two synthase genes from the bacterium *Rhodococcus aetherivorans* I24 (6). *Rhodococcus* species have been shown to synthesize PHA copolymers (41), and analysis of a draft genome of *R. aetherivorans* revealed two putative PHA synthase genes. The strains were further improved by incorporating *phaJ* genes and by increasing gene expression using a stable plasmid system. Our work resulted in the construction of two stable *R. eutropha* strains that accumulate high levels of P(HB-*co*-HHx) when grown on plant oil, in which the HHx content of the PHA is >12mol%.

Materials and methods

Bacterial strains and cultivation conditions

All PHA production experiments in this study were conducted with *Ralstonia eutropha* H16 and mutants derived from this strain (Table 5.1, Fig. 5.1). The rich medium used for growth of *R. eutropha* was dextrose-free Tryptic Soy Broth (TSB) medium (Becton Dickinson, Sparks, MD). The salt concentrations in the *R. eutropha* minimal medium have been reported previously (Chapter 4). Carbon and nitrogen sources were added to the minimal medium as described in the text. The carbon sources used in this study were fructose and palm oil (Wilderness Family Naturals, Silver Bay, MN). All media contained 10 µg/mL gentamicin sulfate. Chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless noted otherwise. *R. eutropha* strains were always grown aerobically at 30°C. In shake flask experiments, 50 mL media was used in 250 mL flasks. The shaker was set to 200 RPM.

Plasmid and strain construction

Plasmids for this study were constructed using standard molecular biology techniques (31). DNA was routinely amplified using high fidelity DNA polymerase (Qiagen, Valencia, CA) and digested using restriction enzymes from New England Biolabs (Ipswich, MA). Plasmids were transformed into *R. eutropha* via transconjugation with *E. coli* S17-1. Markerless gene deletions and insertions in the *R. eutropha* genome were achieved following the protocol described in (7), which is based on the work of York et al. (42). The strains and plasmids used in this study are described in Table 5.1. *R. eutropha* strains whose PHA production was analyzed are illustrated in Fig. 5.1. The sequences of all oligonucleotide primers used in this study are provided in Appendix 5.1.

A *R. eutropha* strain with the *phaC1* gene deleted (Re1034) was previously made in our lab (42). This strain is unable to synthesize PHA. The plasmid used to make the *phaC1* deletion (pGY46) contained a section of DNA in which the region of the genome immediately upstream of *phaC1* was connected to the region of the genome downstream of *phaC1*. In order to insert new synthase genes at the *phaC1* locus, pGY46 was altered via site-directed mutagenesis using the Invitrogen GeneTailor kit (Carlsbad, CA). A SwaI site was inserted between the upstream and downstream sequences, allowing us to clone synthase genes into this site in the mutated plasmid (pJV7). Two novel PHA synthase genes from the organism *R. aetherivorans* I24 were investigated in this study, which were named $phaC1_{Ra}$ and $phaC2_{Ra}$. These genes

were identified by analyzing a draft copy of the *R. aetherivorans* I24 genome, provided by Dr. John Archer. The sequences of these genes can be found in Appendix 5.2, and they have also been deposited in GenBank under accession numbers HQ130734 and HQ130735. When amplifying $phaC1_{Ra}$ from the *R. aetherivorans* I24 genome, a primer was used such that the start codon in the cloned gene was ATG, rather than the TTG found in the genome. A version of the *phaC* gene from *A. caviae* whose sequence had been codon optimized for expression in *R. eutropha* was purchased from Codon Devices (Cambridge, MA). The optimized $phaC_{Ac}$ was designed with SwaI sites on both ends of the gene so that it could also be cloned into pJV7.

Many strains were constructed based on Re2115, a *R. eutropha* strain in which the three *phaB* genes in the *R. eutropha* genome had been deleted (7). New genes were inserted into the *phaB1* locus to alter production of PHA monomers using plasmids based on pCB42. The genes inserted into this locus were *phaB2* from *R. eutropha* (see Chapter 4), *phaJ1* from *Pseudomonas aeruginosa* (38), and a codon optimized version of *phaJ* from *A. caviae* (13). The $phaJ1_{Pa}$ gene was cloned via colony PCR from *P. aeruginosa* PAO1. The codon optimized $phaJ_{Ac}$ was purchased from Integrated DNA Technologies (Coralville, IA) and had EcoRV sites at both ends of the gene, allowing it to be cloned into pCB42.

In order to increase gene expression, the PHA biosynthetic operon from strain Re2152 was amplified via PCR and cloned into plasmid pBBR1MCS-2, creating pCB81. This plasmid was maintained in *R. eutropha* by adding 200 µg/mL kanamycin to the growth media. In order to improve plasmid stability in the absence of kanamycin, strains were constructed in which the *proC* gene was deleted from their genomes. The gene *proC* (locus tag h16_A3106, GeneID: 4250351) encodes pyrroline-5-carboxylate reductase, which is part of the proline biosynthesis pathway. When constructing the Δ*proC* strains, 0.2% proline was added to all selection plates. The region of the *R. eutropha* genome containing *proC* and h16_A3105 was amplified via colony PCR and cloned into pCB81, creating plasmid pCB113. This plasmid was transformed into Δ*proC R. eutropha* strains.

Fermentation conditions

Strains Re2058/pCB113 and Re2160/pCB113 were grown to higher densities than is possible in shake flasks using an Infors Sixfors multiple fermentor system (Bottmingen, Switzerland). Cultures were prepared by first growing the strains overnight in TSB containing 200 µg/mL kanamycin. These cultures were used to inoculate 50 mL minimal medium flask precultures containing 2% fructose and 0.1% NH_4Cl.

The minimal medium precultures were used to inoculate the fermentors so that the initial OD_{600} of each 400 mL culture was 0.1. Each fermentor contained 4% (Re2160/pCB113) or 4.5% (Re2058/pCB113) palm oil and 0.4% NH_4Cl. Neither the fructose nor palm oil minimal medium cultures contained kanamycin.

The temperature of each fermentor was kept constant at 30°C. The pH of each culture was maintained at 6.8 ± 0.1 through controlled addition of 2 M sodium hydroxide. Stirring was provided by two six-blade Rushton impellers at speeds of 500-1,000 RPM. Air was supplied at 0.5-1 VVM and the dissolved oxygen concentration was maintained above 40% through controlled addition of pure oxygen. Silicone oil AR200 was used as an antifoam in these experiments and was added to cultures by hand as necessary.

Analytical methods

The cell dry weights (CDW) of cultures were measured by taking 8-14 mL samples in preweighed plastic test tubes. The samples were centrifuged and the pellets were washed with 5 mL cold water. For experiments using palm oil as the carbon source, 2 mL cold hexane was also included during the wash step to remove unused oil from the samples. Samples were then centrifuged again, resuspended in 1 mL cold water, frozen at -80°C, and lyophilized. The dried samples were weighed and CDWs were determined. Residual cell dry weight (RCDW) values were calculated for each sample, which are defined as the total CDW minus the mass of PHA. Ammonium concentrations in clarified culture supernatants were measured with an ammonium assay kit (Sigma-Aldrich, Cat. No. AA0100) following the manufacturer's instructions.

The PHA contents and compositions of dried samples were determined using a methanolysis protocol adapted from (5). Dried cells were weighed into screw top glass test tubes and reacted with methanol and sulfuric acid in the presence of chloroform for 2.5 h at 100°C. This reaction converts PHA monomers into their related methyl esters. The concentrations of methyl esters were determined via gas chromatography with an Agilent 6850 GC (Santa Clara, CA) equipped with a DB-Wax column (Agilent, 30 m x 0.32 mm x 0.5 µm) and a flame ionization detector. 2 µL of each sample were injected into the GC with a split ratio of 30:1. Hydrogen was used as the carrier gas at a flow rate of 3 mL/min. The oven was held at 80°C for 5 min, heated to 220°C at 20°C/min, and held at 220°C for 5 min. Pure standards of methyl 3-hydroxybutyrate and methyl 3-hydroxyhexanoate were used to generate calibration curves for the methanolysis assay.

PHA was extracted from dried cells using chloroform for measurement of polymer molecular weights. Molecular weight measurements were made via gel permeation chromatography (GPC) relative to polystyrene standards, as described in Chapter 4.

Additional polymer characterization was performed with purified PHA recovered from samples of Re2058/pCB113 and Re2160/pCB113 taken at the end of palm oil fermentations (120 h of growth). PHA was isolated from lyophilized cells by extracting the polymer with methyl isobutyl ketone (MIBK). For each extraction, 100 mL of MIBK was added to 1.5 g of dried cells and stirred at 100°C for 4 h. Cell debris was removed by centrifugation and PHA was precipitated from solution by addition of 3 volumes of hexane. The resulting precipitate was washed with hexane, collected, and dried. The monomer composition of the purified PHA was determined by proton nuclear magnetic resonance (NMR) spectroscopy. Polymer was dissolved in deuterated chloroform and ^{1}H NMR spectra were collected with a Varian Mercury 300 MHz spectrometer.

Table 5.1: Strains and plasmids used in this study.

Strain or plasmid	Description[a]	Reference or source
R. eutropha strains		
H16	Wild type strain, Gm resistant	ATCC 17699
Re1034	H16 Δ*phaC1*	(42)
Re2000	Re1034::$phaC1_{Ra}$, made with pINY3	This study
Re2001	Re1034::$phaC2_{Ra}$, made with pINY4	This study
Re2058	Re1034 Δ*proC*, made with pCB110	This study
Re2115	H16 Δ*phaB1* Δ*phaB2* Δ*phaB3*	Chapter 4
Re2133	Re2115 Δ*phaC1*, made with pGY46	This study
Re2135	Re2133:: $phaC2_{Ra}$, made with pINY4	This study
Re2136	Re2133:: $phaC_{Ac}$, made with pLW484	This study
Re2151	Re2135::*phaB2*, made with pCB66	This study
Re2152	Re2135::$phaJ1_{Pa}$, made with pCB69	This study
Re2153	Re2135::$phaJ_{Ac}$, made with pCB72	This study
Re2154	Re2136::*phaB2*, made with pCB66	This study
Re2155	Re2136::$phaJ1_{Pa}$, made with pCB69	This study
Re2156	Re2136::$phaJ_{Ac}$, made with pCB72	This study
Re2160	Re2133 Δ*proC*, made with pCB110	This study
E. coli strains		
DH5α	General cloning strain	Invitrogen
S17-1	Strain for conjugative transfer of plasmids to *R. eutropha*	(34)
Other strains		
R. aetherivorans I24	Source of $phaC1_{Ra}$ and $phaC2_{Ra}$	(6)
P. aeruginosa PAO1	Source of $phaJ1_{Pa}$	(36)
Plasmids		
pGY46	Plasmid for deletion of *phaC1* from *R. eutropha* genome, backbone was used to make other plasmids for gene deletion/insertion in *R. eutropha* genome, confers Km resistance	(42)
pJV7	pGY46 with SwaI site between regions of DNA upstream and downstream of *phaC1*, used to insert new genes at the *phaC1* locus	This study
pINY3	pJV7 with $phaC1_{Ra}$ cloned into SwaI site	This study
pINY4	pJV7 with $phaC2_{Ra}$ cloned into SwaI site	This study
pLW484	pJV7 with $phaC_{Ac}$ cloned into SwaI site	This study
pCB42	Plasmid for insertion of genes at the *phaB1* locus in the *R. eutropha* genome, confers Km resistance	Chapter 4
pCB66	pCB42 with *R. eutropha phaB2* cloned into SwaI site	Chapter 4
pCB69	pCB42 with $phaJ1_{Pa}$ cloned into SwaI site	This study
pCB72	pCB42 with $phaJ_{Ac}$ cloned into SwaI site	This study
pCB110	Plasmid for deletion of *R. eutropha proC*	This study
pBBR1MCS-2	Vector for plasmid-based gene expression in *R. eutropha*, confers Km resistance	(18)
pCB81	pBBR1MCS-2 with the PHA operon from Re2152 cloned between KpnI and HindIII sites	This study
pCB113	pCB81 with *R. eutropha proC* region cloned into AgeI site	This study

[a]Abbreviations: Gm, gentamicin; Km, kanamycin

Results

Characterization of *R. aetherivorans* I24 synthases

We confirmed that the putative synthase genes from *R. aetherivorans* I24 encoded active enzymes by inserting them into the Re1034 genome at the *phaC1* locus. Insertion of either gene restored production of PHB from fructose (Table 5.2). Re2000 accumulated approximately the same amount of PHB as H16, while Re2001 made significantly less. It has been demonstrated that if a synthase capable of polymerizing MCL monomers is expressed in recombinant *R. eutropha*, the strain will accumulate MCL-PHA when grown on fatty acids (23). (The pathway through which HA_{MCL}-CoA molecules are synthesized in *R. eutropha* has not yet been identified.) We therefore grew Re2000 and Re2001 on a variety of fatty acids in order to characterize the substrate specificities of the *R. aetherivorans* PHA synthases (Table 5.2). The cultures contained 0.05% NH_4Cl and an initial fatty acid concentration of 0.2%. An additional 0.2% fatty acid was added to the cultures after 24 h of growth. We found that both recombinant strains were able to incorporate more HHx into PHA than H16, and that PHA from the strain harboring $phaC2_{Ra}$ also included HHp when heptanoate was used as the carbon source. No PHA monomers longer than HHp were detected in any of the samples.

Table 5.2: PHA produced by H16 and recombinant *R. eutropha* strains harboring $phaC1_{Ra}$ and $phaC2_{Ra}$ was analyzed after the strains were grown for 60 h on 2% fructose or 0.4% fatty acids. All media contained 0.05% NH_4Cl. The values reported are averages from triplicate cultures ± SD.

Carbon Source	Strain	PHA (% of CDW)	PHA Composition (mol%)[a]			
			HB	HV	HHx	HHp
Fructose	H16	75 ± 3	100			
	Re2000	79 ± 2	100			
	Re2001	39 ± 1	100			
Hexanoate	H16	49 ± 2	99.61 ± 0.01		0.39 ± 0.01	
	Re2000	51 ± 1	88.5 ± 0.2		11.5 ± 0.2	
	Re2001	48 ± 2	81.1 ± 0.4		18.9 ± 0.4	
Heptanoate	H16	52 ± 3	62.6 ± 0.5	37.4 ± 0.5		0
	Re2000	62 ± 1	40.4 ± 0.3	59.6 ± 0.3		trace
	Re2001	48 ± 6	25.2 ± 1.1	72.9 ± 1.6		1.9 ± 0.5
Octanoate	H16	66 ± 3	100		trace	
	Re2000	66 ± 2	93.44 ± 0.08		6.56 ± 0.08	
	Re2001	42 ± 4	89.6 ± 0.3		10.4 ± 0.3	

[a]Abbreviations: HB, 3-hydroxybutyrate; HV, 3-hydroxyvalerate; HHx, 3-hydroxyhexanoate; HHp, 3-hydroxyheptanoate

Analysis of *R. eutropha* strains with engineered genomes

Our goal was to produce SCL/MCL PHA copolymers using palm oil as the carbon source. Palm oil is an important agricultural product in Southeast Asia with a high oil yield per acre of land (40). We therefore grew Re2000 and Re2001 in minimal medium with palm oil as the sole carbon source. While these strains accumulated P(HB-*co*-HHx) with significant HHx content when grown on hexanoate and octanoate, there was <2mol% HHx in the PHA made from palm oil (Table 5.3). We hypothesized that high intracellular concentrations of HB-CoA may limit HHx incorporation into the PHA made by the recombinant strains. Our group previously constructed a strain with low acetoacetyl-CoA reductase activity that accumulates significantly less PHB than H16 (Re2115). We deleted the *phaC1* gene from the genome of Re2115, and inserted $phaC2_{Ra}$ (Re2135) and $phaC_{Ac}$ (Re2136) in its place. (The genotypes of the *R. eutropha* strains investigated in this study are illustrated in Fig. 5.1.) These synthase genes were chosen because the strain containing $phaC2_{Ra}$ synthesized PHA with the highest HHx content during fatty acid growth (Table 5.2), while $phaC_{Ac}$ is used in most P(HB-*co*-HHx) production studies in the literature. Both Re2135 and Re2136 made PHA with high HHx content from palm oil, but these strains did not accumulate significant polymer (~25% of CDW after 72 h).

We therefore introduced additional genes into these strains at the *phaB1* locus, with the goal of increasing total polymer accumulation. One of these genes was *phaB2*, which encodes a low activity acetoacetyl-CoA reductase (see Chapter 4). We believed expression of this gene would increase HB-CoA production, but not to the level of H16. We also inserted *phaJ* genes from *P. aeruginosa* and *A. caviae*, which would allow the strains to convert intermediates of fatty acid β-oxidation into HA-CoA molecules. All of these strains exhibited greater PHA production than Re2135 and Re2136 when grown on palm oil (Table 5.3). The strains containing $phaC_{Ac}$ (Re2154-Re2156) made the most polymer, but the HHx content of the PHA was reduced to 4-5mol%. The strains containing $phaC2_{Ra}$ (Re2151-Re2153) made more PHA than Re2135, and the polymer still contained significant HHx. Of these strains Re2152 was the most promising, as it accumulated 40wt% P(HB-*co*-HHx) with 22mol% HHx.

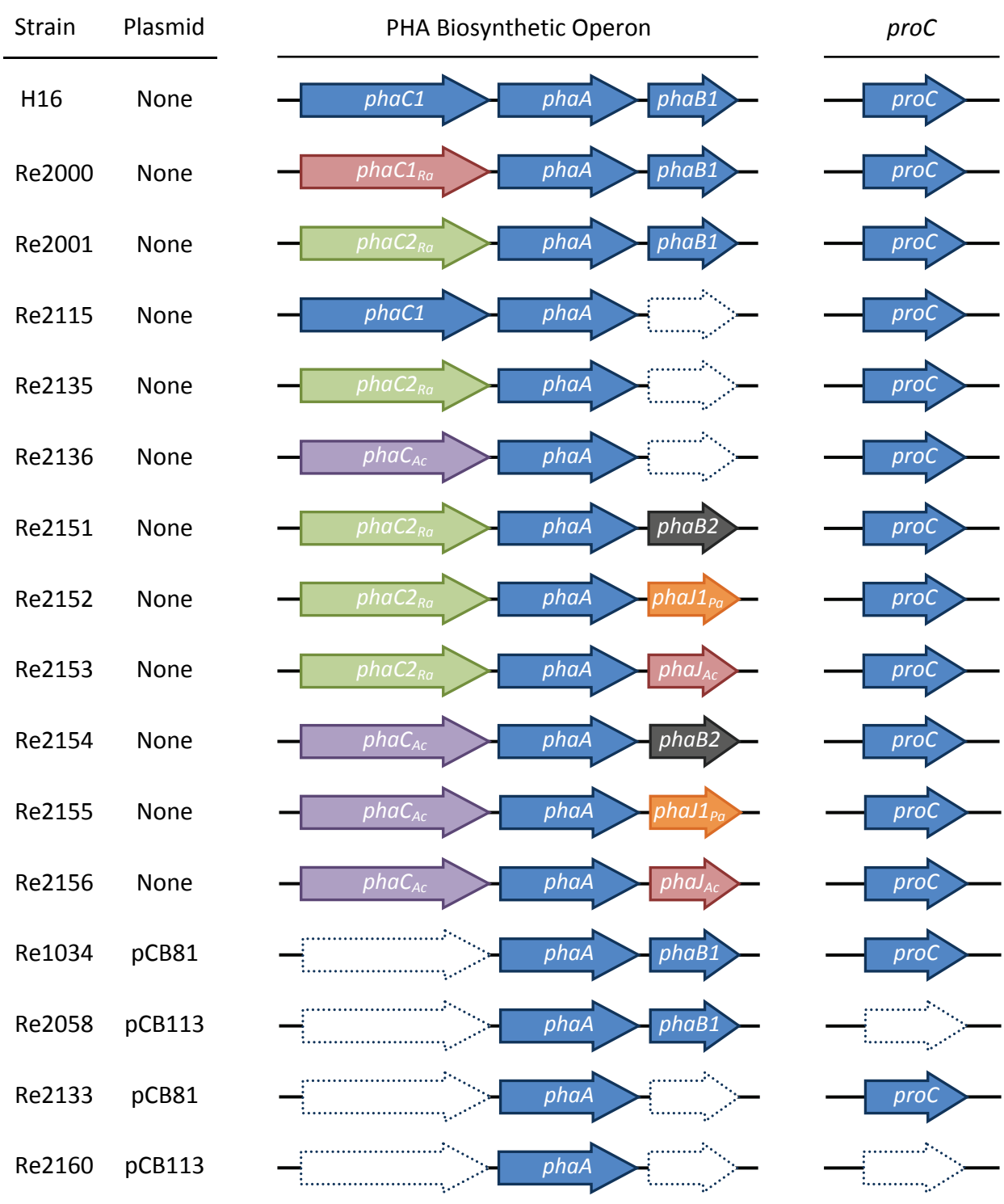

Fig. 5.1: The genotypes of the *R. eutropha* strains used in this study are illustrated above. All strains with numbers 2115 and above are based on Re2115, and therefore also have *phaB2* and *phaB3* deleted from their genomes. Plasmid pCB81 consists of the PHA operon from Re2152 (*phaC2*$_{Ra}$-*phaA*-*phaJ1*$_{Pa}$) cloned into pBBR1MCS-2. Plasmid pCB113 consists of the *proC* region from the *R. eutropha* genome cloned into pCB81.

Table 5.3: *R. eutropha* strains were grown in minimal medium with 1% palm oil and 0.05% NH_4Cl. Samples were harvested after 48 and 72 h of growth to analyze CDW and P(HB-*co*-HHx) content. Re1034/pCB81 and Re2133/pCB81 cultures contained kanamycin. All values represent means from duplicate or triplicate cultures, with the uncertainties indicating the maximum and minimum observed values.

Strain	48 h			72 h		
	CDW (g/L)	PHA (% of CDW)	HHx (mol%)	CDW (g/L)	PHA (% of CDW)	HHx (mol%)
H16	5.3 ± 0.4	71 ± 1	0	6.0 ± 0.2	79.2 ± 0.9	0
Re2000	6.1 ± 0.1	75.3 ± 0.3	1.5 ± 0.1	7.3 ± 0.1	82 ± 4	1.1 ± 0.3
Re2001	1.89 ± 0.04	49 ± 2	1.6 ± 0.2	2.19 ± 0.09	50 ± 3	1.5 ± 0.2
Re2115	0.78 ± 0.04	16.9 ± 0.2	1.68 ± 0.01	1.13 ± 0.06	22 ± 3	1.7 ± 0.3
Re2135	1.0 ± 0.1	22.3 ± 0.2	31.4 ± 0.2	1.22 ± 0.08	26 ± 2	31.4 ± 0.8
Re2136	0.72 ± 0.04	21.3 ± 0.2	15.01 ± 0.02	1.05 ± 0.01	25.5 ± 0.7	13.9 ± 0.5
Re2151	0.83 ± 0.01	28.63 ± 0.01	15.04 ± 0.01	1.01 ± 0.07	33 ± 3	12 ± 1
Re2152	1.15 ± 0.07	35.27 ± 0.07	23.29 ± 0.02	1.40 ± 0.02	40.4 ± 0.4	22.44 ± 0.08
Re2153	1.1 ± 0.1	31.5 ± 0.8	22.29 ± 0.01	1.32 ± 0.09	37 ± 2	22.29 ± 0.07
Re2154	1.26 ± 0.08	45.8 ± 0.8	5.8 ± 0.2	1.92 ± 0.04	53 ± 3	4.83 ± 0.01
Re2155	1.87 ± 0.01	54.9 ± 0.5	3.85 ± 0.07	2.55 ± 0.06	63 ± 3	4.00 ± 0.04
Re2156	2.2 ± 0.2	53 ± 3	3.8 ± 0.3	2.45 ± 0.09	57 ± 2	2.8 ± 0.4
Re1034/pCB81	3.3 ± 0.2	68.8 ± 0.8	13.6 ± 0.2	4.0 ± 0.2	73.0 ± 0.9	11.6 ± 0.2
Re2058/pCB113	3.24 ± 0.03	68 ± 2	15.3 ± 0.4	3.6 ± 0.3	73.1 ± 0.2	12.7 ± 0.3
Re2133/pCB81	2.3 ± 0.1	60 ± 4	24.3 ± 0.8	2.9 ± 0.1	67.0 ± 0.3	23.3 ± 0.2
Re2160/pCB113	2.00 ± 0.01	56.0 ± 0.5	25.32 ± 0.09	2.74 ± 0.06	63.99 ± 0.03	24.13 ± 0.02

Analysis of engineered *R. eutropha* strains harboring plasmids

We hypothesized that polymer accumulation could be increased in the engineered *R. eutropha* strains by increasing expression of the PHA biosynthetic genes. To accomplish this, the engineered PHA operon from Re2152 ($phaC2_{Ra}$-*phaA*-$phaJ1_{Pa}$) was amplified and cloned into pBBR1MCS-2. The cloned region included 460 bp from the genome upstream of the start codon of $phaC2_{Ra}$, so that the operon in the plasmid would be expressed from the native *R. eutropha* promoter. The resulting plasmid (pCB81) was transformed into Re1034 and Re2133, to determine how the different acetoacetyl-CoA reductase activity levels of the two strains would influence PHA synthesis. When these strains were grown in palm oil minimal medium containing kanamycin, both accumulated >65wt% P(HB-*co*-HHx) (Table 5.3). The PHA from Re1034/pCB81 contained 12mol% HHx, while the PHA from Re2133/pCB81 contained 23mol% HHx.

While both strains harboring pCB81 accumulated significant P(HB-*co*-HHx) with high HHx content, these strains were not suitable for industrial PHA production from palm oil. The use of plasmids meant that

expensive antibiotic would have to be added to all cultures in order to achieve consistent results. A common strategy for maintaining plasmid stability without the use of antibiotics is to create an auxotrophic mutant through a genome mutation, and then to complement the mutation with a plasmid containing the deleted gene (19). We deleted the *proC* gene from Re1034 and Re2133. These strains were unable to grow in minimal medium that did not contain proline (data not shown). Plasmid pCB113 was created by cloning the *proC* region of the *R. eutropha* genome into pCB81. When pCB113 was transformed into Re2058 and Re2160, the ability of these strains to grow in minimal medium without proline was restored. PHA production from palm oil in kanamycin-free medium by Re2058/pCB113 and Re2160/pCB113 closely matched the results observed for Re1034/pCB81 and Re2133/pCB81 (Table 5.3). We also observed that high level PHA accumulation by these engineered strains required the use of plant oil or fatty acids as the carbon source. When Re2058/pCB113 and Re2160/pCB113 were grown in fructose minimal medium, these strains accumulated only 40wt% and 17wt% PHA, respectively, and no HHx was detectable in the polymer.

We evaluated the performance of Re2058/pCB113 and Re2160/pCB113 in higher density palm oil cultures by growing these strains in fermentors, using medium with eight times the NH_4Cl concentration as the medium in the flask cultures (Fig. 5.2). No kanamycin was added to the fermentation medium or the minimal medium precultures. Both strains grew in the high nitrogen medium, although Re2160/pCB113 exhibited a lag phase of 24 h. By the ends of the fermentations, Re2058/pCB113 accumulated 71wt% PHA with 17mol% HHx, while Re2160/pCB113 accumulated 66wt% PHA with 30mol% HHx. The PHA contents of the cells in both fermentations closely matched the values measured in the low density flask cultures, suggesting that plasmid loss was not an issue in the fermentations. We made several interesting observations regarding PHA production in these experiments. In both fermentations the HHx content of the polymer was extremely high (>40mol%) early in the cultures. Over time the HHx content decreased and then remained stable over the final 48 h of each experiment. The final HHx content in the PHA was higher in the fermentor cultures than the low density flask cultures (Table 5.3). When analyzing the GC chromatograms of the methanolysis samples from both fermentations, we observed small peaks with the same retention time as methyl 3-hydroxyoctanoate (data not shown). This suggests that the PHA produced in these fermentations contained trace amounts of 3-hydroxyoctanoate.

In order to confirm the HHx content of the PHA produced by these strains, we extracted polymer from dried cells using MIBK and precipitated the PHA by addition of hexane. Proton NMR spectra were taken

for PHA from each strain (Fig. 5.3). The NMR spectroscopy data indicated that PHA from Re2058/pCB113 contained 21mol% HHx, while the PHA from Re2160/pCB113 contained 28 mol% HHx. These values agree well with the methanolysis measurements.

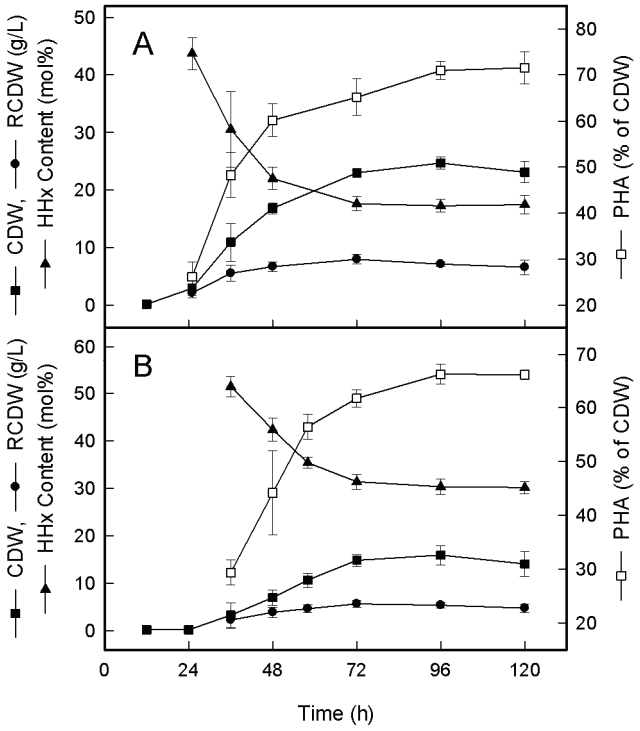

Fig. 5.2: Re2058/pCB113 (A) and Re2160/pCB113 (B) fermentations were carried out using palm oil as the sole carbon source. Plasmid pCB113 was retained by the cells without the use of kanamycin. Both strains accumulated P(HB-*co*-HHx) with higher HHx than was achieved in flask cultures. Data points are means from triplicate fermentations and error bars indicate SD.

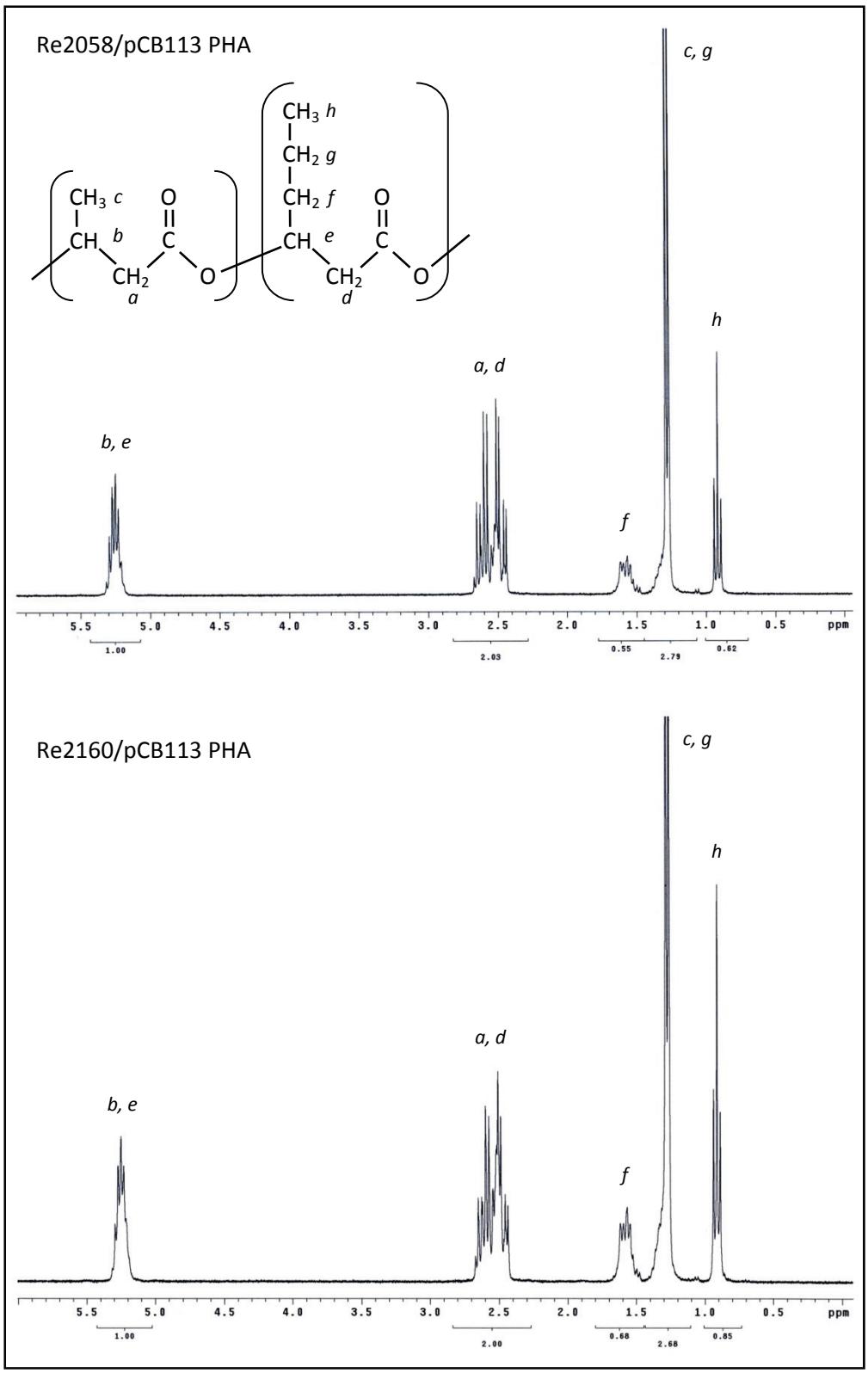

Fig. 5.3: ^{1}H NMR spectra in $CDCl_3$ of P(HB-*co*-HHx) isolated from Re2058/pCB113 and Re2160/pCB113. Based on the area of peak *h*, it was estimated that the Re2058/pCB113 contained 21mol% HHx, while the Re2160/pCB113 PHA contained 28mol% HHx.

Additional characterization experiments were carried out with polymer produced in the fermentations shown in Fig. 5.2. PHA was extracted from lyophilized samples taken after 48 h and 96 h of growth, and the number average (M_n) and weight average (M_w) molecular weights were measured relative to polystyrene standards (Table 5.4). PHA from both Re2058/pCB113 and Re2160/pCB113 had similar molecular weights at each time point. In both cases the polymer was significantly smaller than PHB made by wild type *R. eutropha* H16, which has an M_w of ~3 MDa (see Chapter 4). For both Re2058/pCB113 and Re2160/pCB113, the average PHA molecular weight decreased and the polydispersity increased from 48 to 96 h. This agrees with previous work that showed PHA is continuously turned over by *R. eutropha*, even under PHA storage conditions, and that this turnover is accompanied by a decrease in polymer molecular weight (37). This means that it is important to harvest the biomass from an *R. eutropha* fermentation as soon as maximum PHA accumulation has been reached, as additional time will lead to a decrease in average polymer chain length.

Table 5.4: PHA was extracted from Re2058/pCB113 and Re2160/pCB113 samples with chloroform and the molecular weights were measured by GPC relative to polystyrene standards. Values reported represent means from three independent samples ± SD.

Strain	Time point (h)	M_n (kDa)	M_w (kDa)	PDI
Re2058/pCB113	48	191 ± 27	362 ± 38	1.9
Re2058/pCB113	96	105 ± 40	260 ± 52	2.5
Re2160/pCB113	48	192 ± 15	350 ± 17	1.8
Re2160/pCB113	96	108 ± 12	276 ± 9	2.6

Discussion

We identified and characterized two novel PHA synthases from the bacterium *R. aetherivorans* I24. When these PHA synthase genes were integrated into the Re1034 genome, the recombinant strains accumulated P(HB-*co*-HHx) when grown on even chain length fatty acids, with the strain containing $phaC2_{Ra}$ synthesizing polymer with the highest HHx content (Table 5.2). These strains also accumulated P(HB-*co*-HHx) when grown on palm oil, but the HHx content of the PHA was significantly lower. For example, the PHA from Re2001 contained 10mol% HHx when the strain was grown on octanoate, but only 1.5mol% HHx when the strain was grown on palm oil. It has previously been observed that HHx content in PHA decreases as the lengths of the fatty acids fed to recombinant *R. eutropha* increase (25). As the most abundant fatty acids in palm oil are palmitic acid (C16:0) and oleic acid (C18:1) (30), our results agree with this observation.

In order to increase the HHx content of the PHA, we constructed *R. eutropha* strains expressing recombinant PHA synthases in which normal synthesis of HB-CoA was blocked. We had previously discovered that *R. eutropha* strains in which the acetoacetyl-CoA reductase (*phaB*) genes had been deleted made significantly less PHB than wild type, presumably because the HB-CoA synthesis pathway had been disrupted (7). The PHA made by these strains had high HHx content, but the strains stored little polymer (Table 5.3). Notably, we observed that the strain containing $phaC2_{Ra}$ (Re2135) made PHA with much higher HHx content than the analogous strain containing $phaC_{Ac}$ (Re2136). The $PhaC_{Ac}$ synthase has been the most widely studied enzyme for synthesis of P(HB-*co*-HHx) (11, 12, 22, 24, 25).

In order to increase synthesis of HB-CoA and HHx-CoA from fatty acid β-oxidation intermediates, *phaJ* genes were inserted into the genomes of the recombinant strains. PhaJ enzymes from *A. cavie* and *P. aeruginosa* have been shown to hydrate crotonyl-CoA and 2-hexenoyl-CoA at similar rates (13, 38). We found that insertion of either $phaJ_{Ac}$ or $phaJ1_{Pa}$ into our recombinant strains led to increased PHA accumulation, with the strains expressing $phaJ1_{Pa}$ generating polymer with slightly higher HHx content (Table 5.3).

We hypothesized that PHA production could be improved in our engineered strains by increasing gene expression. This was accomplished by cloning the PHA biosynthetic operon from Re2152 into pBBR1MCS-2, creating pCB81. This plasmid was then transformed into strains with different levels of acetoacetyl-CoA reductase activity. The resulting strains accumulated high levels of P(HB-*co*-HHx), with higher acetoacetyl-CoA reductase activity corresponding to greater HB content in the polymer. Plasmid

stability issues have been reported in high density *R. eutropha* cultures, even in the presence of antibiotics (35). In order to ensure that our strains would produce PHA in high density cultures without the need for kanamycin, we adapted a plasmid stability system that has been used successfully with other species of bacteria (32). The *proC* gene was deleted from the Re1034 and Re2133 genomes, and *proC* was cloned into pCB113, making the plasmid essential for growth of Re2058 and Re2160 in minimal media. One scenario that could lead to plasmid loss in this system is if some cells produce excess proline and excrete it into the medium, which would allow other cells to grow and replicate without pCB113. As both Re2058/pCB113 and Re2160/pCB113 accumulated similar amounts of PHA in low density flask cultures with kanamycin and high density fermentor cultures without kanamycin, we do not believe this is an issue with our strains.

The data presented in Fig. 5.2 shows that the polymer produced by these strains varies over the course of a fermentation culture. The HHx content in the PHA is very high early in the culture, then decreases and eventually stabilizes. This means that late in the cultures, newly synthesized polymer has lower HHx content than the overall average. For example, Re2058/pCB113 produced 10.1 g/L PHA with 22.0mol% HHx by the 48 h time point. By the 96 h time point, this strain had produced 17.5 g/L PHA with 17.3mol% HHx. This means that from 48 to 96 h, 7.4 g/L PHA was accumulated with an average HHx content of 10.9mol%. The reason for higher HHx content in the PHA early in cultures is not completely understood. Some of the HB-CoA made by the strains is produced from acetyl-CoA through the actions of a β-ketothiolase (PhaA) and an acetoacetyl-CoA reductase (PhaB1 in Re2058/pCB113, unknown reductases in Re2160/pCB113). It has been shown that during the *R. eutropha* growth phase, the high intracellular concentration of free CoA inhibits PhaA, slowing the rate of HB-CoA synthesis (27). This suggests that early in the cultures the ratio of HHx-CoA to HB-CoA is high, causing more HHx to be incorporated into the PHA. This could also explain the higher HHx content observed in fermentor cultures relative to flask cultures. The fermentation medium contained more NH_4Cl than the flask medium, leading to a longer growth phase in which more HHx is included in the PHA.

Interesting questions remain concerning MCL-PHA formation in *R. eutropha*. Strains Re2000 and Re2001 expressing *R. aetherivorans* PHA synthases produced P(HB-*co*-HHx). As PhaB1 can reduce 3-ketohexanoyl-CoA (14), this enzyme likely contributed to HHx-CoA formation in these strains. It is unclear, however, how HHx-CoA is synthesized in Re2135 and Re2136, as these strains lack both *phaB* and *phaJ* genes.

Moving forward, we will scale up the size and density of Re2058/pCB113 and Re2160/pCB113 palm oil cultures in order to characterize the PHA made under these conditions. High density fermentations will likely require an oil feeding strategy to prevent excess substrate in the bioreactors. We are also exploring routes to further increase the amount of bioplastic accumulated by our engineered strains.

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Appendix 5.1: Oligonucleotide primer sequences

Primers used in the construction of plasmids and strains are described below. Primers 1-2 were used to insert a SwaI site into pGY46. Primers 3-6 were used to amplify *R. aetherivorans* I24 PHA synthase genes. Primers 7-8 were used to amplify *P. aeruginosa phaJ1*. Primers 9-10 were used to amplify the PHA operon from Re2152 so that it could be cloned into pBBR1MCS-2. Primers 11-16 were used to delete *proC* from the *R. eutropha* genome. Primers 17-18 were used to amplify the *proC* region of the *R. eutropha* genome so that it could be cloned into pCB81.

Primer	Name	Sequence (5' to 3')	Comment
1	pGY46 SwaI F	GGCAGAGAGACAATCAAATCATTTAAATGCTTGCATGAG	SwaI site underlined
2	pGY46 SwaI R	GATTTGATTGTCTCTCTGCCGTCACTATTC	
3	*phaC1* Ra F	ATTTAAATAGGAGATGTCCCATGCTCGACCA	SwaI site underlined
4	*phaC1* Ra R	ATTTAAATCAGCTGAAGACGTACGT	SwaI site underlined
5	*phaC2* Ra F	ATTTAAATAGGAGGAGGCGCATGATGGCCCA	SwaI site underlined
6	*phaC2* Ra R	ATTTAAATCAGCCGGCGGGCAGGTCGCGCA	SwaI site underlined
7	*phaJ1* Pa F	GGCGCGCC*AAGGAGATCTCC*ATGAGCCAGGTCCAGAACATTCC	AscI site underlined, RBS italicized
8	*phaJ1* Pa R	GGTTAATTAAGACGGTAGGGAAAGCCGCTCAGCCGATGCTGATCG	PacI site underlined
9	PHA operon F	GATATCGGTACCCATCCTTCTCGCCTATGCTC	KpnI site underlined
10	PHA operon R	GATATCAAGCTTCTGCCCTGATTCTATGCCCAAC	HindIII site underlined
11	*proC* upstream F	CGGATCCCTACGTCCAGGAAGGCGTCGAC	BamHI site underlined
12	*proC* upstream R	GCCGATTTAAATGCCGATCGAGCATGGAGATCCGTTG	Overlap region with SwaI site underlined
13	*proC* downstream F	CGGCATTTAAATCGGCATTGAGGCGCGGCCAAAC	Overlap region with SwaI site underlined
14	*proC* downstream R	CGGATCCCCGTTCTTCAAGCGCTTCTTTGCG	BamHI site underlined
15	*proC* diag F	GGTCAATATCAGCGGCGAAG	
16	*proC* diag R	CGATCATGCTCTGCTATGCC	
17	*proC* region clone F	GACCGGTGGACATCCTTGTGCGTCATC	AgeI site underlined
18	*proC* region clone R	GACCGGTGGTATCATTACACGCTGATTCGTGAC	AgeI site underlined

Appendix 5.2: Sequences of PHA synthase genes from *R. aetherivorans* I24

The sequences of the PHA synthase genes from *R. aetherivorans* I24 are provided below. Amino acid sequences can be found in Chapter 2. Note that *phaC1* was formerly referred to as the "C09 synthase," while *phaC2* was formerly referred to as the "D12 synthase." The prior names were based on the DNA contigs on which the genes were discovered.

R. aetherivorans* I24 *phaC1

TTGCTCGACCACGTGCACAAGAAGTTGAAGTCGACCCTGGACCCGATCGGCTGGGGTCCCGCGGTGAAGTCGGTGGC
CGGACGCGCCGTCCGCAACCCCCAGGCCGTCACCGCCGCCACGACGGAATACGCGGGCCGGCTGGTGAAGATCCCCG
CGGCGGCCACCCGCGTGTTCAACGCCGACGATCCCAAGCCGCCGATGCCGCTCGACCCGCGGGACCGCCGTTTCTCC
GACACCGCCTGGCGGGAGAACCCCGCGTACTTCTCGCTCCTGCAGAGTTATCTCGCGACGCGGGCCTACGTCGAGGA
ACTCACCGACGCCGGCGCCGGCGATCCGCTGCAGGACGGCAAGGCCCGCCAGTTCGCGAACCTGATGCTCGACGTGC
TGGCCCCGTCGAACTTCCTGTGGAATCCGGGCGTGCTCACCCGTGCATTCGAGACGGGCGGGGCAAGCCTGCTGCGC
GGCGCCCGATATGCCGTGCACGACGTGCTCAACCGCGGCGGCCTGCCGCTGAAGGTGGACTCGGACGCGTTCACCGT
CGGCGAGAACCTCGCGGCCACCCCGGGCAAGGTGGTCTATCGCAACGACCTGATCGAGCTGATCCAGTACACGCCGC
AGACCGAGCAGGTGCATGCGGTGCCGATCCTCGCCGCGCCGCCGTGGATCAACAAGTACTACATCCTCGATCTCGCA
CCCGGTCGCAGCCTCGCCGAGTGGGCGGTCCAGCACGGCCGCACCGTGTTCATGCTCTCGTACCGGAACCCGGACGA
GTCGATGCGGCACATCACCATGGACGACTACTACGTCAACGGCATTGCCGCCGCGCTGGACGTGGTCGAGGAGATCA
CCGGGTCGCCGAAGATCGAGGTGCTGTCCATCTGCCTCGGCGGCGCGATGGCCGCGATGGCCGCCGCGCGCGCATTC
GCCGTCGGCGACAAGCGCGTGACCGCCTTCACCATGCTCAACACCCTGCTCGACTACAGCCAGGTCGGGGAACTCGG
GTTGCTGACCGATCCGTCCACGCTGGACCTCGTCGAGTTCCGGATGCGGCAGCAGGGCTTCCTGTCCGGCAAGGAGA
TGGCCGGCAGCTTCGACATGATCCGCGCGAAGGACCTCGTCTTCAACTACTGGGTCTCGCGGTGGATGAAGGGCGAG
AAGCCTGCGGCCTTCGACATCCTCGCGTGGAACGAGGACAGCACGAGCATGCCCGCGGAGATGCACTCGCACTACCT
CCGGTCGCTGTACGGCCGCAACGAGCTGGCCGAGGGCCTCTACGTGCTCGACGGACAGCCCCTGAACCTGCACGACA
TCACGTGCGACACCTACGTCGTCGGCGCGATCAACGACCACATCGTGCCCTGGACATCGTCGTACCAGGCGGTGAAC
CTGCTGGGCGGCGACGTGCGCTACGTGCTCACCAACGGCGGGCACGTCGCCGGCGCGGTGAACCCGCCCGGCAAGAA
GGTGTGGTTCAAGGCCGTCGGGGCGCCGGACGCCGAGACCGGCTCGCCGCTGCCCGCGGATCCGCAGGTCTGGGACG
ACGCGGCCACCCGCTACGAGCACTCGTGGTGGGAGGACTGGACGGCCTGGTCGAACAAGCGCGCCGGGGAGCTGGTG
CCGCCGCCGGCAATGGGCAGCGCCGCCCACCCGCCGCTCGAGGACGCTCCGGGCACGTACGTCTTCAGCTGA

R. aetherivorans* I24 *phaC2

ATGATGGCCCAGGCACGAACCGTGATCGGTGAGAGCGTCGAGGAGTCGATCGGGGGTGGCGAGGACGTCGCGCCACC
GAGGCTCGGGCCGGCCGTCGGCGCCCTGGCCGACGTGTTCGGTCACGGCCGGGCGGTGGCCCGGCACGGCGTGTCGT
TCGGCAGGGAACTGGCGAAGATCGCCGTCGGCCGGTCGACGGTGGCTCCGGCGAAGGGAGACCGCCGGTTCGCCGAC
TCGGCGTGGAGTGCGAACCCCGCCTACCGCCGGCTCGGGCAGACCTACCTGGCGGCAACCGAGGCCGTCGACGGAGT
CGTCGACGAGGTCGGTCGCGCGATCGGCCCGCGACGCACGGCCGAGGCCAGGTTCGCCGCCGACATCCTCACCGCGG
CCCTGGCCCCGACGAACTACCTGTGGACCAACCCCGCGGCGCTGAAGGAGGCGTTCGACACCGCCGGACTCAGCCTC
GCACGCGGCACCAAGCACTTCGTCTCCGATCTGATCGAGAACCGGGGCATGCCGTCGATGGTCCAGCGCGGCGCCTT
CACCGTCGGGAAGGACCTTGCGGTGACCCCGGGTGCGGTGATCTCCCGCGACGAGGTCGCCGAGGTGCTGCAGTACA
CCCCGACCACGGAGACGGTCCGCCGCCGGCCGGTGCTCGTGGTGCCCCCGCCGATCGGCCGGTACTACTTCCTGGAC
CTGCGGCCGGGACGCAGCTTCGTCGAGTACAGCGTGGGCCGGGGCCTGCAGACCTTCCTGCTGTCGTGGCGCAATCC
CACCGCCGAGCAGGGCGACTGGGACTTCGACACGTACGCGGGCCGGGTGATCCGGGCGATCGACGAGGTGCGGGAGA
TCACCGGCAGCGACGACGTGAACCTGATCGGTTTCTGCGCCGGCGGGATCATCGCCACCACGGTGCTCAATCACCTT
GCCGCGCAGGGCGACACCCGAGTGCACAGCATGGCCTATGCGGTGACGATGCTGGACTTCGGCGATCCGGCACTGCT
CGGCGCGTTCGCCCGGCCCGGCCTGATCCGGTTCGCCAAGGGCCGGTCCCGCCGCAAGGGCATCATCAGCGCCCGCG
ACATGGGGTCCGCGTTCACCTGGATGCGCCCGAACGACCTGGTGTTCAACTACGTCGTCAACAACTACCTCATGGGT
CGCACCCCACCGGCCTTCGACATCCTCGCCTGGAACGACGACGGCACCAACCTGCCCGGCGCCCTGCACGGTCAGTT
CCTCGACATCTTCCGTGACAACGTGCTCGTCGAGCCCGGCCGGCTCGCCGTGCTGGGCACGCCCGTCGACCTGAAGT
CGATCACCGTGCCCACGTTCGTCTCGGGCGCCATCGCCGACCATCTGACCGCATGGCGCAACTGCTACCGCACCACC
CAATTGCTCGGTGGAGAAACAGAATTCGCGCTCAGCTTCTCCGGGCACATCGCCAGCCTGGTCAACCCGCCGGGCAA
TCCGAAGGCACACTACTGGACCGGGGGCACACCCGGCCCGGACCCGGATGCCTGGCTCGAGAACGCCGAGCGGCAGC
AGGGCAGCTGGTGGCAGGCCTGGTCCGACTGGGTGCTCGCCCGCGGCGGGGAGGAAACCGCCGCGCCGGACGCACCC
GGCAGTGCGCAGCATCCCGCGCTCGACGCCGCTCCCGGCCGGTACGTGCGCGACCTGCCCGCCGGCTGA

Chapter 6

Determination of Genes Involved in *Ralstonia eutropha* Fatty Acid Metabolism by Whole Cell Gene Expression Analysis

Many of the results presented in this chapter were also reported in the paper "Elucidation of β-oxidation Pathways in Ralstonia eutropha H16 by Examination of Global Gene Expression" (3), which has been accepted for publication in the Journal of Bacteriology.

Introduction

Despite the fact that many groups are interested in conversion of plant oils to polyhydroxyalkanoates (PHAs) using *Ralstonia eutropha* H16 (14, 18, 22, 23), relatively little is known about the fatty acid metabolism of this bacterium. Plant oils are attractive carbon sources for industrial PHA production due to their high carbon content and the fact that intermediates in fatty acid metabolism can be converted into PHA precursors (1). Plant oils consist of triacylglycerol (TAG) molecules, in which three fatty acids are connected to a glycerol backbone. In our lab the focus is on oils derived from the oil palm tree, a major agricultural crop in Southeast Asia. Palm oil contains mainly palmitic (C16:0) and oleic (C18:1) acids, while palm kernel oil contains shorter fatty acids, with lauric acid (C12:0) being the primary constituent (29).

Most knowledge of fatty acid metabolism in bacteria is based on studies conducted with *Escherichia coli* and *Bacillus subtilis* (9, 13). Fatty acids are transported into the cell and are then activated for degradation by a fatty acyl-CoA synthetase (FadD, also referred to as a fatty acid CoA ligase), which converts the acid into a fatty acyl-CoA. (If plant oil is used as the carbon source, fatty acids must first be released from the TAGs by a lipase (17)). The fatty acyl-CoA is catabolized in the β-oxidation cycle, through the actions of an acyl-CoA dehydrogenase, an enoyl-CoA hydratase, a (*S*)-3-hydroxyacyl-CoA dehydrogenase, and a β-ketothiolase (see Fig. 6.1). With each round of β-oxidation acetyl-CoA is released and the fatty acyl-CoA is reduced in length by two carbons. Bacteria must then use the acetyl-CoA to build up the larger molecules necessary for cell growth and division. In most species this is made possible by the glyoxylate bypass, which allows intermediates from the tricarboxylic acid (TCA) cycle to be diverted for synthesis of other metabolites (8). The enzymes that make up the glyoxylate bypass are

isocitrate lyase (encoded by *icl*) and malate synthase (encoded by *aceB*). While the glyoxylate bypass seems to be the most widespread method used by bacteria to grow on C2 carbon sources, recent work has revealed alternate anaplerotic pathways (10, 15).

The genes encoding fatty acid metabolism enzymes in *R. eutropha* have never been identified. Examination of the published *R. eutropha* genome reveals many potential proteins for each step in β-oxidation (25). For example, 64 genes in the genome are annotated as acyl-CoA dehydrogenases, while 51 genes are annotated as enoyl-CoA hydratases. It would therefore de difficult to predict which genes are important for fatty acid metabolism simply based on sequence homology. We decided to perform whole genome microarray experiments in order to study gene transcription levels when *R. eutropha* was grown on different carbon sources, both before and after nutrient limitation. We hypothesized that genes involved in oil and fatty acid metabolism would increase in expression during growth on TAGs, relative to growth on sugar. These experiments have provided important insights into the fatty acid metabolism of *R. eutropha*, and hopefully the results will allow for development of new strains that grow more quickly on palm oil and produce PHA more efficiently.

Materials and methods

Bacterial strains and cultivation conditions

All experiments were performed with *Ralstonia eutropha* H16 (ATCC 17699) and mutants derived from this strain (Table 6.1). *R. eutropha* strains were grown aerobically at 30°C in both rich and minimal media. The rich medium was dextrose free tryptic soy broth (TSB) medium (Becton Dickinson, Sparks, MD). The minimal medium was identical to the one used in previous work (5). Ammonium chloride was used as the nitrogen source in the minimal medium and was added in the concentrations indicated in the text. The carbon sources used in the minimal medium were fructose, trioleate, palm oil (Wilderness Family Naturals, Silver Bay, MN), crude palm kernel oil (CPKO, gift from Dr. K. Sudesh Kumar), and oleate. The lipid carbon sources were frequently emulsified using 0.3% gum arabic, a glycoprotein that is not metabolized by *R. eutropha* (refer to Chapter 3 for details on emulsified oil media). All media contained 10 µg/mL gentamicin. Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless noted otherwise.

Cultures were grown for expression analysis in minimal media containing either 2% fructose or 1% emulsified trioleate. Trioleate was used instead of palm oil because it is a pure TAG carbon source, unlike natural oils which may contain contaminants. Triplicate cultures were prepared for each carbon source. Colonies were initially picked from TSB agar plates and used to inoculate 5 mL TSB cultures. 0.5 mL aliquots from these cultures were used to inoculate fructose and trioleate minimal media cultures containing 0.1% NH_4Cl (50 mL media in 250 mL flasks). All flask cultures in this experiment were shaken at 200 RPM. Cells were harvested from these cultures and used to inoculate fructose and trioleate minimal media cultures with 0.05% NH_4Cl (50 mL media in 250 mL flasks) to an initial optical density at 600 nm (OD_{600}) of 0.1. These cultures were incubated 12 h. The last set of cultures was then inoculated from the precultures to an initial OD_{600} of 0.05. These were again fructose and trioleate minimal media cultures with 0.05% NH_4Cl (50 mL media in 250 mL flasks). Ammonium concentrations in these cultures were monitored using a Sigma-Aldrich Ammonium Assay Kit following the manufacturer's instructions. When approximately half the ammonium remained in the media (~250 mg/L NH_4Cl), cells were harvested for later RNA isolation. These samples were used to represent cells in exponential growth phase. The harvesting procedure was to take a volume of culture with a total OD_{600} equivalent of 2.5 and immediately pipette the cells into two volumes of Bacteria RNAprotect Reagent (QIAgen). The cells were then pelleted, the supernatant was removed, and the cell pellets were stored at -80°C until the

RNA was purified. Culture samples were also taken using the same procedure two hours after ammonium in the media was depleted, to represent cells in the PHB storage phase.

RNA isolation and microarray analysis

For RNA isolation, cell pellets were first thawed at room temperature. They were then resuspneded in a solution containing lysozyme and proteinase K and incubated for 10 minutes at room temperature, with vortexing applied every two minutes. RNA was isolated from the lysed cells using the RNEasy Mini Kit (QIAgen) following the manufacturer's instructions. The isolated RNA was quantified based on its absorbance at 260 nm. Samples of RNA were delivered to the MIT BioMicroCenter and the quality of the RNA was measured using an Agilent 2100 BioAnalyzer. Only RNA with a RNA Integrity Number (RIN) above 9 was used for microarray analysis (RIN's are calculated on a 1-10 scale) (30).

The custom *R. eutropha* H16 microarray chips used in this study were purchased from Affymetrix (Santa Clara, CA). Probes for 6626 protein-encoding genes and 3 rRNA genes from the published *R. eutropha* genome were printed on an 11 μm array (Affymetrix 49-5241 format). Each probe set included 15 exact match 25-mer oligonucleotide probes and 15 mismatch 25-mer probes (7). After submitting the design, chips were manufactured following standard Affymetrix protocols.

Biological triplicates were used for all microarray experiments. For each sample, 100 ng of total isolated RNA was amplified and labeled using the MessageAmp II-Bacteria prokaryotic RNA Kit (Ambion, Cat. No. AM1790). Samples were then hybridized to the *R. eutropha* H16 microarray chips for 16 hours at 45°C. Chips were scanned at the MIT BioMicroCenter using an Affymetrix 7G scanner. Data was scaled to an average intensity of 500 per chip, and base 2 logarithms of expression values were calculated (note that $\log_2(500) = 9.0$).

Microarray data was extracted with Affymetrix GCOS version 1.4 and analyzed with ArrayStar (DNAStar, Madison, WI) and Microsoft Excel. All data were normalized by Robust Microchip Average (RMA, ArrayStar software, Madison, WI) with quantile normalization. The statistical significance of expression differences between conditions was determined by the Student's *t*-test and ANOVA testing. Genes with statistically significant differences in expression between conditions ($p < 0.1$) were selected for further analysis. The microarray data reported in this study have been deposited in the NCBI Gene Expression

Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) and can be accessed through the GEO series accession number GPL10276.

Strain construction

Markerless deletions of gene clusters and individual genes were made in the *R. eutropha* genome using established methods (5, 37). All strains and the plasmids used in their construction are described in Table 6.1. Gene deletion fragments used to knockout gene clusters were constructed by overlap extension PCR, as described previously (5). Oligonucleotide primer sequences are listed in Table 6.2. Gene deletion fragments used to knockout individual genes were synthesized by Integrated DNA Technologies (Coralville, IA). These sequences consist of ~250 bp upstream of a given gene connected to ~250 bp downstream of the gene. The synthesized sequences are provided in Appendix 6.1.

Growth and analysis of mutant *R. eutropha* strains

Colonies were picked from TSB agar plates and used to inoculate 5 mL test tube cultures of TSB, which were incubated overnight at 30°C. Aliquots from these cultures were harvested, washed, and resuspended 1:10 in sterile saline. 50 µL from these saline suspensions were used to inoculate minimal medium cultures containing either 2% fructose or 1% palm oil, CPKO, or oleate. The lipid carbon sources were typically emulsified unless noted otherwise. The minimal medium was the same as described above and contained 0.1% NH_4Cl. Cultures were shaken at 200 RPM and 30°C, and samples were taken at various time points for CFU counting and measurement of PHB accumulation.

To determine cell dry weight (CDW) and PHB content, 5 – 10 mL of culture were taken in preweighed glass tubes and cells were pelleted. Pellets were washed with 5 mL cold water and 2 mL cold hexane. The hexane was added to remove residual oil, so it was not used to wash samples from fructose cultures. Samples were dried under vacuum at 80°C and the mass was measured to determine CDW. PHB was quantified by treating dried cell pellets with concentrated sulfuric acid, which leads to conversion of PHB to crotonic acid, as has been described previously (19, 38).

Table 6.1: Strains and plasmids used in this study.

Strain or plasmid	Description[a]	Reference or source
R. eutropha strains		
H16	Wild type, Gm resistant	ATCC 17699
Re2300	H16 Δ(A0459-A0464)	This work
Re2302	H16 Δ(A1526-A1531)	This work
Re2303	Re2300 Δ(A1526-A1531)	This work
Re2304	H16 Δ*aceB*	This work
Re2306	H16 Δ*iclA*	This work
Re2307	H16 Δ*iclB*	This work
Re2312	H16 Δ*fadD3*	This work
Re2313	H16 ΔA1322 (putative lipase gene)	This work
E. coli strains		
S17-1	Strain for conjugative transfer of plasmids to *R. eutropha*	(33)
Plasmids		
pGY46	*oriT, traJ*; confers Km resistance; based on pJQ200mp18; all other deletion plasmids use backbone from this plasmid	(27, 37)
pCJB4	pGY46 with Δ*phaC1* allele removed by *Bam*HI digestion, and replaced with Δ(A0459-A0464) allele	This work
pCJB5	pGY46 with Δ*phaC1* allele removed by *Bam*HI digestion, and replaced with Δ(A1526-A1531) allele	This work
pCB86	pGY46 with Δ*phaC1* allele removed by *Bam*HI digestion, and replaced with Δ*aceB* allele	This work
pCB94	pGY46 with Δ*phaC1* allele removed by *Bam*HI digestion, and replaced with Δ*iclA* allele	This work
pCB95	pGY46 with Δ*phaC1* allele removed by *Bam*HI digestion, and replaced with Δ*iclB* allele	This work
pCB96	pGY46 with Δ*phaC1* allele removed by *Bam*HI digestion, and replaced with Δ*fadD3* allele	This work
pCB97	pGY46 with Δ*phaC1* allele removed by *Bam*HI digestion, and replaced with ΔA1322 allele	This work

[a]Abbreviations: Gm, gentamicin; Km, kanamycin

Table 6.2: Oligonucleotide primers used in this study. Restriction sites (BamHI and PacI) in the primers are underlined.

Name	Sequence
A0459upstreamF	5’-CGGATCCAACGTCGCCGTTTATGATGCGG-3’
A0459upstreamR	5’-GCCGTTAATTAAGCCGGTTTTTTCTCCGTTCTGCGG-3’
A0459downstreamF	5’-CGGCTTAATTAACGGCGGCCCACGTCTGTCCAATCC-3’
A0459downstreamR	5’-CGGATCCACCACACACCGTCATGAACGCTCC-3’
A0459diagF	5’-CGAGTATGCCGAGAGCTTCC-3’
A0459diagR	5’-GCGCGATTGTCGCAGAGTTC-3’
A1526upstreamF	5’-CGGATCCCTTGAGCCTGCGCTTGAGGTG-3’
A1526upstreamR	5’-GCCGTTAATTAAGCCGGCTGGTTCCTTTGGTGTCAA-3’
A1526downstreamF	5’-CGGCTTAATTAACGGCCGGATTGCCCTCAAACTTGG-3’
A1526downstreamR	5’-CGGATCCCGGCTTGAATACTGCGTCGGGA-3’
A1526diagF	5’-GCTGTAGGTGACGAAGGAGC-3’
A1526diagR	5’-GCGCTTGAACGTTTTGGACA-3’
aceBdiagF	5’-GCTGCTGTCGGTCATCTGGA-3’
aceBdiagR	5’-CTTTGTCTCGTCCAACGGCTGG-3’
iclAdiagF	5’-CGTCTGCCATTGGCATCTACC-3’
iclAdiagR	5’-GACGATCATCCCATCCGTGC-3’
iclBdiagF	5’-GGACACATGGACTGCGCTGA-3’
iclBdiagR	5’-CAACGCGGGAGGGTTCTCTAC-3’
fadD3diagF	5’-GCTTTGCCTTCGACCTGAGC-3’
fadD3diagR	5’-TCCTCTACACCTGGCATGAACC-3’
A1322diagF	5’-GTCACCATCGATGGCTGGATC-3’
A1322diagR	5’-CGGATATCGTCGTACACCAGCC-3’

Results

Microarray expression analysis

Microarrays were used to analyze the *R. eutropha* transcriptome during growth on fructose and trioleate. RNA was isolated from samples harvested from cultures during their exponential growth phase and also after nitrogen limitation had occurred, which stimulates PHB accumulation. Our initial focus was to determine which genes were differentially expressed during growth on the two carbon sources. We analyzed all genes in which expression values were at least two fold different between carbon sources at the 99% confidence level. We eliminated all genes in which $\log_2$ of the expression values were below 6 in all conditions, as we found that this level of expression was within the background noise of the experimental system. Note that a $\log_2$(expression) of 6 corresponds to 12.8% of the average expression. Genes that are thought to be unexpressed (e.g. *phaB2* and *phaC2*) all showed expression levels in this range. (When expression values are mentioned in this study, we always refer to $\log_2$ of the measured value.) A total of 787 genes were found to be differentially expressed according to this analysis. 418 genes were upregulated during trioleate growth (relative to fructose) and 369 genes were upregulated during fructose growth (relative to trioleate). The predicted functions of the differentially expressed genes are summarized in Table 6.3.

Examining the data in Table 6.3, we find that for trioleate growth 46 genes involved in lipid metabolism are upregulated, while for fructose growth 34 genes involved in carbohydrate metabolism are upregulated. These findings provide a good indication that our analysis is capturing the changes in transcription that occur during growth on the two carbon sources. In both cases several genes involved in transcription are differentially expressed, which may be due to different transcriptional regulators directing metabolism in response to the carbon source. Both conditions also show upregulation of many genes annotated as being involved in inorganic ion transport and metabolism. It is not known how accurate all of these annotations are, so other transporters may have incorrectly been included in this category based on their amino acid sequences. Many energy production and conversion genes are upregulated during fructose growth, primarily because numerous genes involved in hydrogen utilization on megaplasmid pHG1 (*hox* and *hyp* genes) are upregulated. It has been shown that expression of hydrogenase genes in *R. eutropha* H16 does not depend on the presence of H_2, because the native *hoxJ* regulatory gene encodes an inactive histidine protein kinase (21). Numerous genes of unknown function

are also differentially expressed, suggesting there is much left to learn about the metabolism of *R. eutropha*.

Table 6.3: A summary of the 787 genes that are differentially expressed during exponential growth on trioleate or fructose.

Code	Functional Category[a]	Upregulated on trioleate[b]		Upregulated on fructose[b]	
		2-4 Fold	>4 Fold	2-4 Fold	>4 Fold
Information storage and processing					
J	Translation, ribosomal structure, and biogenesis	1 (0.6)	0 (0.0)	4 (2.2)	1 (0.6)
K	Transcription	32 (4.1)	9 (1.1)	10 (1.3)	6 (0.8)
L	DNA replication, recombination, and repair	5 (3.1)	0 (0.0)	1 (0.6)	1 (0.6)
Cellular processes					
D	Cell division and chromosomal partitioning	3 (10.8)	0 (0.0)	3 (10.8)	0 (0.0)
O	Post-translational modification, protein turnover, chaperones	8 (5.1)	2 (1.3)	7 (4.5)	2 (1.3)
M	Cell envelope biogenesis, outer membrane	11 (4.7)	4 (1.7)	9 (3.9)	2 (0.9)
N	Cell motility and secretion	0 (0.0)	0 (0.0)	16 (8.9)	12 (6.7)
P	Inorganic ion transport and metabolism	17 (16.7)	5 (4.9)	18 (17.6)	9 (8.8)
T	Signal transduction mechanisms	9 (1.1)	5 (0.6)	2 (0.2)	1 (0.1)
Metabolism					
C	Energy production and conversion	20 (4.1)	7 (1.4)	45 (9.3)	31 (6.4)
G	Carbohydrate metabolism and transport	11 (7.4)	4 (2.7)	20 (13.3)	14 (9.4)
E	Amino acid metabolism and transport	8 (2.7)	2 (0.7)	30 (10.1)	13 (4.4)
F	Nucleotide metabolism and transport	0 (0.0)	0 (0.0)	3 (4.0)	0 (0.0)
H	Coenzyme metabolism	3 (1.9)	2 (1.3)	2 (1.3)	1 (0.6)
I	Lipid metabolism	27 (8.2)	19 (5.7)	11 (3.3)	6 (1.8)
Q	Secondary metabolite biosynthesis, transport, and catabolism	3 (3.1)	2 (2.0)	2 (2.0)	1 (1.0)
Poorly characterized					
R	General function prediction only	32 (4.3)	9 (1.2)	26 (3.5)	10 (1.3)
S	Function unknown	117 (6.4)	41 (2.2)	35 (1.9)	15 (0.8)
	TOTAL	307	111	244	125

[a]Functional categories follow the work of Tatusov, *et al*. (35).

[b]The numbers in parentheses indicate the percentage of genes in a given category that are upregulated, based on the total number of genes in the *R. eutropha* genome that have been assigned to a given category. For example, 9 genes out of 785 total transcription genes are upregulated >4 fold on trioleate, equaling 1.1%.

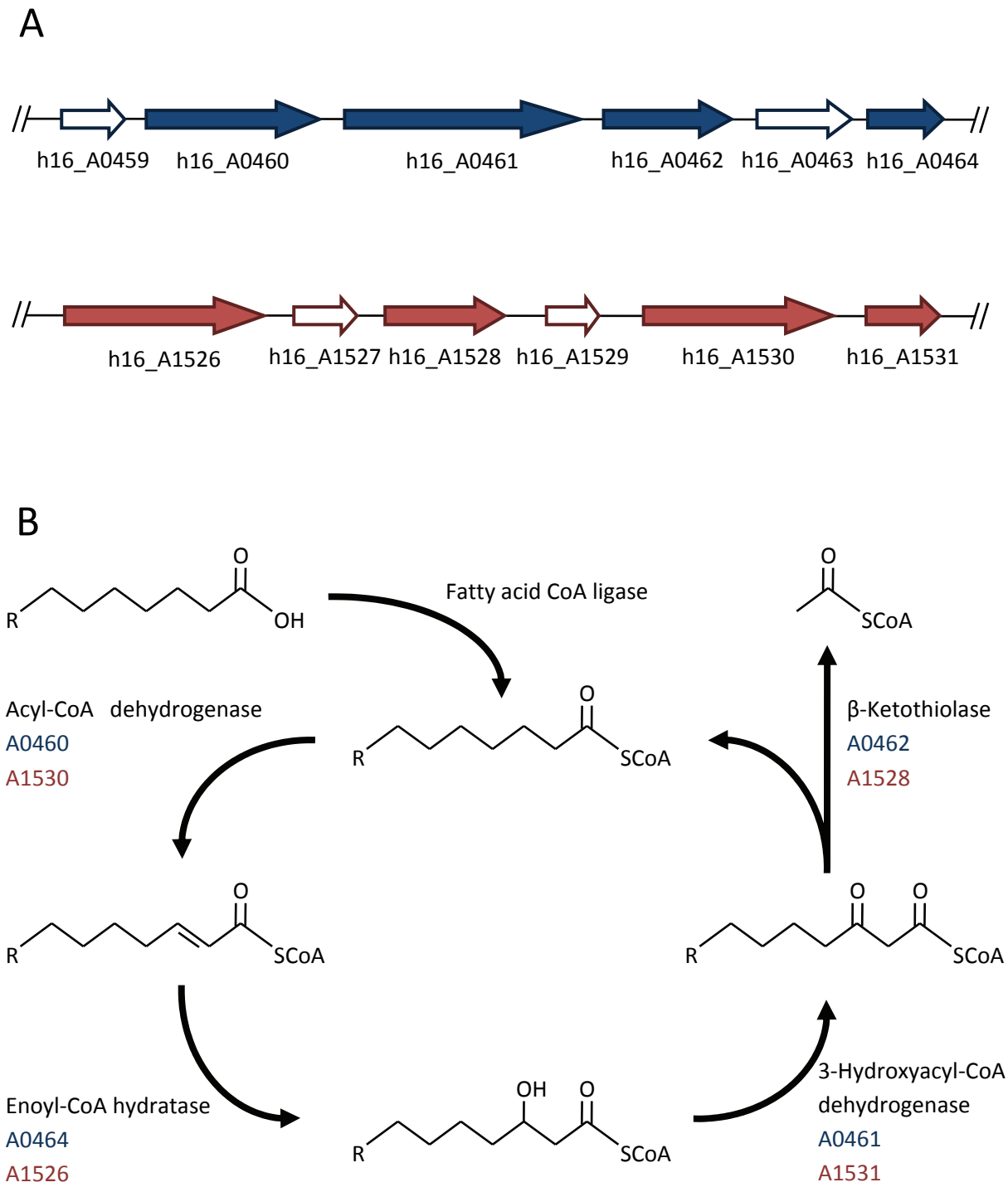


Fig. 6.1: (A) Based on microarray expression data, two gene clusters were identified as potentially encoding all enzymes necessary for fatty acid β-oxidation (closed arrows), as well as other proteins of unknown function (open arrows). (B) A schematic of the β-oxidation cycle showing which reaction each of the identified enzymes is predicted to catalyze. Note that the 3-hydroxyacyl-CoA molecule generated in β-oxidation is the (*S*) form.

Table 6.4: Gene clusters and genes whose expressions are upregulated during exponential growth on trioleate.

Gene locus tags	GeneID numbers[a]	Description	Fold increase[b]
A3732-A3736	4246691, 4247741, 4247742, 4247743	Function unknown, genes possibly encode outer membrane associated proteins	184
A0459-A0464	4247875, 4247128, 4247876, 4247877, 4247878, 4247879	Fatty acid β-oxidation operon	36
A1526-A1531	4249355, 4250030, 4249356, 4249357, 4249358, 4249320	Fatty acid β-oxidation operon	5
A2507-A2509	4247547, 4247548, 4247471	First steps in glycerol metabolism	4
A2227	4250182	Isocitrate lyase, *iclB*	40
A2211	4250181	Isocitrate lyase, *iclA*	36
A2217	4247136	Malate synthase, *aceB*	9
A1322	4249488	Triacylglycerol lipase	7
A3288	4246987	Acyl-CoA synthetase, *fadD3*	6
A3742	4249675	Lipase	4

[a]NCBI GeneID numbers for gene clusters are given in ascending order of gene locus tags (i.e. A3732, A3733, A3734…).

[b]Fold increases reported for gene clusters are the mean increase for all genes in the set.

Closer examination of the genes that are upregulated during trioleate growth revealed several that could be involved in fatty acid metabolism (Table 6.4). Three clusters of successive genes were identified in which all genes showed similar expression patterns, leading us to believe that these are co-transcribed operons. The cluster of genes from A3732-A3736 was the most highly upregulated, although this was partially due to the fact that their expression levels during fructose growth were extremely low. (Note that the nomenclature "A*xxx*" and "B*xxx*" refer to the locus tags of genes discussed in this work, where A indicates the gene is on chromosome 1, while B indicates the gene is on chromosome 2.) The functions of the genes in this cluster are not known, but it is predicted that some of the gene products are associated with the outer membrane. Gene clusters A0459-A0464 and A1526-A1531 were of great interest because both are predicted to include enzymes that could catalyze all the steps in fatty acid β-oxidation (see Fig. 6.1 for details). Genes A2507-A2509 appear to encode the first steps in glycerol metabolism. When all fatty acids are released from a TAG molecule, glycerol is the byproduct. These genes may be upregulated in response to the presence of glycerol in the trioleate medium. Other genes of interest include lipases, an acyl-CoA synthetase, and genes encoding the

enzymes of the glyoxylate bypass (*iclA*, *iclB*, and *aceB*). While the main focus of this study was not on sugar metabolism, we did identify a putative fructose utilization operon (B1497-B1505), in which gene expression was upregulated an average of 15 fold during growth on fructose relative to trioleate.

The glyoxylate bypass allows TCA cycle intermediates to be diverted for synthesis of cellular metabolites. One of these crucial compounds is phosphoenolpyruvate (PEP), a precursor to glycolytic intermediates. A recent study examined PEP formation from TCA cycle intermediates in *R. eutropha* and proposed two routes for PEP synthesis: (i) formation of PEP from oxaloacetate by PEP carbokykinase (Pck) and (ii) conversion of malate to pyruvate by the malic enzyme (Mae), followed by conversion of pyruvate to PEP by PEP synthetase (Pps) (4). Oxaloacetate is synthesized from malate by the TCA cycle enzyme malate dehydrogenase (Mdh). Interestingly, none of the genes encoding these enzymes were upregulated during growth on trioleate, although several were highly expressed in both fructose and trioleate cultures (Table 6.5).

Table 6.5: Expression values for genes encoding enzymes that participate in conversion of TCA cycle/glyoxylate bypass intermediates to PEP. The mean log_2(expression) of all genes was 9.0.

Locus tag	GeneID	Encoded enzyme	Trioleate expression[a]	Fructose expression[a]	Fold change[b]
A1002	4250044	Malic enzyme, Mae	9.98	9.73	+1.19
A3153	4250043	Malic enzyme, Mae	9.38	10.91	-2.89
A2634	4250055	Malate dehydrogenase, Mdh	11.56	11.23	+1.26
B0942	4456280	Malate dehydrogenase, Mdh	7.36	6.99	+1.26
A3711	4249772	PEP carboxykinase, Pck	8.78	9.86	-2.11
A2038	4250263	PEP synthetase, Pps	11.25	11.15	+1.07

[a] Log_2 of measured expression is reported.

[b] Fold change of trioleate expression relative to fructose expression.

We also examined the expression patterns of several genes involved in PHA metabolism in *R. eutropha*. The key genes in PHB biosynthesis (*phaA*, *phaB1*, *phaC1*), as well as the regulatory gene *phaR*, are all constitutively expressed, as has been shown previously (20). Transcript levels for *phaB2* and *phaC2*, which overlap in the *R. eutropha* genome, were always at low levels suggesting that these are unexpressed pseudo-genes. Expression of *phaP1* increased dramatically during PHB storage conditions, which agrees with previous results (20). Interestingly, several putative PHA depolymerases were

expressed as PHB accumulated, and some (*phaZ1*, *phaZ2*, *phaZ6*, *phaY1*) actually showed increased transcription under PHB storage conditions. The reasons for these transcription patterns are unclear, but expression of these genes may help explain the observation that there is turnover of accumulated PHB even during nutrient limited conditions (34).

Expression of *phaP3* and *phaB3*, which are adjacent to one another in the genome, was significantly higher during growth on fructose than growth on trioleate. This agrees with the results presented in Chapter 4, in which I showed that a *R. eutropha* strain with *phaB1* and *phaB2* deleted from the genome accumulated significant PHB when grown on fructose, but not when grown on palm oil. Expression of *phaP3* and *phaB3* also decreased once nitrogen limitation occurred. We observed that the Δ*phaB1,2* strain made approximately the same amount of PHB as wild type early in fructose minimal medium cultures, but that PHB synthesis in this strain stopped late in the cultures, while PHB accumulation continued in H16. Based on the microarray results, we believe that *phaB3* is expressed when nitrogen is present in fructose medium, but that expression stops after nitrogen limitation occurs. The PhaB3 level in the Δ*phaB1,2* strain then eventually drops to the point that there is not sufficient acetoacetyl-CoA reductase activity for normal PHB synthesis late in the culture. Our finding that *phaP3* expression decreases during nitrogen starvation contradicts a previous report which suggested that this gene is regulated by PhaR, meaning expression should increase in response to PHB storage (26).

Growth and PHB production of fatty acid metabolism mutants

R. eutropha mutants were constructed in which genes and entire gene clusters identified by microarray analysis were deleted from the genome. These mutants were then grown on a number of carbon sources, including palm oil, CPKO, and oleate. The first mutants examined were those in which the putative fatty acid β-oxidation operons had been deleted (A0459-A0464 and A1526-A1531). These mutants showed the same phenotypes on all lipid carbon sources: strains in which a single operon had been deleted grew at the same rate as the wild type organism, while the mutant with both operons deleted (Re2303) was unable to grow on oils or long chain fatty acids (Fig. 6.2). Interestingly, Re2303 was still able to grow on shorter chain fatty acids, such as octanoate (data not shown), indicating that there are other genes that encode enzymes capable of catabolizing fatty acids shorter than those used in the experiments shown in Fig. 6.2. Deletion of *fadD3* did not cause a growth defect in oil or oleate

cultures. All strains grew identically in TSB and fructose minimal medium, indicating that the mutations affect only fatty acid catabolism (data not shown).

Glyoxylate bypass mutants were also grown in emulsified oil (Fig. 6.3). The Δ*aceB* strain (Re2304) grew more slowly than wild type on oil and fatty acids, but eventually reached the same cell density as H16. Both the Δ*iclA* strain (Re2306) and Δ*iclB* strain (Re2307) grew indistinguishably from H16. This suggests that only one of these isocitrate lyases is required for the pathway to function normally. Several attempts were made to construct a strain with both *icl* genes deleted, but these were unsuccessful. The reasons for this problem are still under investigation.

Deletion of the putative lipase gene A1322 led to an interesting phenotype. We normally emulsified the plant oils used in our growth experiments with gum arabic. This led to formation of a "milky" liquid in which tiny oil droplets were suspended throughout the medium. In our experience, emulsification of the oil with gum arabic led to more reproducible growth with a shorter lag phase (see Chapter 3). When *R. eutropha* is grown in non-emulsified oil minimal medium, the oil is initially in a separate phase on top of the aqueous solution. As the cells grow, the oil is eventually broken down and becomes emulsified. The mechanism by which this emulsification occurs is not completely understood. When the ΔA1322 strain (Re2313) was grown in emulsified oil, it grew at the same rate as wild type (Fig. 6.4A, B). When Re2313 was grown in non-emulsified palm oil medium, however, the medium never became emulsified (Fig. 6.4C). Re2313 exhibited some growth in this medium, but significantly less than H16.

The abilities of all mutants constructed in this study to accumulate and catabolize PHB were also examined (Table 6.6). When grown on fructose, all strains accumulated ~70wt% PHB. Cells with stored PHB were then transferred to medium with 0.1% NH_4Cl and no carbon source, triggering PHB utilization. The amount of stored PHB decreased for all strains, while the CFU/mL increased, indicating that stored PHB was being used for cell growth and division. These findings show that the gene deletions made in this study do not prevent PHB synthesis or utilization.

Table 6.6: PHB production and utilization by fatty acid metabolism mutants. Cell viability counts were made 0 and 24 h after cultures had been transferred to fresh medium with no carbon source.

Strain	Genotype	PHB (% of CDW), production[a]	PHB (% of CDW), utilization[b]	Cell count, 0 h (CFU/mL × 10^5)	Cell count, 24 h (CFU/mL × 10^5)
H16	Wild type	75.5 ± 3.5	33.2 ± 3.8	2.2 ± 0.4	85 ± 5
Re2300	H16 Δ(A0459-A0464)	75.3 ± 1.1	33.1 ± 0.2	4.9 ± 2.6	135 ± 60
Re2302	H16 Δ(A1526-A1531)	70.6 ± 5.4	34.7 ± 0.3	2.2 ± 0.8	90 ± 20
Re2303	Re2300 Δ(A1526-A1531)	72.2 ± 1.5	27.9 ± 3.1	6.2 ± 2.3	90 ± 30
Re2304	H16 Δ*aceB*	67.5 ± 3.8	41.9 ± 1.5	3.1 ± 1.0	150 ± 50
Re2306	H16 Δ*iclA*	69.1	29.9	1.2 ± 0.2	160 ± 60
Re2307	H16 Δ*iclB*	70.4	40.3	2.1 ± 0.1	100 ± 25
Re2312	H16 Δ*fadD3*	66.9 ± 0.1	33.8 ± 3.0	2.3 ± 0.3	120 ± 30
Re2313	H16 ΔA1322 (lipase)	71.5 ± 2.6	33.0 ± 1.5	2.8 ± 0.2	95 ± 15

[a]PHB content was measured after cells had been incubated 72 h in minimal medium with 2% fructose and 0.05% NH_4Cl.

[b]PHB content was measured after cells had been transferred to minimal medium with no carbon source and 0.1% NH_4Cl and incubated 24 h.

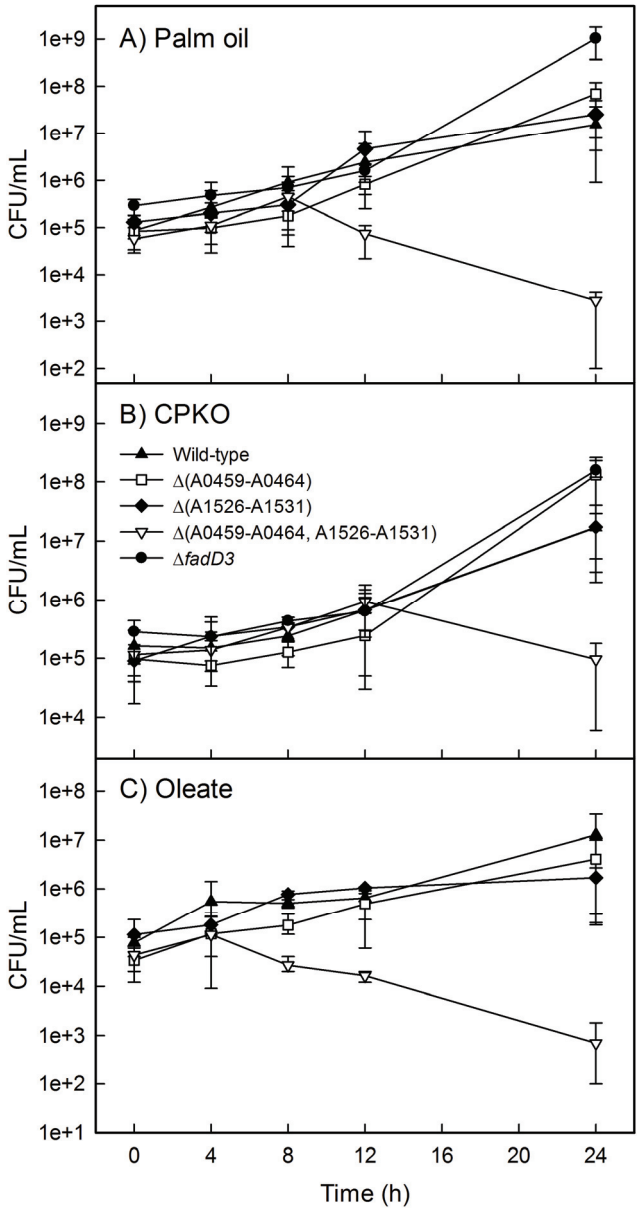


Fig. 6.2: Deletion of the A0459-A0464 and A1526-A1531 operons prevented growth of *R. eutropha* on palm oil (A), CPKO (B), and oleate (C). Data points are means from triplicate cultures and error bars indicate the maximum and minimum CFU measurements.

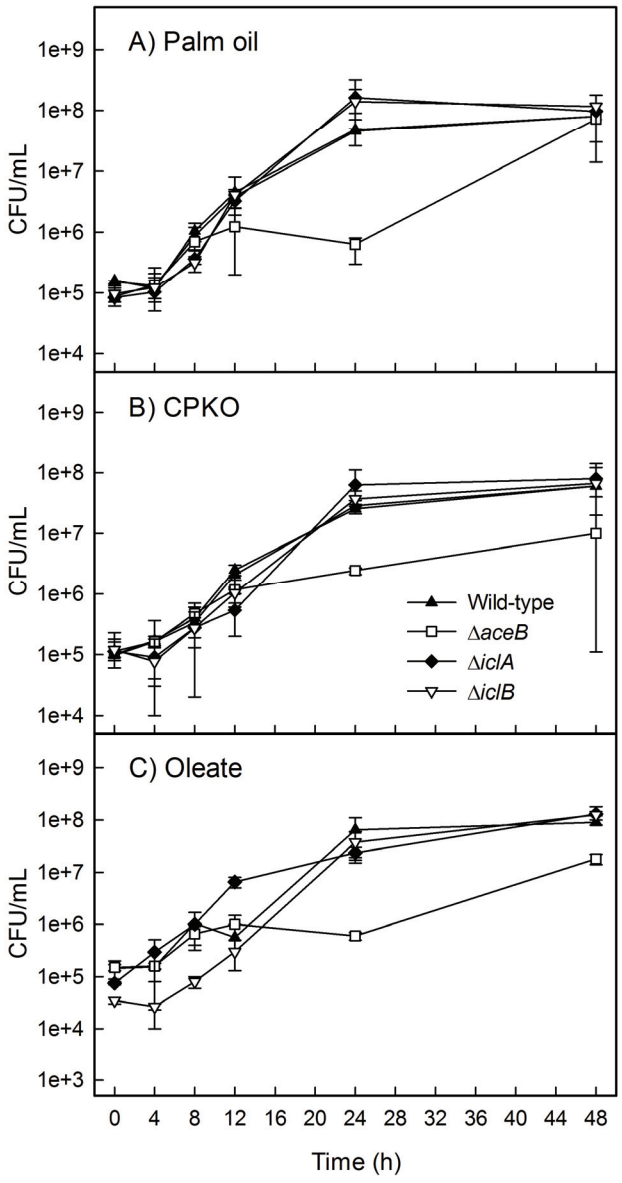
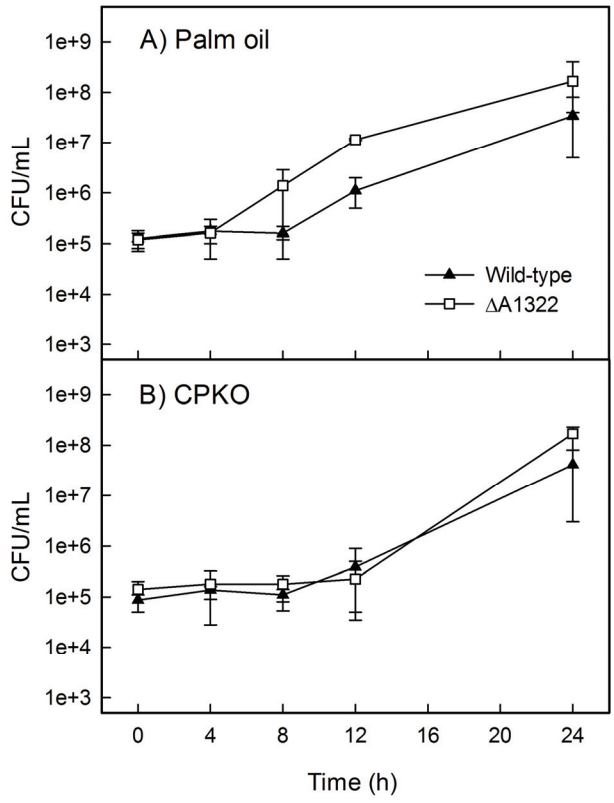


Fig. 6.3: Deletion of *aceB* slowed growth of *R. eutropha* on palm oil (A), CPKO (B), and oleate (C). Deletion of a single *icl* gene did not affect growth on any of the carbon sources. Data points are means from triplicate cultures and error bars indicate the maximum and minimum CFU measurements.



C) Non-emulsified oil

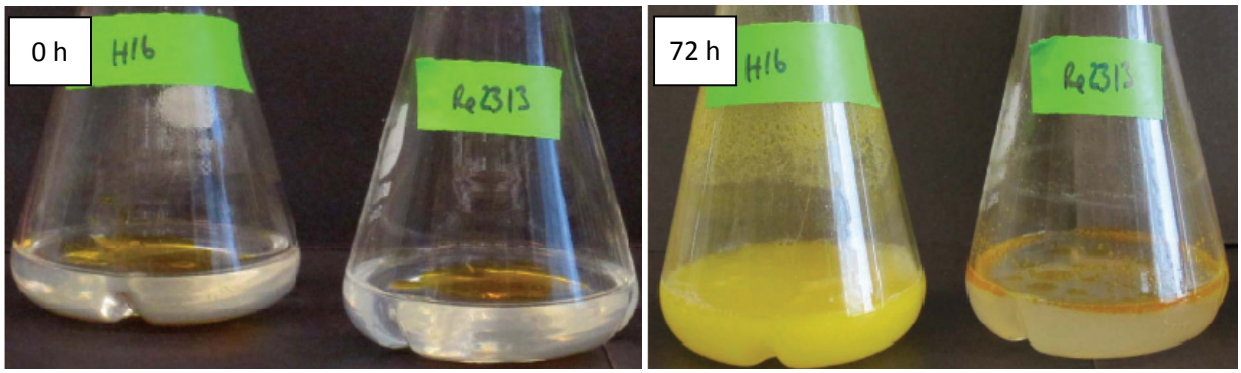


Fig. 6.4: Deletion of the putative lipase gene A1322 did not affect growth of *R. eutropha* on emulsified palm oil (A) or CPKO (B). Deletion of this gene did significantly reduce growth on non-emulsified palm oil, as seen in photos of cultures taken 0 and 72 h after inoculation (C). Data points in (A) and (B) are means from triplicate cultures and error bars indicate the maximum and minimum CFU measurements.

Discussion

Microarray experiments performed using *R. eutropha* H16 showed that two gene clusters encoding all steps necessary for fatty acid β-oxidation were upregulated when trioleate was provided as the carbon source (Fig. 6.1). As all genes in each cluster showed similar expression patterns, we believe that each gene cluster is a co-transcribed operon. Mutant strains were constructed in which the operons were deleted individually and in combination. If one operon was deleted, the strains grew at the same rate as H16 on oils and oleate, but if both operons were deleted no growth was observed (Fig. 6.2). This demonstrates that the *R. eutropha* genome contains redundant genes for oxidation of long chain fatty acids. Given that the genes encoding each β-oxidation enzyme are in different orders in the two operons, and that each operon contains distinct genes whose potential roles in fatty acid metabolism are unclear, it seems unlikely that the two operons resulted from a recent DNA duplication event.

Of the two proposed operons, A0459-A0464 is more upregulated and its genes are expressed more highly during trioleate growth. This operon contains two genes that do not have obvious roles in fatty acid oxidation. A0459 is annotated as a transcriptional regulator, so it may be involved in altering expression of other genes during growth on fatty acids. A0463 is a hypothetical protein that belongs to the DegV family, a class of proteins found exclusively in bacteria. The functions of these proteins are currently unknown, but a structural study revealed that a *Thermotoga maritime* DegV protein binds fatty acids (32), indicating a possible role for these proteins in fatty acid metabolism. The A1526-A1531 operon also contains two genes whose products do not have obvious roles in fatty acid β-oxidation. A1527 is annotated as a bifunctional pyrazinamidase/nicotinamidase (*pncA*). PncA is part of the NAD salvage pathway and may therefore play a role in controlling NADH/NAD^+ levels in *R. eutropha* during growth on fatty acids (12). A1529 has sequence similarity to the phenylacetic acid degradation protein PaaI, although the function of this protein in the degradation pathway is unknown (11). The number of fatty acyl-CoA synthetases (*fadD* genes) expressed in *R. eutropha* was unclear. The gene *fadD3* was upregulated during growth on trioleate, but deletion of this gene did not produce a growth phenotype (Fig. 6.2). Two other genes on the megaplasmid pHG1 are annotated as *fadD* genes (*fadD1*, PHG399; *fadD2*, PHG398), but their transcript levels were low enough that we cannot confidently say whether they were expressed or not. The gene A2794, which is annotated as an acyl-CoA synthetase and is upregulated 6 fold on trioleate, is another potential *fadD* that deserves further investigation.

We observed that strain Re2303 (the double operon deletion mutant) could still grow on fatty acids shorter than those found in palm oil and CPKO. It has been established that in eukaryotes there are multiple enzymes for each step in the β-oxidation pathway, with different enzymes having different substrate specificities (2). In *E. coli* there are two acyl-CoA dehydrogenases with different specificities (*fadF* and *fadG*), while other steps in the pathway are catalyzed by single enzymes (9). *R. eutropha* must have genes in addition to the A0459-A0464 and A1526-A1531 operons that allow for metabolism of fatty acids shorter than C12. Examination of the genome reveals additional gene clusters that appear to encode all or most steps necessary for fatty acid oxidation, including A2455-A2461, B0354-B0362, B0378-B0386, and B0387-B0391. The genes A2455-A2461 are annotated as encoding the 2-aminobenzoate degradation pathway, which includes several reactions that are analogous to the steps in fatty acid β-oxidation (6, 31). Further work is required to determine if these enzymes are truly involved in 2-aminobenzoate metabolism or if they may actually degrade fatty acids. Little can be predicted about the three gene clusters on chromosome 2 based solely on amino acid sequences. The products of these genes could participate in fatty acid oxidation, or they may be involved in degradation of other organic compounds. None of these gene clusters were upregulated during growth on trioleate, so if one or more of them is indeed responsible for oxidation of octanoate and other fatty acids, it seems that they are only upregulated in response to these substrates, or they are constitutively expressed. It is also possible that there are additional genes that participate in medium and short chain length fatty acid β-oxidation that we have been unable to identify.

Wang *et al*. previously studied the glyoxylate bypass of *R. eutropha* grown on acetate and gluconate (36). Our results confirm their finding that deletion of *aceB* slows, but does not eliminate, growth on acetate (or fatty acids in our study). They found that malate synthase activity was still detectable in their Δ*aceB* strain, but the source of this activity was not determined. When Wang *et al*. deleted *iclB*, growth on acetate was not possible, and strangely the growth rate on gluconate decreased. Deletion of *iclA* had no impact on growth with either carbon source. These isocitrate lyase results differ from ours, as deletion of either *icl* gene did not affect growth on oils or fatty acids (Fig. 6.3). It is possible that *iclA* is not expressed during growth on acetate, but is expressed when fatty acids are used as the carbon source. No genes that encode enzymes for synthesis of PEP were clearly upregulated during trioleate growth (Table 6.5). Microarray data indicates that these genes are expressed in the presence of both fructose and trioleate, which agrees with previously published data showing enzymatic activity during growth of *R. eutropha* on a variety of carbon sources (4).

Deletion of the putative lipase gene A1322 resulted in a strain (Re2313) with a unique phenotype (Fig. 6.4). Re2313 grew at the same rate as H16 in emulsified oil medium. When non-emulsified oil was used, however, very little growth was observed. When H16 is grown using non-emulsified oil, the oil is broken down and eventually becomes emulsified. It is unclear how this emulsification takes place. Other species of bacteria are known to synthesize surfactants that can emulsify hydrophobic compounds (28), but there is no evidence that *R. eutropha* is a surfactant producer. Another possibility is that as TAGs are broken down, the resulting polar lipids (diacylglycerols, monoacylglycerols, and free fatty acids) emulsify the remaining oil. Based on our results, this seems to be the case for *R. eutropha*, with A1322 being the key lipase for release of fatty acids from TAGs. When oil is emulsified with gum arabic, we believe that other non-lipase esterases are able to cleave fatty acids from the suspended oil droplets, and that these esterases are unable to act on non-emulsified oil. We plan to conduct additional research on this lipase, which will include studying the function of the gene immediately downstream of A1322, which is annotated as a lipase chaperone.

The results of this study also shed some light on the PHB utilization pathway. When PHB is depolymerized *in vivo*, (*R*)-3-hydroxybutyrate is released (16). The next steps in the pathway have not been fully determined. It is likely that a ligase converts the free hydroxyacid to (*R*)-3-hydroxybutyryl-CoA. It has been proposed that this product is then sequentially converted to acetoacetyl-CoA, (*S*)-3-hydroxybutyryl-CoA, and finally crotonyl-CoA (24). If this hypothesis is correct, the crotonyl-CoA could then be converted into two molecules of acetyl-CoA by one round of fatty acid β-oxidation. It is unclear, however, why formation of crotonyl-CoA would be more advantageous to the organism than direct conversion of acetoacetyl-CoA to acetyl-CoA by a β-ketothiolase. PHB utilization is unaffected by the deletion of the two fatty acid β-oxidation operons identified in this study (Table 6.6), providing evidence that the products of different genes are involved in catabolism of long chain fatty acids versus short chain length organic acids.

The results of our microarray experiments provided valuable insights into the lipid metabolism of *R. eutropha*. Two operons encode the enzymes responsible for β-oxidation of long chain fatty acids. Given that deletion of a single operon does not decrease the growth rate relative to H16, β-oxidation must not be the step that limits growth on fatty acids. Additional research is required to determine what limits the growth rate of *R. eutropha* on fatty acids, so that this restraint can be removed by genetic engineering. Additional studies will be performed to characterize the genes that make up the two β-oxidation operons, in order to understand the roles of the individual enzymes and how products of β-

oxidation could be diverted to PHA synthesis. Some genes encoding enzymes of the glyoxylate bypass are upregulated during growth on trioleate, but there must be at least one additional gene that provides malate synthase activity that we have not yet identified. The gene product of A1322 encodes a lipase that is essential for robust growth of *R. eutropha* on non-emulsified plant oil. We will further characterize this enzyme to determine its kinetic parameters and substrate specificity. It is possible that overexpression of A1322 could improve growth on non-emulsified oil. Microarrays are a useful tool for determining genes involved in different *R. eutropha* metabolic pathways, and future microarray experiments will be performed to elucidate other pathways in this organism, such as PHB utilization and catabolism of other carbon sources.

Acknowledgements

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Appendix 6.1: Gene deletion sequences

DNA sequences for deletion of individual genes were ordered from Integrated DNA Technologies. Each sequence consisted of ~250 bp upstream of a given gene connected to ~250 bp downstream of the gene. BamHI restriction sites were present at both ends of each sequence so that the gene deletion fragments could be ligated into the pCB46 backbone. The sequences to delete each gene are given below.

aceB deletion sequence

GGATCCGCCGCCTGTACAGGAGCATTGTAGTGCGCAAGCGATGCGTGTGGCCCCCGTCATCCAGCGCCCGTAAGTTGGCGGC
TTCGACGCAGAAGGCTTACGTTCTGCGCACATTTGGAGGCTGTGCCAGTGAGTGACCGCCTTCCTGAACGACTCCAACAGTTG
AGTGGACACTCGATCGGCATCCCAAAAGAAAAAACCGCAGCCAAAGCTGCGGTTTTTTCATCCTGCTAGCACCCTACCCGCTTA
ATTAACAGTCTTCTCCTGTGATCGATAGCGGTAAGGCTTGGTTGAAGGGACCCTGCTACACCGCCACGCCACGGACGCGGACG
AATTCAAGCAGGTCGTTCATGTCGTGCCCGGTGCCGGAGGGGGCTACGTCGAGCCGCTCCGCCGGATGGCCGGCACGGTTGA
TCCAGAACGTGGTGTAGCCGAACCACGTGGCGCCGCAGGCGTCCCAGCCGTTGGACGAGACAAAGAGCATCTCTTCGGCGGG
ATCC

iclA deletion sequence

GGATCCGTGGAATTTGCTCCGGCTTTCCCCCGGCGCTATCTTTCGTTCCTTGAAAATGCTTTCCACATTGCGGAATTTAGTGTTT
ATCTCATTGAAAAATAAGGAAAAATCAACTCTTATGTCTTATATAAGACTTTGCCGCGACGCATCAAGAACGCCCTACAATGAA
CCCACGCTGCTCGCCTCGACATCCGCCAGCACACTCAACTATTTTTTTCAAACGCGCTTTCTTCTTAGGAAATTCCCTTAATTAAT
ACCAGTAATACCCCAAGCAAAACGCCAACCGATACGGGTTGGCGTTTTTTTGTCCGGCGAGATCGCTCGCGGGGTTCTCATCA
AACATATTCTTGTGCGCTTCCATGCAGAAGCTGTACGTGTGGCTTGAAATCAGCGTGCTGGGTTGAGACGCGGATGCGTCTCC
TCTCCCCAGTGATACAGTACAAGGCCAGACAGTCCCATCGCGATGGCGACGAACCAGAATGCACTCTCGACGCGGATCC

iclB deletion sequence

GGATCCATGGAAGATCATTTCCCGCATCGTGAAATAAAATCTATAAGCCATTGAATTATTGTAAGAAAAAAACTAAGCGAGGA
CTGCAGATCAATGTTGTTGCAACGCGGGAGGGTTCTCTACACTCTTATATAAGACTCATGACATGAGGCGCGAAAAACAGCAG
GCGACGCAGCCACCGCGACGTAGCCCAGGTCAGGTGATCAACAGATTCTCAGCCATCCCCGCATCATCAGGAGAGTGACCTTA
ATTAACTCTCCTGGTCACCCCCGCCGGAACGTGGGTCCGGCGGTTAAGGAAGGCGTGCGACCATCACGCCTTCTGATTTAGGC
GCCGCGCCGTTCTCTCTCAGGGACGGCGCGTTTTTTTATTTTCCAATGCTGGGGGCGGGTTAGCGGGTAGCTGTTCAACGGGT
GTTCAAAACTCAAACTCCATGCAGAGTTACCGCCCAAGCCTGGCCGGGTTCACGCCGGCTATTTAGAAGCCTGACGCGGGGAT
CC

fadD3 deletion sequence

GGATCCGCAGGAGCTTGCCGCCAGCATGATGAAACTGGCGCTGCCCAGCGGCGGCTGAGTGTGGATTTGCCTCACTAGGGGT
TATCCCCCTACTTGCGCCCGATCGGTGGCGCATAATGCCCGTCAGGGGCACCAAGGCCTGTCCGGTGGCCCCGAGGACTTCGT
CTAAGTTGTTGTTTTAACCGGGGAGCACGTTTAGCATCTCGGCAAAATCGAACGACCATTCAGAAATCAGGAGACGGTGCTTT
AATTAAGCGGCAGCCGCTGCCAACGACAACGGCCTCCCGAGTGGAGGCCGTTGTGCTTTCAGGCTGCGCCGCGGCGGCTCAG
CACTCGTTCGATGGCGCCAGGCGGGCTTCCTGGCTGCCGTCGGGGTAGATCACCCGCACGCACGCGCCTGGCTCCAGCCCCG
GCGCCGCCGGCGCGTTGGCCGCCACGATCGTGCCGTCCATGGTTCGCACCTTGTACTGGAACAGGTCCACCTGCTGCACCGGG
GATCC

A1322 (lipase) deletion sequence

GGATCCCGCAGGTTGCCTTGCGCGTCGAGTTCGGGGCCCGCGGGCACGTCGACGCCGGACAGCGATGGCAAGGCAGCCACG
CCGGCGGGTCCGGCGTCAGCCGATGTCGCCGAAGGACTCGCAGCGGTGGTGATCGCTGCCGGGCGCTGCGGCACGGCAACC
GGGCCAGACGGCATCGTCAGCCAGTACACCGCGGCCGCCGCCGCGCCGGCCGGCAGCAAGGCCAGCCAGGGCCGCGGCGAG
CGCTTAATTAACTTGTCTCCTCGGTGGTGGGTCTTTTTATGTTGGAAGCAGTAAAGACTGGCCGCCCCGACGAGACAATCAAAA
CATTAGCGGATTCCCTCTAAACGGCCGCTACAGCCGCGGGCTGCAAGGGCATGCGGGTGCTGGAAAGCAGCTGTTCGTAGAT
GTAGCACTGCAGCAGCGTCAGCTCGCGCTCGGCCAGCACGTCGAAGGCCACGCCGAAGGCCGGAAAGTCGGCATCGTCGCTG
GCCGGATCC

Chapter 7

PHA Recovery from Bacterial Biomass and Simulation of a PHA Production Process

Introduction

There are several requirements that must be met for a PHA production process to be economically viable. A strain must be engineered that can convert an industrially relevant carbon source into a useful type of PHA (see Chapter 5). A fermentation strategy must then be developed for growing dense microbial cultures with high productivities and yields (for examples refer to (15, 18)). Finally, there must be an efficient recovery process that allows for consistent isolation of high purity polymer. There may be different purity requirements for the PHA depending on its intended application, and these requirements will influence the design of the recovery process. For example, there are stricter regulations for plastic that will be used in medical applications compared to plastic used to make other products. Several PHA purification methods have been described in the academic and patent literature that could potentially be adapted for recovery of P(HB-*co*-HHx) from palm oil cultures.

There are two classes of recovery processes: those that utilize organic solvents to dissolve the PHA, and those that do not use solvents. When he first discovered PHB, Lemoigne found that the polymer could be dissolved in chloroform and later precipitated by addition of alcohol (e.g. methanol) to the solution (11). When groups became interested in the commercial potential of bioplastics, additional solvents that could be used to extract PHB were identified, including dichloromethane, 1,4-dioxane, and pyridine (1, 2). It was later determined that PHA copolymers containing medium chain length monomers are soluble in a wider range of chemicals, including a variety of ketones, esters, and alcohols (9, 12, 17). The dissolved polymer can be recovered by evaporating the solvent, cooling the solution, or adding a chemical precipitant. Evaporation is problematic because the polymer will coat the vessel after the solvent is removed. Additionally, any contaminants that also dissolve in the solvent will co-purify with the PHA. Decreasing the temperature of the solution may require significant cooling costs and can result in gelation as the polymer comes out of solution. Addition of a chemical precipitant is therefore the most common strategy. The precipitant must be a liquid that is miscible with the solvent, but that cannot dissolve PHA itself. Alkanes have frequently been employed as precipitants in the literature (13,

17). A solvent/precipitant recovery process has been shown to produce polymer that is >99% pure with 98% recovery of the PHA present in the bacterial biomass (see Example XII in (17)). In some proposed processes, the biomass may be pretreated before solvent is added, in order to disrupt the cells and speed extraction. Pretreatments include addition of acetone (although this is a solvent for some PHAs), exposure to a mild acid or base, and mechanical disruption.

Non-solvent recovery methods use more severe disruption techniques to completely break down the bacterial cells, so that the PHA granules can be isolated from the other biomass. The key advantage of this strategy is that it avoids the costs of purchasing and recycling solvent and precipitant. Disruption methods for non-solvent recovery processes include chemical treatment, enzymatic digestion, and comminution (5), although most focus has been on chemical methods. It was discovered in 1958 that PHB granules could be recovered from *Bacillus* cells after treatment of the biomass with sodium hypochlorite (19). Other alkaline chemicals can also be used (e.g. NaOH and KOH), as can a variety of detergents (5). After digestion, the PHA is separated from the remaining biomass by centrifugation, and in many implementations the polymer then goes through an additional wash step. Achieving high purity polymer (>98%) via chemical disruption can be challenging, especially if the biomass does not have extremely high PHA content. Problems have also been reported with removal of bacterial endotoxins from PHA in chemical disruption methods, which is a key issue for polymer that will be used to make medical products (10). Polymer purity can be increased by using harsher chemical treatments (e.g. more concentrated base, higher temperature, and/or longer treatment) (5, 10), but these conditions can then lead to degradation of the polymer, as evidenced by decreased PHA molecular weight (3, 14).

An additional concern for a recovery process using our strains is the possibility of residual palm oil associated with the biomass. In a solvent based process, the oil would likely remain in solution after addition of the precipitant, but in a non-solvent process it would become saponified and be difficult to separate from the plastic. Because of this issue, as well as the other challenges associated with non-solvent recovery discussed above, we focused our attention on solvent based processes. An overview of the steps in a solvent based recovery process is shown in Fig. 7.1. Much of the water from the fermentation broth is first removed by centrifugation, and the resulting wet biomass slurry is pumped to the extraction step. PHA is extracted from the biomass using as organic solvent, and is then precipitated and recovered. The solvent and precipitant are separated so that they can be reused in the process.

The first goal of this study was to find an effective solvent for dissolving the P(HB-*co*-HHx) synthesized by the strains described in Chapter 5. We next sought to find a suitable precipitant to force the PHA out of

solution. Finally, we designed a process for recovering polymer using recycled solvent and precipitant. Fig. 7.1 includes a compounding operation in which additional components would be mixed with the polymer, which could include fillers, pigments, plasticizers, stabilizers, and other additives. While compounding is an important step in manufacturing a commercially viable plastic, we have not conducted research in this field, so it will not be addressed further in this chapter.

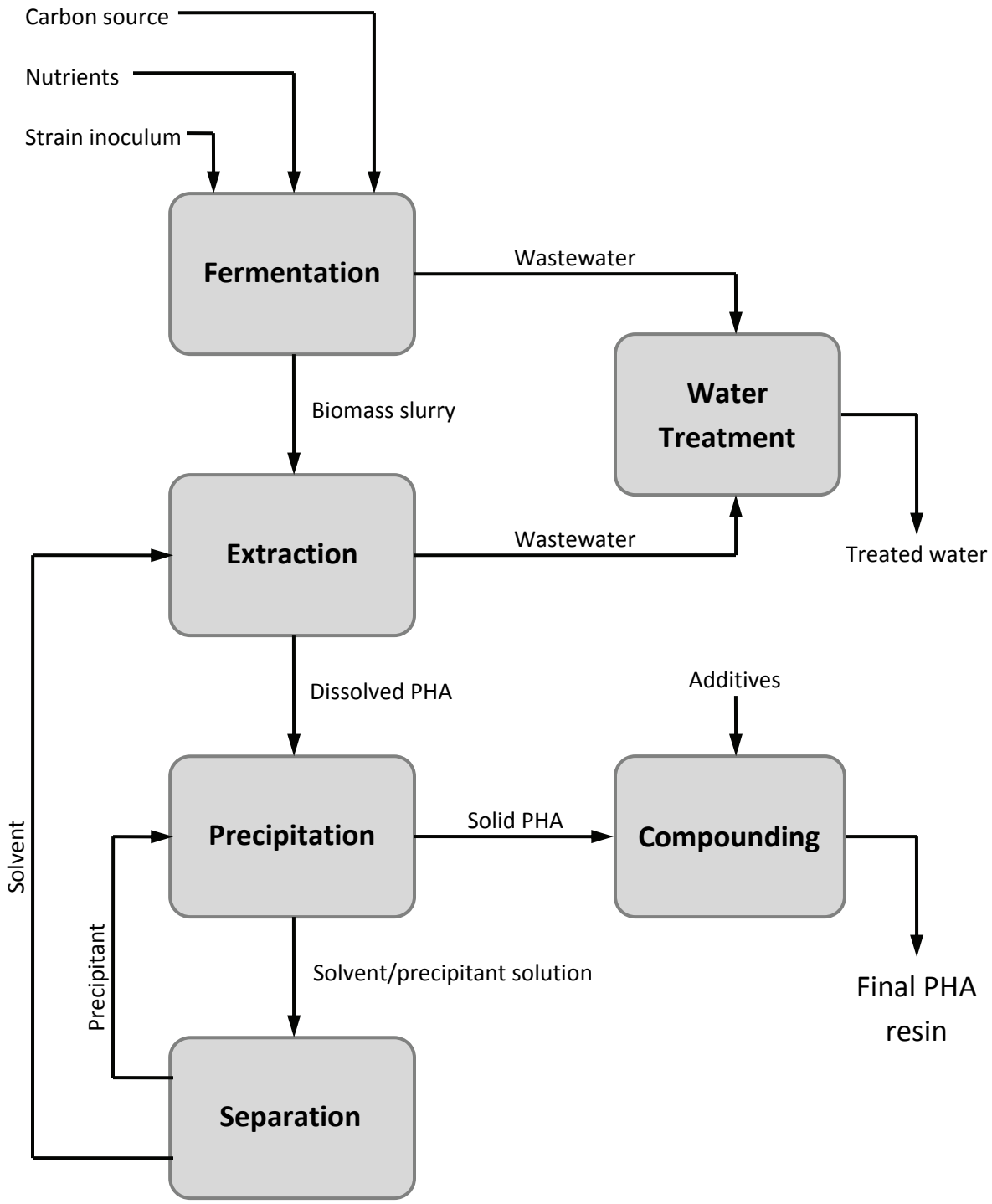

Fig 7.1: A simplified block diagram showing the steps in an industrial PHA production process utilizing a solvent based recovery process.

Materials and methods

Chemicals used in the study

The PHA used in this study was P(HB-*co*-HHx). This PHA was produced in a high density fermentation of Re2133/pCB81 (see Chapter 5) using palm oil as the carbon source. Polymer was initially extracted from the biomass with chloroform, and then precipitated by the four fold addition of cold methanol. GC-methanolysis analysis of this material revealed that it was 86% pure and that the polymer contained 39mol% HHx, while GPC showed that it had a peak molecular weight of 150,000 g/mol. Additional purification steps later increased the polymer to 92% purity, as discussed in the Results section. A variety of potential solvents and precipitants were used in the study, all of which were purchased from Sigma-Aldrich (St. Louis, MO) and had minimum purities of 99%. The solubilities of a number of lipids were also tested, including palm oil (Wilderness Family Naturals, Silver Bay, MN) and several fatty acids (Sigma-Aldrich).

Testing PHA solubility and precipitation

The solubility of PHA in various solvents was tested by adding solvent to solid PHA such that a 10% solution would be formed if all polymer went into solution. The solutions were stirred for 2 h at 50°C and then filtered through 0.2 µm polytetrafluoroethylene syringe filters into preweighed glass tubes. The solvent was removed by evaporation and the tubes were weighed to determine the amount of PHA that had dissolved. Precipitation of PHA from butyl acetate (BA) and methyl isobutyl ketone (MIBK) solutions was tested with hexane and heptane. The PHA was first dissolved by stirring at 70°C to form 5% solutions. 1 mL aliquots of the solutions were transferred to preweighed glass tubes and different volumes of hexane or heptane were added. Solid PHA that came out of solution was pelleted by centrifugation, the supernatant was removed, and the polymer was dried under vacuum. The tubes containing the PHA pellets were then weighed to determine the amount of polymer recovered. Similar experiments were carried out with palm oil, oleic acid (C18:1), palmitic acid (C16:0), and lauric acid (C12:0) in order to determine if these lipids would also come out of solution upon addition of precipitant. 5% solutions of the different lipids were prepared in BA and MIBK, three volumes of hexane were added, and the solutions were observed after incubation at room temperature and 4°C.

Process modeling

Simulations of PHA recovery processes were carried out with Aspen Plus (Aspen Technology, Burlington, MA) process modeling software. PHA recovery was modeled as a continuous process in which the input was 100 kg/h of a biomass slurry containing 30 wt% solids (conditions adapted from (17)). The solids were taken to be bacterial cells containing 65wt% high HHx PHA, which is similar to the results we achieved in Chapter 5. Sufficient solvent was added to the biomass to form a 4% PHA solution, and the PHA was recovered after addition of three volumes of precipitant. The Aspen model only included water, solvent, and precipitant as components. It was assumed that all PHA would be dissolved by the solvent and would be recovered after precipitation. Similarly, it was assumed that all residual biomass would be suspended in the wastewater after PHA extraction and that no residual palm oil would enter the system. The NRTL thermodynamic model was employed in all simulations.

Initial simulations were carried out in which the solvent and precipitant were not recycled, in order to test the performance of different solvents and precipitants. The process was identical to the one shown in Fig. 7.5, except without the splitters (units B7 and B8) and recycle streams. A simulation was then performed using BA as the solvent and n-hexane as the precipitant, in which the BA and hexane were separated by distillation and recycled. This process is shown in Fig. 7.5 and details can be found in Tables 7.4 and 7.5.

Results

We identified numerous solvents from the academic and patent literature that groups have proposed to use for PHA extraction (9, 12, 13, 17). We then gathered data on the physical properties and safety characteristics of these chemicals, in order to determine which had the most potential for use in a large scale industrial process (Table 7.1). Each property can influence the process in different ways. Compounds with lower viscosities will require less energy to pump (4), while lower specific heats mean less energy will be required to heat and cool streams. Solvents that are more hydrophobic (i.e. have lower solubility in water and higher log(K_{ow})) will be easier to separate from the water that enters the system in the biomass slurry. Solvent and precipitant combinations with large differences in boiling points will be easier to separate via distillation than pairs with similar boiling points. Chemicals with high Permissible Exposure Limits (PEL's) are better for worker safety. If the PHA manufactured in the process will be used for medical applications, it is desirable to use chemicals that are rated as Class 3 (least hazardous) by the FDA. It is obviously preferable to use low cost materials in a process, but due to the volatility of chemical prices we did not include pricing information in Table 7.1. There are clearly many points to consider when evaluating potential solvents and precipitants, but it must first be determined which combinations are useful for recovery of PHA.

The abilities of butyl acetate (BA), chloroform, ethyl acetate, isoamyl alcohol, methyl ethyl ketone (MEK), and methyl isobutyl ketone (MIBK) to dissolve PHA were examined (Fig. 7.2). The PHA used in this study contained 39mol% HHx and had 86% purity. The PHA was initially isolated from lyophilized biomass using the chloroform/methanol procedure. Recovering high purity PHA from this biomass was challenging, as the cells had low PHA content (<50%) and there was significant residual palm oil associated with the cells. To test the solubility of the PHA, solvent was added to the polymer such that 10% solutions would be formed if all the solid dissolved, the resulting solution was filtered, and the amount of polymer recovered was determined. We found that all solvents performed similarly, except for isoamyl alcohol, in which no dissolution was observed and filtration was impossible. BA and MEK showed lower average recovery than the other solvents, but the high variations suggest that this was due to experimental issues. The highest PHA recoveries observed were 95%. We believe the reason that 100% of the polymer could not be recovered was because some of the polymer solution was trapped in the syringe filters and could not be transferred.

Table 7.1: Property data for chemicals that could potentially be used in a PHA recovery process. The top group of compounds consists of potential PHA solvents, with water included as a reference. The bottom three compounds (hexane, heptane, and octane) are potential PHA precipitants.

Compound	CAS Number	Formula	Molar mass (g/mol)	Boiling Point (°C)[a]	Density (g/cm^3)	Viscosity (cP)	Kinematic Viscosity (cSt)
Water	7732-18-5	H_2O	18.02	100	1.03	0.911	0.884
Chloroform	67-66-3	$CHCl_3$	119.38	61	1.48	0.539	0.364
Acetone	67-64-1	C_3H_6O	58.08	56	0.79	0.308	0.390
Methyl acetate	79-20-9	$C_3H_6O_2$	74.08	57	0.93	0.353	0.380
Ethyl acetate	141-78-6	$C_4H_8O_2$	88.11	77	0.89	0.421	0.473
Butyl Acetate (BA)	123-86-4	$C_6H_{12}O_2$	116.16	126	0.88	0.677	0.769
Methyl isobutyl ketone (MIBK)	108-10-1	$C_6H_{12}O$	100.16	117	0.80	0.598	0.748
Methyl ethyl ketone (MEK)	78-93-3	C_4H_8O	72.1	80	0.81	0.396	0.489
Cyclohexanone	108-94-1	$C_6H_{10}O$	98.14	156	0.94	2.054	2.185
Isobutanol	78-83-1	$C_4H_{10}O$	74.12	108	0.80	3.269	4.086
Isoamyl alcohol	123-51-3	$C_5H_{12}O$	88.15	131	0.81	3.688	4.553
n-Hexane	110-54-3	C_6H_{14}	86.18	69	0.66	0.296	0.448
n-Heptane	142-82-5	C_7H_{16}	100.2	98	0.68	0.390	0.574
n-Octane	111-65-9	C_8H_{18}	114.23	126	0.70	0.511	0.730

Compound	Specific Heat ($J\ mol^{-1}\ K^{-1}$)	Specific Heat ($J\ g^{-1}\ K^{-1}$)	Solubility in water (ppmw)	$\log(K_{ow})$[b]	PEL (ppm)[c]	FDA Class[d]
Water	75.60	4.20	N.A.	N.A.	Safe	Safe
Chloroform	112.49	0.94	7.50e+03	1.97	50	2
Acetone	127.53	2.20	Miscible	-0.24	1,000	3
Methyl acetate	138.99	1.88	2.44e+05	0.18	200	3
Ethyl acetate	170.66	1.94	7.37e+04	0.73	400	3
Butyl Acetate (BA)	228.25	1.96	6.80e+03	1.78	150	3
Methyl isobutyl ketone (MIBK)	212.42	2.12	1.90e+04	1.31	100	3
Methyl ethyl ketone (MEK)	159.75	2.22	2.48e+05	0.29	200	3
Cyclohexanone	190.32	1.94	9.32e+04	0.81	50	N.D.
Isobutanol	163.33	2.20	8.10e+04	0.76	100	3
Isoamyl alcohol	164.90	1.87	2.70e+04	1.16	100	3
n-Hexane	192.63	2.24	1.33e+01	3.90	500	2
n-Heptane	230.42	2.30	2.24e+00	4.66	N.D.	3
n-Octane	254.71	2.23	4.31e-01	5.20	500	N.D.

[a]Physical property data is from Yaw's Chemical Properties Handbook, measured at 20 or 25°C and 1 atm.

[b]$\log(K_{ow})$ refers to the 1-octanol/water partition coefficient.

[c]PEL is the Permissible Exposure Limit established by the United States Occupational Safety and Health Administration (OSHA Standard 1910.1000 TABLE Z-1), N.D. indicates no data was available.

[d]The FDA rates chemicals for use in manufacturing of biomedical products, where class 1 is most toxic and class 3 is least toxic (http://www.fda.gov/RegulatoryInformation/Guidances/ucm128290.htm), N.D. indicates no data was available.

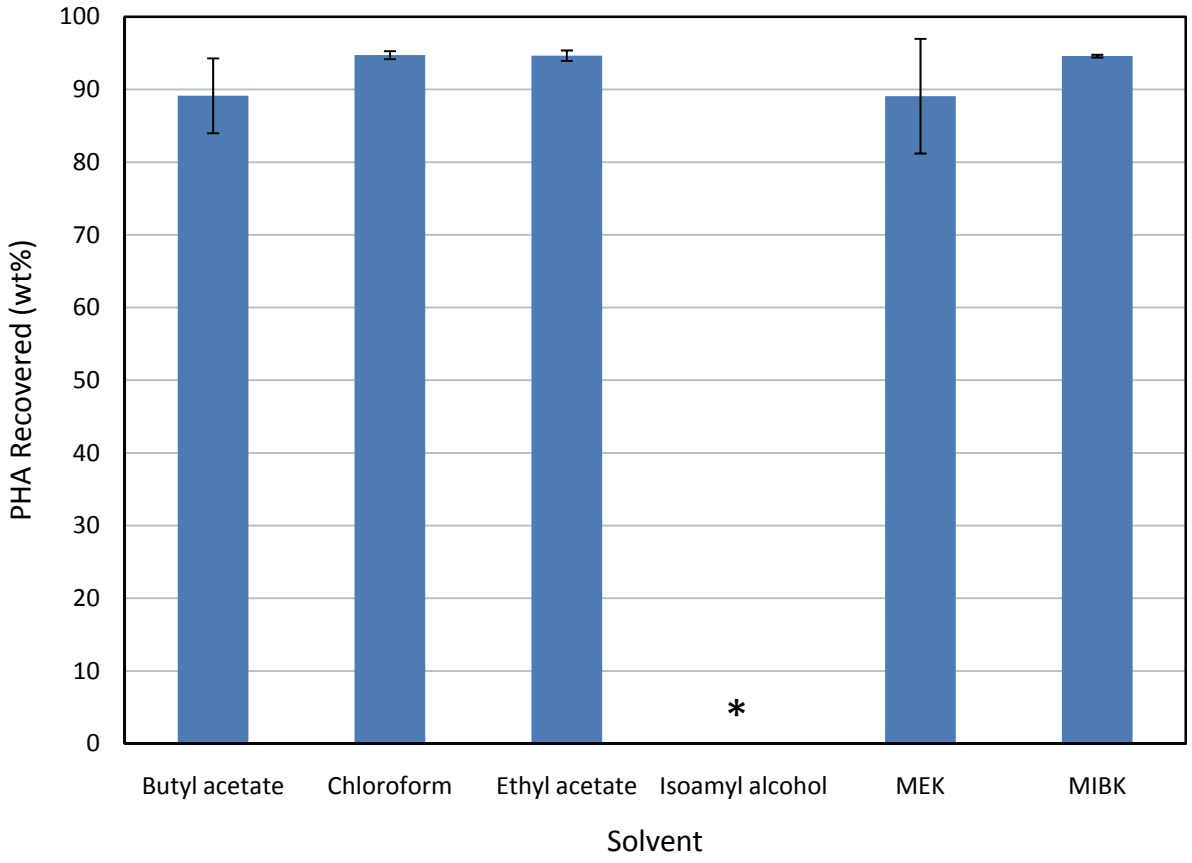

Fig. 7.2: Amounts of PHA recovered after dissolving polymer in various solvents, filtering, and removing the solvents by evaporation. Values are means of two replicates, with the maximum and minimum recoveries indicated by error bars. *The polymer did not dissolve in isoamyl alcohol.

We next tested precipitation of PHA out of solution. For these experiments we focused on BA and MIBK. While chloroform is the best known PHA solvent, halogenated compounds are severe environmental pollutants and prolonged exposure can seriously damage human health (European Union Commission Directive 2001/59/EC). MEK has been shown to dissolve several types of PHA copolymers (Dr. Daniel Yang, personal communication), but has high water solubility. BA and MIBK are both good PHA solvents that are hydrophobic and pose less of a health risk than chloroform. Before conducting the precipitation study, the 86% pure PHA used in the experiments in Fig. 7.2 was dissolved in MIBK and precipitated by the addition of four volumes of hexane. After this treatment, the purity of the PHA was increased to 92%. 5% solutions of this PHA were made in BA and MIBK, then the plastic was recovered after addition of different volumes of hexane or heptane (Fig. 7.3). (Octane was not tested because its boiling point is close to the boiling points of BA and MIBK.) The results in Fig. 7.3 show that the amount of PHA recovered increased as the volume of precipitant was increased from 0.5 fold to 3 fold. For the samples in which 3 or more volumes of precipitant were added, nearly all the PHA was recovered when either hexane or heptane was used. Interesting differences emerge when the 1 and 2 fold samples of the different solvent/precipitant combinations are compared. In both cases more polymer could be recovered from the BA solutions with a given precipitant, relative to the MIBK solutions. We also observed that heptane was a more effective precipitant than hexane with both solvents.

It is possible that there may be some residual palm oil and fatty acids associated with the biomass at the end of a high density fermentation. While economics dictate that almost all of the carbon source must be converted to plastic, it may be impossible to completely utilize the oil and fatty acids in a fermentation. These lipids could potentially co-purify with the PHA in a recovery process. To examine the behavior of palm oil and several fatty acids in a recovery process, we first made 5% solutions of palm oil, oleic acid, palmitic acid and lauric acid in BA and MIBK (a significantly higher concentration than one would expect to be present at the end of a fermentation). All lipids went into solution at room temperature, but palmitic acid precipitated when incubated at 4°C (Fig. 7.4A, B). We then added 3 volumes of hexane to the solutions. No lipid precipitation was observed at room temperature, but when the solutions were incubated at 4°C, palmitic acid once again came out of solution (Fig. 7.4C, D). These findings indicate that co-purification of oil and fatty acids with the PHA should not be a significant issue as long as residual oil/fatty acid concentrations are kept low and process temperatures are maintained at room temperature and above.

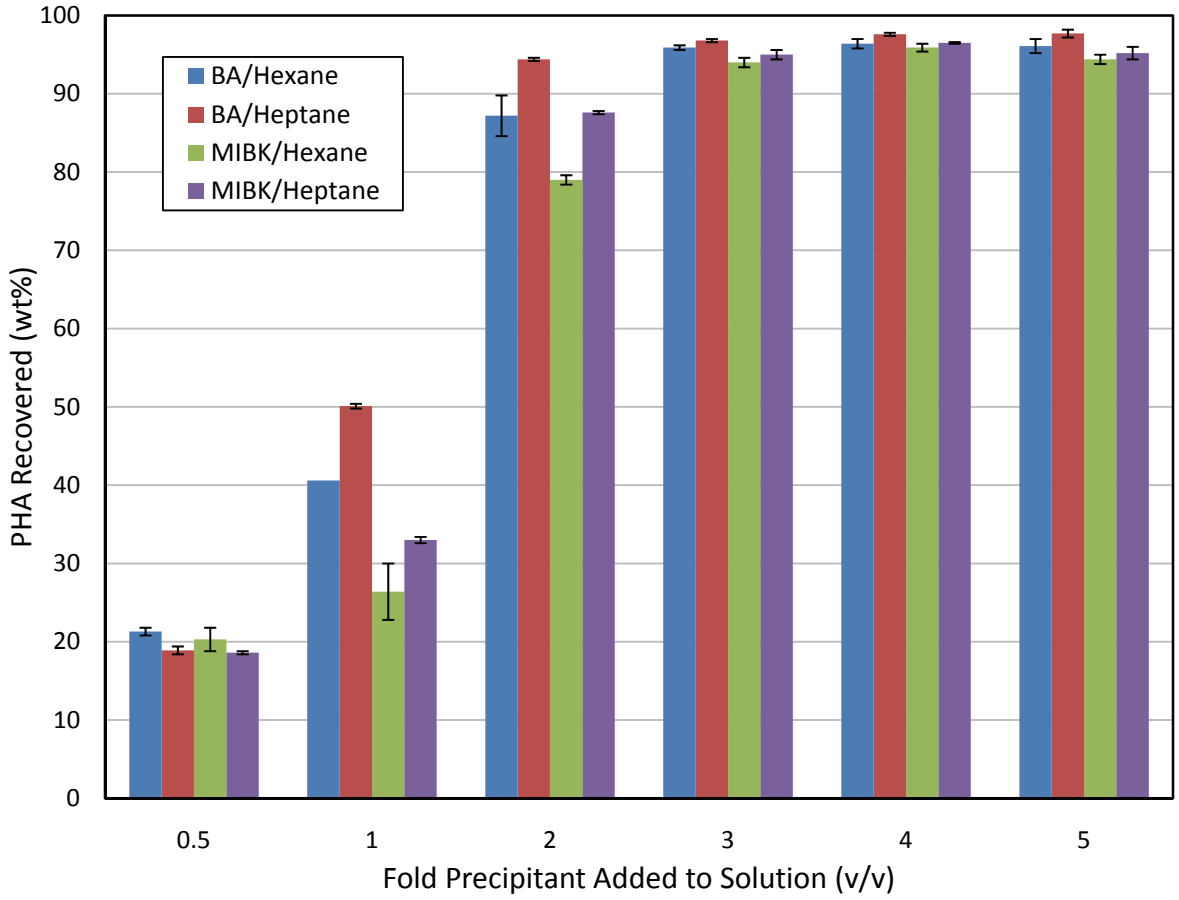

Fig. 7.3: The precipitants hexane and heptane were added to 5% PHA solutions made with BA and MIBK. The solid precipitant was collected and the amount of PHA recovered was determined. Values are means of two replicates, with the maximum and minimum recoveries indicated by error bars.

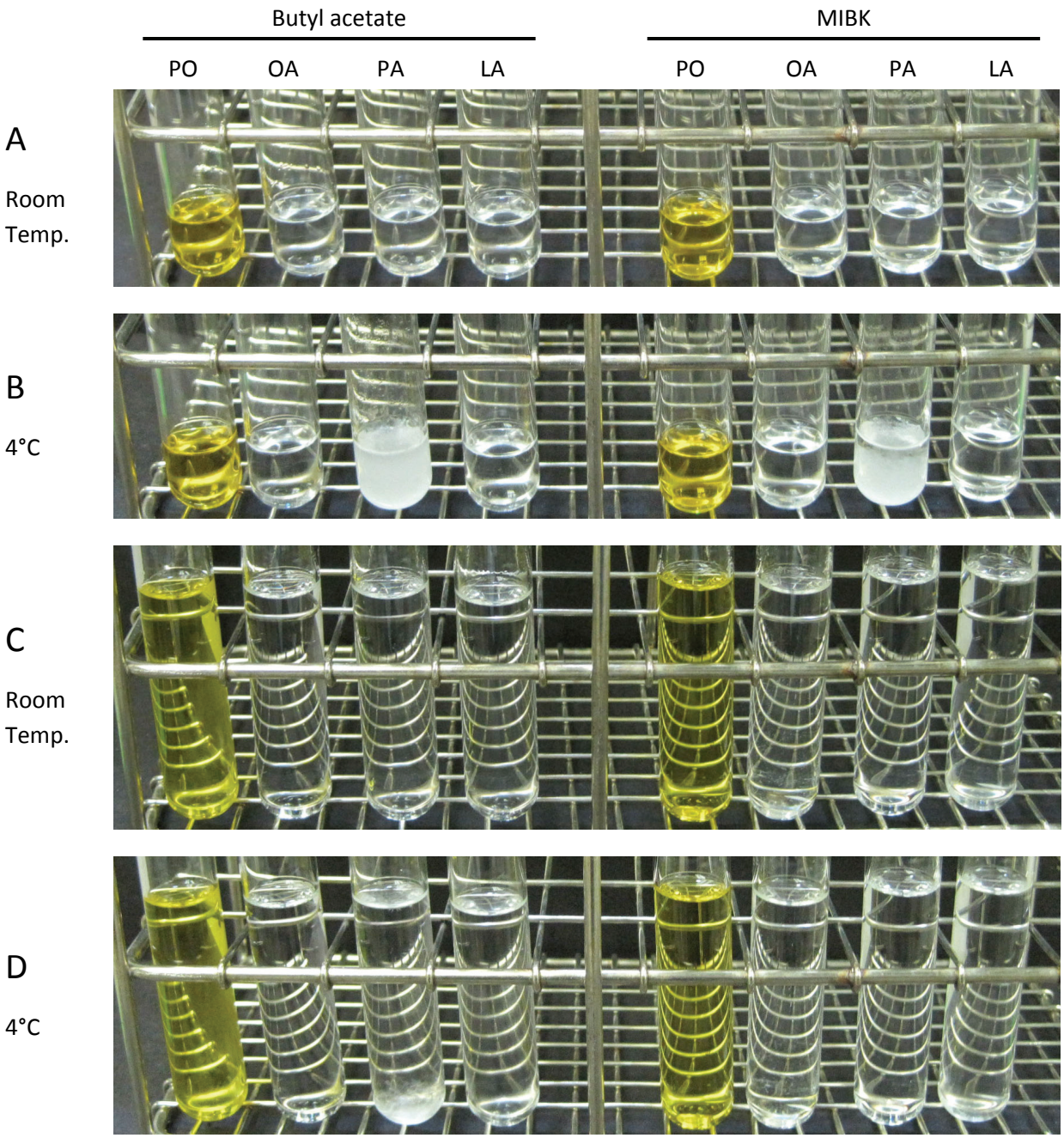

Fig. 7.4: 5% solutions of palm oil (PO), oleic acid (OA), palmitic acid (PA), and lauric acid (LA) were made in butyl acetate and MIBK. The solutions were made at room temperature (A) and then incubated overnight at 4°C (B). The solutions were then warmed back to room temperature and three volumes of hexane were added to check for precipitation. The new solutions were again incubated at room temperature (C) and overnight at 4°C (D). Palmitic acid precipitates from BA and MIBK at 4°C, and from BA/hexane at 4°C.

Process modeling results

Our proposed continuous recovery process uses a biomass slurry as the starting material. While many academic publications use lyophilized cells in PHA recovery studies, we believe that this is not a realistic scenario. Significant energy would be required to dry the biomass, either by evaporation or lyophilization. The process would also then require an additional solids handling operation, which is more difficult than working with a liquid. A consequence of starting with wet biomass, however, is that there will be water present in the system. It is desirable to remove as much of this water as quickly as possible after extracting the PHA from the biomass slurry. Our first simulations were therefore to determine how easily the solvents BA, MEK, and MIBK could be separated from water in a simple phase separation (i.e. decanting) unit operation. The input to this operation is water and residual biomass mixed with the organic PHA solution (B2 in Fig. 7.5). The results these simulations are given in Table 7.2. For BA and MIBK, almost 90% of the water can be removed by a decanting step, while only half the water is removed from the MEK stream. (Note that because solvent is added on a volumetric basis and the results in Table 7.2 are calculated on a mass basis, there are some unexpected results, such as more water being removed from the input with MIBK than BA.) Additionally, when MEK is used the resulting wastewater still has high organic content, which will raise downstream treatment costs. Based on these results, we concluded that MEK is not a suitable solvent for PHA recovery.

Table 7.2: Aspen results showing separation of water from various PHA solutions achieved by decanting.

Solvent	Water removed from input stream (wt%)	Organic content of wastewater (wt%)
BA	87%	0.6%
MIBK	89%	1.9%
MEK	46%	7.2%

We next modeled separation of solvent from precipitant via distillation (B6 in Fig. 7.5). Recycling materials in a solvent based recovery process is essential for operating an economically viable plant. We tested BA and MIBK as solvents with n-hexane and n-heptane as precipitants. A single feed, two product distillation column (DSTWU column) was used in Aspen to simulate the separations. The most desirable distillation procedure would be the one that requires the fewest stages (i.e. the smallest column) and has the lowest condenser and reboiler duties (i.e. lowest energy consumption). Results of the simulations are shown in Table 7.3. We found that for both solvents, distilling hexane requires less energy and a smaller column. If heptane is used as the precipitant, BA is easier to separate than MIBK. The easiest solvent/precipitant system to separate for recycling is BA/hexane.

Table 7.3: The energy and tray requirements for separating various solvent/precipitant combinations were determined with Aspen simulations. The feed to the column also contains a small amount of water that cannot be removed by decanting.

Solvent	Precipitant	Reboiler Heating (GJ/h)	Condenser Cooling (GJ/h)	Trays Required
MIBK	Hexane	0.66	-0.55	11.6
MIBK	Heptane	1.89	-1.73	19.8
BA	Hexane	0.63	-0.49	10.2
BA	Heptane	1.18	-0.97	18.8

After determining that BA/hexane was the most attractive combination for PHA recovery, we developed a process simulation that included recycling of chemicals (Fig. 7.5). The unit operations making up the process are described in Table 7.4 and stream information is given in Table 7.5. The feed was the same as used previously (100 kg/h biomass slurry, 30wt% solids containing 65wt% PHA), meaning that the facility would produce 19.5 kg/h PHA (170 metric tons/year, if run continuously). In the process, PHA is extracted from the biomass (represented by mixer B1) and water is removed by decanting (B2). Hexane is added to precipitate the PHA (B3). The hexane makes the solution more hydrophobic and allows additional water to be removed by decanting (B4). At this point the solid PHA also would be recovered, although a separate unit for this step has not been included in the model. The solvent and precipitant are separated by distillation (B6) and recycled, while the wastewater streams are combined for further treatment.

In the model we arbitrarily decided to recycle 99wt% of the streams coming out of the distillation tower and discarded the remaining 1wt%. (It is a general rule of process engineering that recycling 100% of a component should not be attempted, as contaminants will build up in the recycle stream over time if there is no discharge.) Input streams of BA and hexane were therefore necessary to replace the discharged material. The BA recycle stream (BA-RE1) is 444.8 kg/h and contains 97.8wt% BA. This stream is at 122°C exiting the distillation tower, therefore it had to be cooled with a heat exchanger before the PHA extraction step, to prevent vaporization of water when it was added to the biomass slurry. The hexane recycle stream is 966.0 kg/h and contains 99.3wt% hexane. Almost all the water that enters the process is collected in the wastewater output stream (H2O-OUT). This stream contains 0.5wt% BA and would also contain the residual biomass that was separated from the PHA. Therefore further treatment of the wastewater would be necessary before it could be discharged into the environment.

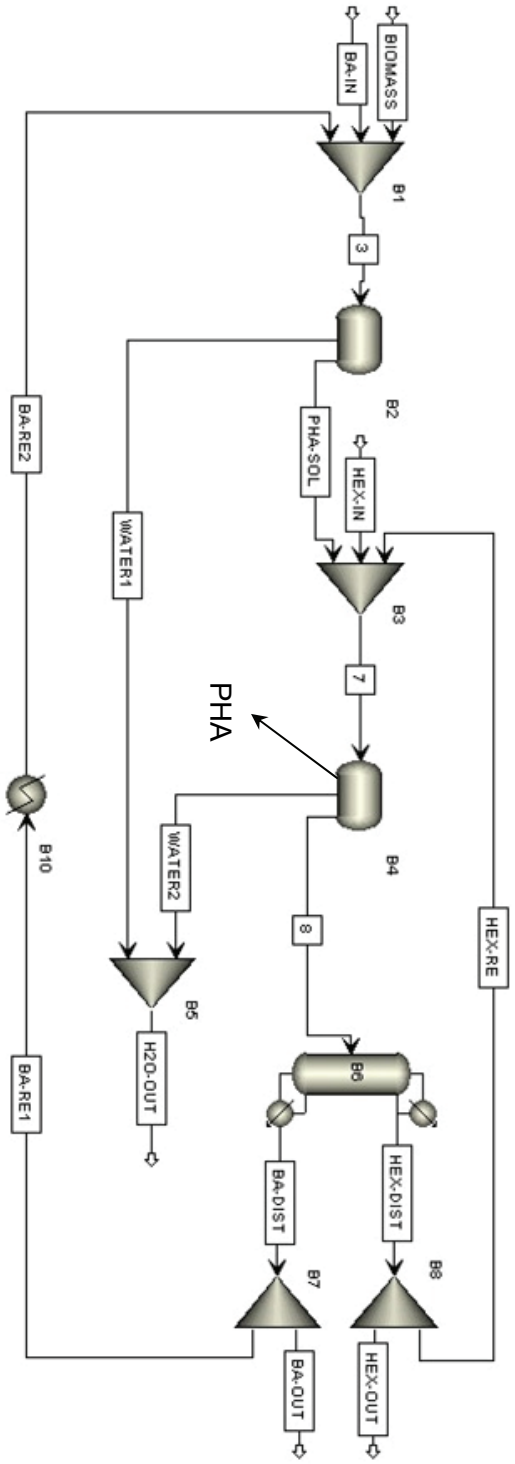

Fig. 7.5: A diagram of the Aspen model developed to simulate a PHA recovery process in which BA is the PHA solvent and hexane is the precipitant. The BA and hexane are separated by distillation and recycled to make the process more efficient. Solid PHA would be recovered after precipitation, although a specific unit for this operation is not shown in the model.

Table 7.4: Details of the unit operations used in the PHA recovery process shown in Fig. 7.5. The outputs (T, P, composition) of the mixers in the simulation (B1, B3, B5) are based on the combined input streams. Negative heat duties indicate that a unit is removing energy. Note that the duties of the distillation tower are slightly different from those in Table 7.3, because the addition of recycling to the process changes the input to the distillation tower.

Unit ID	B2	B4	B6	B7, B8	B10
Unit Type	Decanter	Decanter	DSTWU Distillation Tower	Splitters	Heat Exchanger
Inputs	P = 1 bar T_{out} = 30°C	P = 1 bar No heat in/out	Reflux ratio = 2 × minimum Reboiler P = 1 bar Condenser P = 1 bar Light key recovery = 99% Heavy key recovery = 1%	99wt% of input goes to recycle streams	P = 1 bar T_{out} = 60°C
Results	Duty = -23.2 MJ/h	T_{out} = 44.9°C	Reflux ratio = 0.40 Stages = 10.2 Feed stage = 5.6 Reboiler duty = 0.58 GJ/h Condenser duty = -0.50 GJ/h		Duty = -60.9 MJ/s

Table 7.5: Temperatures, flow rates, and compositions of the streams in the PHA recovery process shown in Fig. 7.5. All streams are at 1 bar and are completely liquid.

Stream Name	3	7	8	BA-DIST	BA-IN	BA-OUT
Temp (°C)	46.0	42.3	44.9	122.2	30.0	122.2
Mass Flow (kg/h)	519.6	1433.6	1425.0	449.3	4.8	4.5
Water (wt%)	13.5	0.7	0.1	0.0	0.0	0.0
BA (wt%)	84.7	31.0	31.2	97.8	100.0	97.8
Hexane (wt%)	1.9	68.3	68.7	2.2	0.0	2.2

Stream Name	BA-RE1	BA-RE2	BIOMASS	H2O-OUT	HEX-DIST	HEX-IN
Temp (°C)	122.2	60.0	30.0	31.8	49.1	30.0
Mass Flow (kg/h)	444.8	444.8	70.0	70.4	975.7	9.8
Water (wt%)	0.0	0.0	100.0	99.5	0.2	0.0
BA (wt%)	97.8	97.8	0.0	0.5	0.5	0.0
Hexane (wt%)	2.2	2.2	0.0	0.0	99.4	100.0

Stream Name	HEX-OUT	HEX-RE	PHA-SOL	WATER1	WATER2
Temp (°C)	48.6	48.6	30.0	30.0	44.9
Mass Flow (kg/h)	9.8	966.0	457.8	61.8	8.6
Water (wt%)	0.2	0.2	1.9	99.4	99.8
BA (wt%)	0.5	0.5	96.0	0.6	0.2
Hexane (wt%)	99.3	99.3	2.1	0.0	0.0

Discussion

In this study we designed a process for recovery of P(HB-*co*-HHx) from bacterial biomass. We found several solvents that were able to effectively dissolve our polymer (Fig. 7.2). We decided to focus primarily on BA and MIBK due to their low miscibilities with water (Tables 7.1 and 7.2). Both hexane and heptane could be used to precipitate PHA from BA and MIBK solutions when three or more volumes of precipitant were added (Fig. 7.3). Palm oil and fatty acids remained in solution with these precipitation conditions, as long as the solutions were maintained at room temperature or above (Fig. 7.4).

Initial Aspen simulations indicated that the combination of BA and hexane would be the easiest to separate so that the solvent and precipitant could be recycled. There are additional considerations that make BA more attractive than MIBK. BA is less flammable than MIBK, has a higher PEL, and is less expensive (as of November 2009). One potential issue with BA is that it can degrade by hydrolysis in the presence of water (16), which is clearly a concern given that the solvent would be continuously recycled. The rate of hydrolysis is increased in the presence of acid or base, which may be present in the recovery process if there is a pretreatment step before PHA extraction. It is also possible that the BA could degrade the PHA via a transesterification reaction. These issues should be examined before a commitment is made to use BA.

Additional recovery studies of P(HB-*co*-HHx) performed with polymer that had 17mol% HHx showed that MIBK could extract most of this PHA, while less than 60% of the polymer was recovered with BA (data not shown). We also found that MIBK cannot be used to effectively extract P(HB-*co*-HHx) with <10mol% HHx. Therefore the PHA being recovered must also be considered when choosing a solvent. BA is effective with high HHx (≥30mol%) material, while MIBK should be used with PHA that has lower HHx content.

We found that hexane was easier to separate from both BA and MIBK than heptane, and therefore decided it had more potential as a precipitant. Hexane is rated as a class 2 chemical by the FDA, while heptane is class 3, and is therefore considered safer for use in manufacturing biomedical products. This means that if the PHA being produced is destined for biomedical applications, heptane may be the preferred precipitant. One advantage of heptane is that because it is more hydrophobic than hexane, more water can be removed from stream 7 by decanting (unit B4).

The design illustrated in Fig. 7.5 is a continuous process. While there is great academic interest in continuous microbial cultures, most industrial fermentations are still done in batch. In order to run the recovery process continuously, the plant would have to be designed such that the biomass generated in the fermentations could be stored in a holding tank and continuously withdrawn. In order to prevent catabolism of the PHA during storage, the cells would need to be killed before transfer to the holding tank. This could be accomplished by a heat treatment or by adjusting the pH to acidic or basic conditions.

While the process shown in Fig. 7.5 provides a good starting point for design of a PHA recovery facility, additional details would need to be worked out before a plant could be built. One would first want to minimize the amounts of solvent and precipitant needed. When these chemicals are used in greater volumes, more energy is required for heating, cooling, and pumping. Our process calls for solvent to be added such that a 4% PHA solution is formed after all polymer is extracted. While there are several benefits to using less solvent, this will also result in a more concentrated, and therefore more viscous, PHA solution (17). Viscous solutions are more difficult to pump, which will limit the maximum viable concentration. The viscosity of polymer solutions is dependent on polymer type, polymer molecular weight, concentration, solvent type, and temperature (6), so additional experiments would be needed to determine how viscosity varies for a given PHA/solvent pair. One would similarly want to use the minimum amount of precipitant that causes all PHA to come out of solution. We found that a threefold addition of hexane causes all PHA to come out of BA and MIBK solutions (Fig. 7.3). Over 75% of the PHA precipitates with only a twofold addition of hexane, so the optimal amount of precipitant is somewhere between these two points. Another way to reduce energy costs is to determine how pure the recycled solvent and precipitant need to be. If more hexane in the recycled BA can be tolerated, less energy will be required for the distillation.

Additional steps could also be added to the process. As mentioned earlier, it has been proposed that the biomass should be treated before PHA extraction in order to disrupt the bacterial cells. This could be done in conjunction with the holding tank that is necessary to run the recovery process continuously. If a chemical pretreatment (e.g. acid or base) was used, studies would need to be performed to confirm that long term exposure to the treatment conditions did not degrade the polymer. Another step that may need to be added to the process is a wash step after the solid polymer has been recovered. This would be important if there was some residual palm oil associated with the PHA after it came out of

solution. The plastic could be washed with fresh precipitant to remove material on the surface of the PHA pellets.

Efficient heat integration would be necessary in the design of an economical process. In the current design most operations are performed at 30-50°C, with the distillation tower being the obvious exception. The one stream whose temperature was actively controlled was the BA recycle (see streams BA-RE1 and BA-RE2), so that the hot BA from the distillation tower would not cause vaporization of water when added to the biomass slurry. Running the PHA extraction at a higher temperature should speed the rate of polymer dissolution (it is currently at 46°C). The temperature could easily be controlled by adjusting the cooling of the BA recycle stream. The heat removed from the BA recycle stream could be used elsewhere in the process, for example it could preheat the stream going into the distillation tower (stream 8).

It is also crucial to determine what actual equipment would be used to carry out the different operations in the process. Extraction of PHA can be modeled as a liquid-liquid extraction, even though the PHA is actually a solid suspended in water. Efficient extraction of the PHA requires intimate contact between the organic solvent and the aqueous suspension. In the simplest implementation, this can be accomplished in a stirred tank, although this unit would inherently be limited to a single equilibrium stage and would require a decanter to separate the organic and aqueous phases. A variety of static and agitated extraction columns are available that improve contact between phases and allow for multiple stages (7). Another option is centrifugal extraction (7), which has been used in the patent literature to effectively extract PHA (17). An advantage of centrifugal extraction is that the two phases are separated in the operation after extraction has occurred. Whatever extraction system is chosen, it must be ensured that the presence of solids in the aqueous phase will not clog or otherwise disable the equipment.

PHA precipitation is likely to occur in a simple stirred tank. An appropriate residence time would have to be determined that would allow for all polymer to come out of solution. The solid PHA could then be separated from the solvent/precipitant mix by continuous centrifugation or filtration (8). One intriguing option is a three phase decanter centrifuge, whose outputs would be an aqueous stream, an organic stream, and solid PHA (8). After recovering the PHA it could be washed (if necessary) and dried by either a continuous press or a belt drier. Once the PHA has been removed from the system, the remaining equipment will only handle streams that are completely liquid, so these units can be designed using standard chemical engineering principles. When appropriate equipment has been chosen for each

operation, the design would first need to be validated in a pilot plant, then scaled up for an industrial process, in which recovery would be integrated with industrial scale fermentations.

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Chapter 8
Exploration of Polylactic Acid Synthesis by PHA Synthases

Introduction

Polyhydroxyalkanoates (PHAs) are not the only biodegradable plastics being commercialized. Another bioplastic that has received significant attention is poly(lactic acid) (PLA). PLA is biodegradable and biocompatible, and was initially studied for its potential in biomedical applications (12). PLA is currently manufactured at commercial scale by NatureWorks LLC, an independent company wholly owned by Cargill. NatureWorks was initially a joint venture between Cargill and Dow, and later became a joint venture between Cargill and Teijin, before Cargill assumed sole ownership. In the NatureWorks process, lactic acid is produced by fermentation of corn sugar. The lactic acid is then converted to cyclic lactides and finally polymerized to PLA by a tin catalyzed, ring opening polymerization reaction (5, 25). A key to adjusting the properties of PLA is controlling the levels of (*R*) and (*S*) monomers in the polymer. Introducing the alternate monomer into a PLA chain (i.e. (*R*)-lactate into (*S*)-PLA or (*S*)-lactate into (*R*)-PLA) decreases the melting temperature, the rate of crystallization, and the final extent of crystallization, which helps reduce the brittleness of the plastic (5). A critical finding was that certain PLA compositions produce material that is completely amorphous, and therefore transparent (5). This opens a range of applications for PLA and represents a crucial difference with PHAs, which are semi-crystalline plastics that cannot be made fully transparent.

A number of studies have been conducted investigating blends of PHA and PLA. Making miscible blends of two polymers is a method used to create alloys with properties intermediate to the two pure compounds. Blending PLA and PHA is an attractive way to produce plastics with unique properties, while using only biobased and biodegradable materials. Thermodynamic calculations predicted that polyhydroxybutyrate (PHB) should be miscible with low molecular weight PLA (M_n ~ 1,800), but immiscible with higher molecular weight PLA (2). Immiscible polymers phase separate when blended, but proper dispersion of the two phases can still lead to improved properties, as is the case with polybutadiene toughened polystyrene (also known as high impact polystyrene). While immiscible blends of P(HB-*co*-HV) and (*S*)-PLA did not have desirable mechanical properties, this can be attributed to the brittle nature of both polymers (7). Later studies conducted with P(HB-*co*-HHx) and (*S*)-PLA showed that blending 10 to 20% PHA with PLA dramatically improved the tensile energy to break and

Izod impact strength relative to pure PLA (17, 20). Conflicting data was presented in the two studies concerning the long term transparency of these blends.

We were interested in enzymatically synthesizing PLA and P(HB-*co*-LA) using a PHA synthase. Making these polymers enzymatically is appealing for a number of reasons. Enzymatic synthesis of PLA could represent a new process for PLA production in which sugar is converted directly into polymer in a single fermentation. Introducing LA units into a PHB chain could improve the polymer properties by disrupting crystallization, as has been observed for a number of other monomers (14, 16). P(HB-*co*-LA) could also potentially serve as a compatibilizer for preparing blends of PHA and PLA (20).

Two *in vitro* studies suggested that the class III synthase from *Allochromatium vinosum* had activity towards (*R*)-lactyl-CoA, based on release of CoA when $PhaEC_{Av}$ was incubated with LA-CoA (24, 29). No formation of PLA was detected in these studies. (Note that throughout this chapter the abbreviation LA-CoA refers to (*R*)-lactyl-CoA, unless the (*S*) form is specifically mentioned.) We envisioned constructing a recombinant *Escherichia coli* strain in which pyruvate would be converted to lactate by a lactate dehydrogenase (Ldh), the lactate would be converted to LA-CoA by a propionate CoA transferase (Pct), and finally the LA-CoA would be polymerized by PhaEC, as illustrated in Fig. 8.1. Ldh enzymes catalyze formation of either (*R*) or (*S*)-lactate from pyruvate and typically use NADH as the electron donor (6). The *E. coli* fermentative Ldh produces (*R*)-lactate and is encoded by the gene *ldhA* (Gene ID: 946315). It has been shown that *E. coli* can be engineered to make substantial amounts of (*R*)-lactate from sugar (30). Pct enzymes are thought to produce LA-CoA during fermentation of alanine, although the enzymes accept a number of different CoA donors and monocarboxylate substrates (21, 22). Thus far Pct's from *Clostridium propionicum* (21, 22) and *Megasphaera elsdenii* (4) have been described in the literature. In our proposed *E. coli* strain, acetyl-CoA (as the most abundant intracellular CoA thioester) would serve as the CoA donor for synthesis of LA-CoA. (*R*)-3-hydroxybutyryl-CoA (HB-CoA) could be synthesized from acetyl-CoA by also expressing *phaA* and *phaB* from *R. eutropha* in the strain.

As we began to develop our strain, groups led by Seiichi Taguchi (23, 27) and Sang Yup Lee (11, 28) published reports of PLA synthesis using schemes similar to the one we proposed, leading us to halt work on this project. Their findings are summarized in the Discussion section of this chapter.

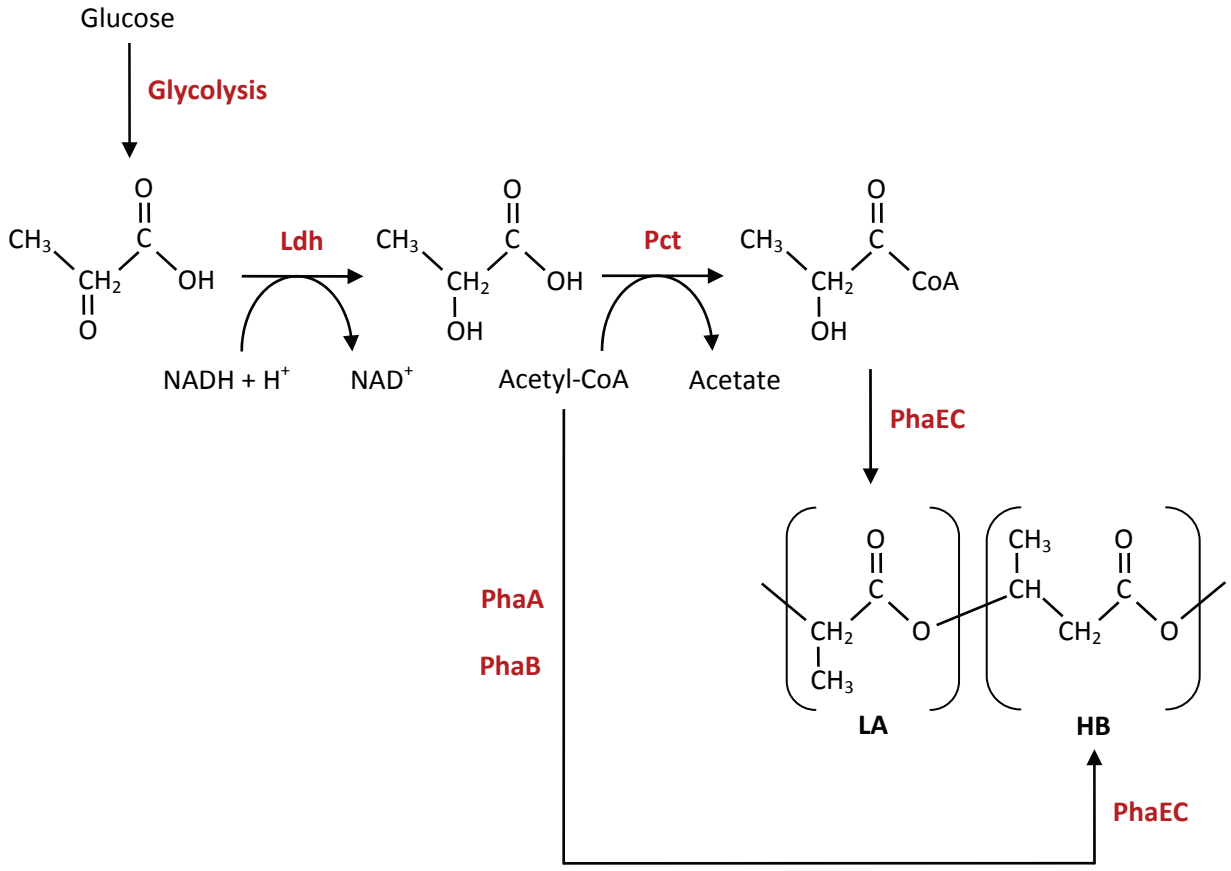

Fig. 8.1: The proposed schemes for synthesizing PLA and P(HB-*co*-LA) from sugar in engineered *E. coli*. Enzymes and pathways are shown in red. Acetyl-CoA can be converted to HB-CoA by PhaA and PhaB, and then polymerized by PhaEC. Both chiral centers in P(HB-*co*-LA) would be the (*R*) forms.

Materials and methods

Bacterial strains and growth conditions

The *E. coli* strains and plasmids used in this study are described in Table 8.1. DH5α was used for DNA cloning and plasmid preparations. Tuner(DE3) was used for recombinant protein expression. This strain is based on the common lab strain BL21(DE3), which allows for protein expression from plasmids carrying the T7 promoter when induced with isopropyl-1-thio-β-D-galactopyranoside (IPTG). A limitation of this strain is that low levels of uniform induction are impossible, because uneven expression of the *lacY* permease leads to cells taking up different amounts of IPTG (18). The Tuner(DE3) strain has a Δ*lacYZ* mutation, which ensures that IPTG enters all cells in a population uniformly, in a concentration dependent manner. We transformed Tuner(DE3) with the plasmid pRARE2 (Novagen), which supplies tRNAs for seven codons that are rare in *E. coli*. It is known that this plasmid is necessary for high level recombinant expression of PCT (Thorsten Selmer, personal communication). We refer to Tuner(DE3) harboring pRARE2 as the TunerRARE strain, or TR. *E. coli* strains were routinely grown aerobically at 37°C in lysogeny broth (LB) (19). Antibiotics were added to LB in the following concentrations, as necessary: ampicillin, 100 μg/mL; chloramphenicol, 34 μg/mL; kanamycin, 50 μg/mL.

Induction of recombinant protein expression in TR carrying various plasmids was carried out by first growing the strains in LB with appropriate antibiotics and monitoring the optical density at 600 nm (OD_{600}). Once the OD_{600} reached ~0.5, IPTG was added to a final concentration of 0.5 mM. Cells were generally harvested 3 h after induction, pelleted, and either lysed immediately or stored at -80°C.

Plasmid and strain construction

The *E. coli ldhA* gene and the *C. propionicum pct* gene were cloned into the pETDuet-1 vector individually and in combination for expression in *E. coli*. The *ldhA* gene was amplified from *E. coli* DH5α by colony PCR using primers that added an NdeI site to the 5' end of the gene and an XhoI site to the 3' end (see Table 8.2). The *pct* gene was amplified from plasmid pET-*pct* (provided by Dr. Thorsten Selmer) by PCR using primers that added an NcoI site to the 5' end of the gene and an EcoRI site to the 3' end. Note that adding the NcoI site to *pct* changed the beginning of the gene, such that it encoded MARK… instead of MRK… After amplification, the resulting DNA fragments were TOPO cloned, sequenced, and cloned into pETDuet-1 using the restriction enzymes indicated above (see Table 8.1). A vector for

expressing Strep-tagged Pct was constructed by amplifying the gene by PCR with primers that added KpnI sites to both ends of the gene. The DNA fragment was TOPO cloned, sequenced, cloned into the pET-51b(+) vector at the KpnI site, and the orientation of the gene was determined by a diagnostic HindIII digest. DNA was amplified and manipulated using high fidelity Taq polymerase (Qiagen) and restriction enzymes from New England Biolabs following standard protocols (19). All plasmids for recombinant protein expression were transformed into TR by electroporation.

Table 8.1: Strains and plasmids used in this study.

Strain or plasmid	Description[a]	Reference or source
E. coli strains		
DH5α	General cloning strain	Invitrogen
Tuner(DE3)	Strain for tunable gene expression from pET vectors	Novagen
TunerRARE	Tuner(DE3) harboring pRARE2, referred to as TR	Novagen
Plasmids		
pRARE2	Provides tRNAs for seven rare codons in *E. coli*, confers Cm resistance	Novagen
pCR2.1-TOPO	Vector for cloning and sequencing PCR products	Invitrogen
pET-*pct*	Plasmid containing *C. propionicum pct*	Dr. Thorsten Selmer
pETDuet-1	Vector for IPTG inducible expression of two proteins, confers Ap resistance	Novagen
pET-51b(+)	Vector for expressing proteins with N terminal Strep-tag II, confers Ap resistance	Novagen
pCB32	pETDuet-1 containing *E. coli ldhA*	This study
pCB33	pETDuet-1 containing *C. propionicum pct*	This study
pCB34	pETDuet-1 containing *E. coli ldhA* and *C. propionicum pct*	This study
pCB46	pET-51b(+) containing *C. propionicum pct* with N terminal Strep-tag II	This study
pAGL28	Plasmid for constitutive expression of *A. vinosum phaEC* in *E. coli*, confers Km resistance	Dr. Adam Lawrence

[a]Abbreviations: Cm, chloramphenicol; Ap, ampicillin; Km, kanamycin

Table 8.2: Oligonucleotide primers used in this study. The genes cloned and the intended vectors are described in the primer names. All sequences are written in the 5' to 3' direction.

Name	Sequence	Comment
ldhA pETDuet F	GGCATATGAAACTCGCCGTTTATAGCAC	NdeI site underlined
ldhA pETDuet R	GGCTCGAGTTAAACCAGTTCGTTCGGGCA	XhoI site underlined
pct pETDuet F	GGCCATGGCAAGAAAGGTTCCCATTATTACCGCA	NcoI site underlined
pct pETDuet R	GGGAATTCTCAGGACTTCATTTCCTTCAGACC	EcoRI site underlined
pct pET51b F	GAGGTACCGAGAAAGGTTCCCATTATTA	KpnI site underlined
pct pET51b R	GAGGTACCTCAGGACTTCATTTCCTTCA	KpnI site underlined

Synthesis of LA-CoA and HB-CoA

CoA thioester derivatives of (*R*)-lactate and (*R*)-3HB were synthesized enzymatically following the method of Dalluge *et al*. (4). Reactions were carried out at the 10 mL scale and contained 50 mM potassium phosphate buffer (pH 7), 1 mM acetyl-CoA, 100 mM of the organic acid sodium salt, and 4.5 U/mL of Pct. The source of the Pct was either purified enzyme or the soluble lysate fraction of TR/pCB33 that was grown and induced as described earlier, then lysed by sonication in an ice water bath. The reaction was started by addition of Pct, incubated 5 minutes at 25°C, then immediately stopped by transferring the test tube to an ice water bath and adding 0.1% trifluoroacetic acid (TFA). CoA compounds were purified at 4°C using Sep-Pak C18 cartridges (Waters, 6 mL volume, 1 g stationary phase, part no. WAT036905). The column was initially soaked with methanol, and was then equilibrated with 3 column volumes of 0.1% TFA in water. The reaction mixture was loaded onto the column, then washed with 40 mL 0.1% TFA, which removed free organic acids. CoA compounds were eluted using 0.1% TFA in a water/acetonitrile solution (1:1 by volume). The acetonitrile was removed with a rotary evaporator, and the resulting solution was frozen at -80°C and lyophilized. The dried products were stored at -20°C.

The products of the reactions were analyzed by HPLC (Waters 515 HPLC Pumps, Waters 2487 Dual Absorbance Detector). An Econosil C18 column was used for the analysis (Alltech, 10µm, 250 x 4.6mm, Part No. 60147). Solvent A was 25 mM potassium phosphate buffer (pH 4.7) and solvent B was pure methanol. The method started with 0% B and increased to 10% B over the first 10 min, then increased to 30% B over the next 30 min, then increased to 90% B over the next 10 min, then returned to 0% B over the next 10 min, and then remained at 0% B for the last 5 min of the run (total of 65 min per run, all

changes in mobile phase composition occurred over linear gradients). The flow rate remained constant at 1 mL/min throughout the method. Species eluting from the column were detected by their absorbances at 210 and 260 nm. Free organic acids eluted during the first 10 min of the run, if they were not removed earlier by Sep-Pak purification. The observed retention times for the CoA compounds of interest were free CoA, 18.5 min; LA-CoA, 21.7 min; acetyl-CoA, 23.7 min; HB-CoA, 25.1 min. Peaks were identified using pure standards of the CoA compounds. Free CoA and acetyl-CoA were purchased from Sigma Aldrich, while HB-CoA and LA-CoA were chemically synthesized by Dr. Ping Li.

Enzymatic assays and protein purification

PHA synthase activities of *R. eutropha* PhaC (9) and *A. vinosum* PhaEC (8) with LA-CoA as the substrate were measured by discontinuous assay procedures that have been previously described. The primary difference between the two assays is that the PhaC reaction contains the detergent Hecameg (9). The PhaC used in this study was a Strep-tagged version isolated from *R. eutropha* grown in tryptic soy broth ($PhaC_{Re}$, provided by Dr. Chris Brigham). The PhaEC featured a His-tag at the N terminus of PhaE and a 27 amino acid truncation at the C terminus of PhaE, and was purified from recombinant *E. coli* ($PhaEC_{Av}$, provided by Rachael Buckley). The mutations to PhaE do not affect synthase activity (Rachael Buckley, personal communication). We used 1.7 mM LA-CoA in all assays, which is five times the reported K_m (29). Due to the low activities of the synthases with LA-CoA as substrate, the enzyme concentrations used in theses assays were significantly higher than when HB-CoA is provided as substrate. The enzyme concentrations used in each experiment are noted in the Results section.

Ldh activity was measured using the assay described in the Sigma Aldrich product literature. The reaction mixture contained 94 mM potassium phosphate buffer (pH 7), 0.2 mM NADH, and 0.75 mM sodium pyruvate. The reaction was carried out at 25°C and started by addition of enzyme. The disappearance of NADH was monitored continuously at 340 nm. The extinction coefficient of NADH at this wavelength is 6.22 mM^{-1} cm^{-1}. One unit of activity was defined as the amount of enzyme needed to reduce 1 µmol of pyruvate per minute at 25°C.

Pct activity was measured using the coupled assay developed by Schweiger and Buckel (3, 21). The reaction mixture contained 100 mM potassium phosphate buffer (pH 7), 200 mM sodium acetate, 1 mM oxaloacetate, 1 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 20 µg/mL citrate synthase (Sigma Aldrich), and 0.1 mM propionyl-CoA. In the assay, CoA is transferred from propionyl-CoA to acetate by

Pct, the resulting acetyl-CoA reacts with oxaloacetate to form citrate and free CoA (via the citrate synthase), and the free CoA then cleaves the disulfide bond in DTNB. Cleavage of DTNB was monitored continuously at 412 nm. The amount of CoA released was calculated using an extinction coefficient at 412 nm of 13.6 mM^{-1} cm^{-1} for TNB.

Pct with an N terminal Strep-tag II was purified using a Strep-Tactin column (IBA GmbH, Göttingen, Germany). TR/pCB46 was grown and induced as described above. The culture was grown at 30°C instead of 37°C, which seemed to improve Pct activity, although this observation needs to be confirmed. Buffers used in the purification were buffer W (100 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 8), buffer E (buffer W with 2.5 mM desthiobiotin), and buffer R (buffer W with 1 mM hydroxy-azophenyl-benzoic acid). 4.5 g wet cell mass was resuspended in 23 mL buffer W and two protease inhibitor tablets (Roche) were added. Cells were lysed by two passes through a French press at 14,000 psi. Cell debris was removed by centrifuging the lysate in an ultracentrifuge at 100,000×*g* for 30 min. Insoluble particulates were removed from the resulting supernatant by filtering through a 0.45 µm low protein binding Supor filter (Pall). The soluble lysate was then loaded onto a 10 mL Strep-Tactin column, washed with 50 mL buffer W, and eluted in eight 5 mL fractions with buffer E. Fractions with significant Pct (determined by a SDS-PAGE gel) were pooled (30 mL total) and dialyzed against 4 L of storage buffer (25 mM KPi, 1 mM DTT, 1 mM EDTA, 1mM $MgCl_2$, pH 7). The dialyzed material was then concentrated to 20 mg/mL using an Amicon spin concentrator (30 kDa molecular weight cut off). Concentrated Pct was aliquoted and stored at -80°C. After the purification the column was regenerated using buffer R.

Absorbance measurements for all enzymatic assays were made using an Agilent 8453 spectrophotometer. Protein concentrations for PHA synthases were found by measuring the absorbance at 280 nm and using previously determined extinction coefficients (8, 9), while all other protein concentrations were determined using a modified Bradford assay (31).

CoA cycling reaction

CoA cycling is a technique that can be used to polymerize multiple monomer units with a PHA synthase *in vitro*, while using only a catalytic amount of CoA (10). When the CoA thioester of an organic acid is used as the substrate in a polymerization reaction, free CoA builds up in solution as the reaction proceeds, potentially inhibiting the reaction. The CoA cycling strategy avoids build up of free CoA, while also eliminating the need to separately synthesize substrate. In the cycling reaction, CoA reacts with

acetate via an acetyl-CoA synthetase, the CoA is transferred to an organic acid ((*R*)-lactate or (*R*)-3HB) by Pct, and the resulting CoA thioester is polymerized by a PHA synthase, thereby releasing the CoA (10). The CoA cycling reaction included 100 mM PIPES buffer (pH 7.5), 20 mM $MgCl_2$, 5 mM sodium acetate, 30 mM (*R*)-lactate, 20 mM ATP, 0.5 mM CoA, 4.6 U/mL acetyl-CoA synthetase, 56 U/mL Pct, and 10 µM $PhaEC_{Av}$. The positive control reaction used 30 mM (*R*)-3HB in place of (*R*)-lactate and only 1 µM PhaEC, while the negative control reaction did not include any PHA synthase. The acetyl-CoA synthetase (from Sigma Aldrich) uses ATP over the course of the experiment, so the ATP concentration can be used to monitor the progress of the reaction. The reactions were incubated at 30°C for 20 h and samples were periodically taken and stored at -80°C for later ATP determination. ATP concentrations were measured using the ATPlite kit (Perkin Elmer) following the manufacturer's instructions. A FLUOstar OPTIMA plate reader (BMG LabTech) was used to measure luminescence in the ATPlite assay.

Results

PHA synthase catalyzed CoA release from LA-CoA

Although other groups have reported PHA synthase activity with LA-CoA (24, 29), our first goal was to determine if these results could be replicated. CoA release from LA-CoA was monitored using a discontinuous assay with purified $PhaC_{Re}$ (Fig. 8.2A) and purified $PhaEC_{Av}$ (Fig 8.2B, C). Greater CoA release was clearly observed in the presence of synthase than in the no synthase controls. Furthermore, additional CoA release occurred with higher enzyme concentrations (Fig. 8.2C), indicating that the observed reaction is indeed dependent on the synthase. Based on the data in Fig. 8.2, we calculated the specific activity of $PhaC_{Re}$ with LA-CoA to be 0.05 U/mg (0.3% of the activity with HB-CoA) and the specific activity of $PhaEC_{Av}$ to be 0.04 U/mg (0.03% of the activity with HB-CoA). Complete utilization of LA-CoA was never observed, even when the reactions were allowed to proceed for over an hour. Approximately 80 molecules of substrate were reacted per molecule of $PhaC_{Re}$ in 120 min (Fig. 8.2A), while 96 molecules of substrate were reacted per $PhaEC_{Av}$ in 60 min (Fig 8.2B). This is significantly different than when HB-CoA is provided as the substrate, in which case turnover of >10,000 substrate molecules per synthase in a 5 min reaction has been reported (8). Our data indicates that an enzymatically catalyzed reaction of LA-CoA occurred in the presence of two different PHA synthases, but unfortunately insufficient material was generated for characterization of the resulting product.

Recombinant expression of *pct* and *ldhA* in *E. coli* and Pct purification

Formation of PLA *in vivo* would first require synthesis of LA-CoA. We therefore set out to express *ldhA* and *pct* in *E. coli*. Plasmids were constructed for IPTG inducible expression of *ldhA* (pCB32) and *pct* (pCB33). An additional plasmid was also made that would allow for expression of both genes simultaneously (pCB34). Expression gels showed that all desired proteins were produced at high levels using these plasmids (Fig. 8.3). Note that LdhA has a molecular weight of 36.5 kDa and Pct has a molecular weight of 56.5 kDa. LdhA and Pct specific activities were measured using the soluble fractions of crude lysates from the induced strains to confirm that active protein was made (Table 8.3). The specific activities of the over expressed proteins were significantly higher in the induced strains than in control strains harboring empty vectors, which had nearly undetectable levels of activity. Interestingly, the LdhA and Pct activities measured for TR/pCB34 were less than half the activities found

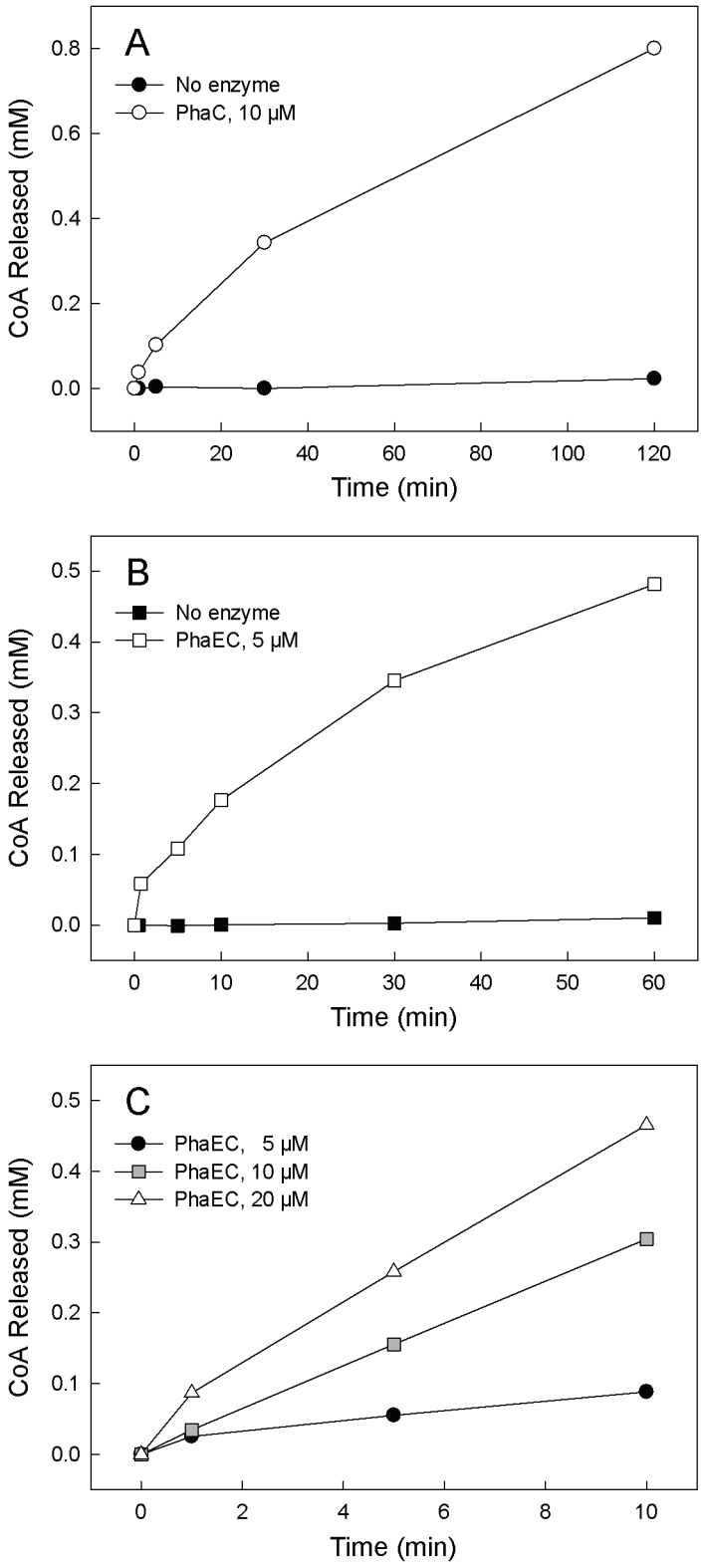

Fig. 8.2: Greater CoA was released when LA-CoA was incubated with $PhaC_{Re}$ (A) and $PhaEC_{Av}$ (B) than in no synthase controls. Higher synthase concentrations led to more CoA release (C).

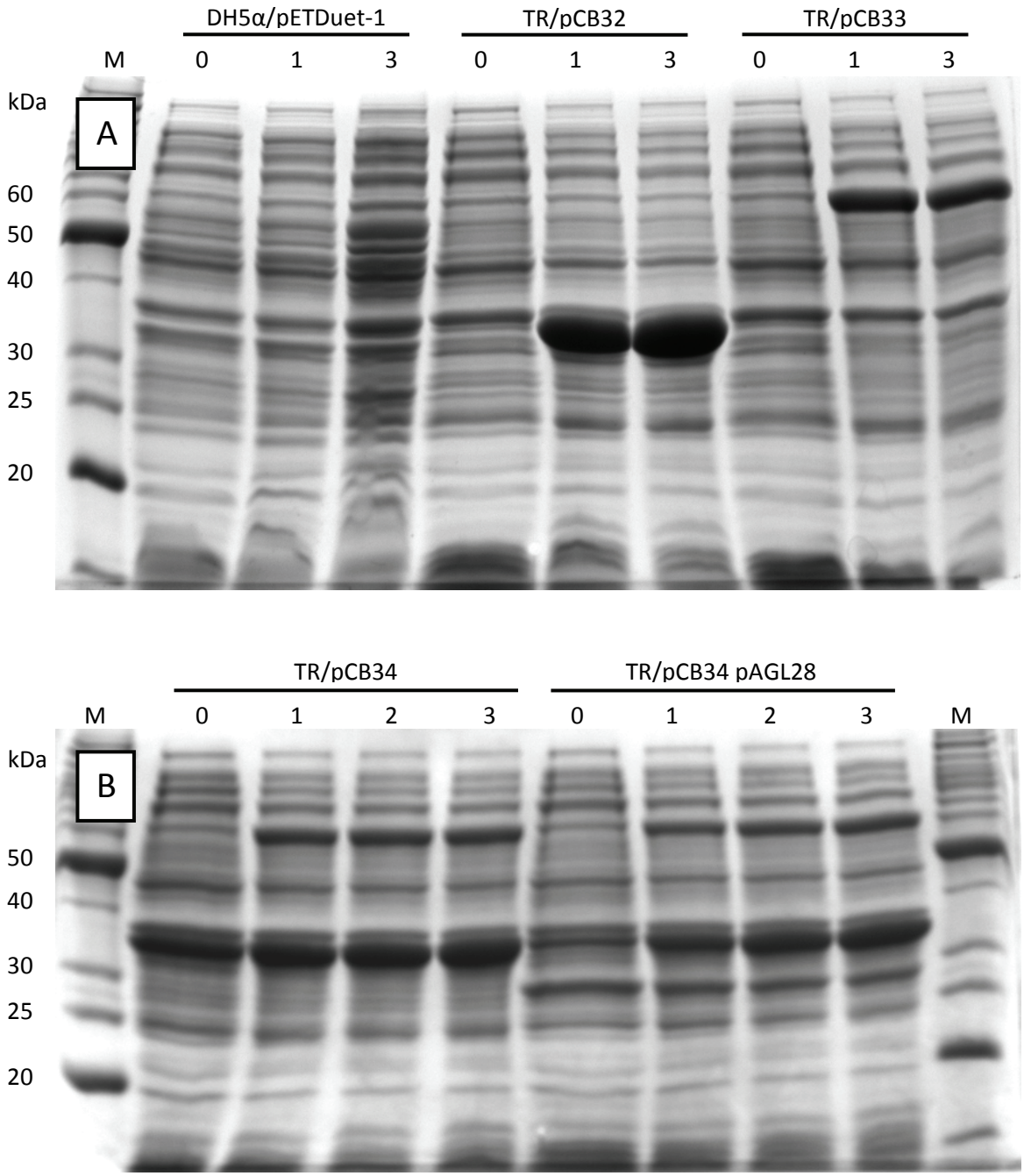

Fig. 8.3: Whole cell SDS-PAGE protein gels showing expression of LdhA (36.5 kDa) and Pct (56.5 kDa). Proteins were expressed individually (A) and in combination (B). The numbers above each lane indicate the number of hours after IPTG induction a sample was taken, while M is the molecular weight marker. DH5α/pETDuet-1 is a negative control.

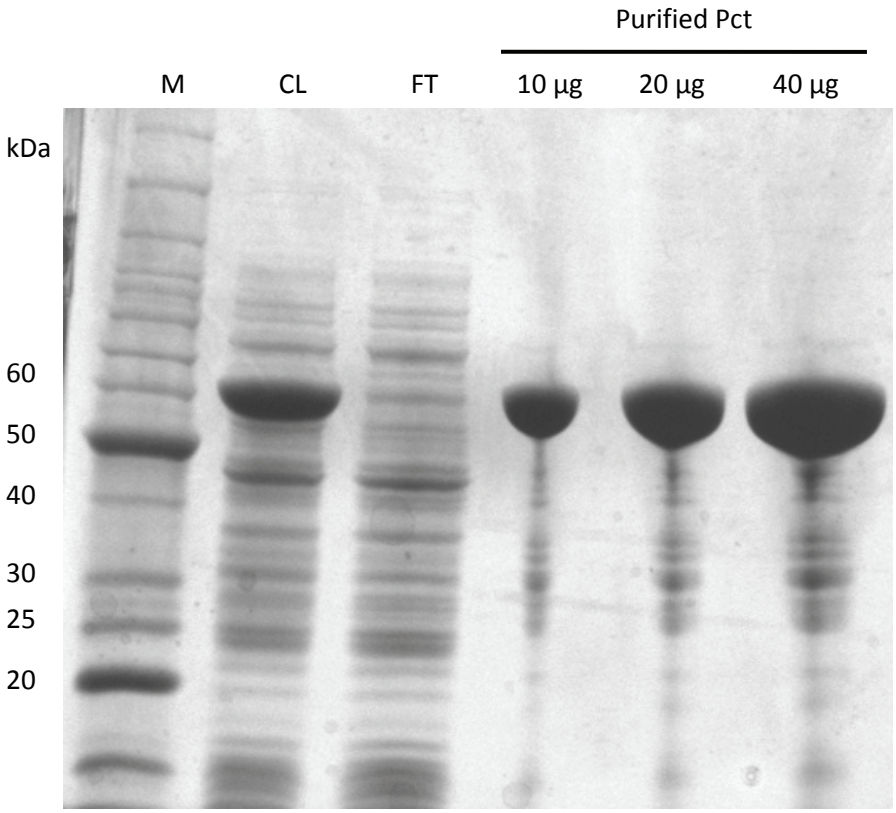

Fig 8.4: SDS-PAGE gel showing the purification of Strep-Pct (58.7 kDa). The lanes are the molecular weight marker, M; soluble fraction of the crude lysate, CL; flow through from column loading, FT; and purified Pct with the amount of protein loaded indicated above each lane. For the CL and FT 20 µg of total protein were loaded.

in the strains expressing only one of the enzymes. This suggests that under the induction conditions used in this study (0.5 mM IPTG) nearly all of the cells' protein synthesis capacity goes toward production of the protein(s) encoded on the plasmids. An *E. coli* strain harboring both pCB34 and pAGL28 (for constitutive $phaEC_{Av}$ expression) was also tested, and it was determined that there was still substantial expression of *pct* and *ldhA* even with the added burden of the additional plasmid. This strain should have theoretically expressed enzymes catalyzing all reactions necessary for PLA synthesis, but no PLA was detected via a methanolysis-GC assay when TR/pCB34 pAGL28 was grown in LB supplemented with 2% glycerol and induced with IPTG (data not shown).

Table 8.3: Plasmids containing *ldhA* and *pct* allowed for significant expression of these genes, as shown by specific activity measurements made using soluble lysate fractions from induced strains. N.D. indicates a value was not determined. Negative control strains had specific activities <1 U/mg for both assays.

Strain	Genes expressed	LdhA Sp Act (U/mg)	Pct Sp Act (U/mg)
TR/pCB32	*ldhA*	47.9	N.D.
TR/pCB33	*pct*	N.D.	50.4
TR/pCB34	*ldhA*, *pct*	19.0	18.9
TR/pCB34 pAGL28	*ldhA*, *pct*, $phaEC_{Av}$	16.3	21.3

After demonstrating expression of *pct* in *E. coli*, we decided that purified enzyme would be necessary for conducting additional experiments, such as CoA cycling reactions. The plasmid pCB46 was constructed for expression of Pct with an N terminal Strep-tag II. Protein expression was induced in TR/pCB46, and the resulting Strep-Pct was purified with a Strep-Tactin column. An SDS-PAGE gel showed a substantial increase in the Strep-PCT purity of the final preparation, relative to the crude lysate (Fig. 8.4). The Pct specific activity of the purified enzyme was 149 U/mg, compared to 42 U/mg for the crude lysate, indicating that the purity of the Pct was increased 3.5 fold.

Synthesis of LA-CoA and HB-CoA using Pct

LA-CoA was necessary for conducting several different experiments in this study. While some chemically synthesized LA-CoA was initially provided by Dr. Ping Li, we wanted to develop a method that would allow for rapid synthesis of additional compound. Pct is able to transfer CoA from commercially available acetyl-CoA to (*R*)-LA *in vitro*, which provided a convenient route for synthesis of LA-CoA. A large excess of (*R*)-LA was included in the reaction to drive CoA transfer from acetyl-CoA to LA. Purified

Strep-Pct was the preferred source of enzymatic activity for carrying out this reaction, but we found that crude lysate from TR/pCB33 could be used as well. After performing the transfer reaction, the CoA compounds were isolated using a Sep-Pak column and analyzed via HPLC (Fig 8.5A). Retention times of the compounds of interest were determined by first running pure standards on the HPLC. We discovered that after 5 min, almost all acetyl-CoA had reacted, producing LA-CoA and a small amount of free CoA. We believe that the free CoA was released during the reaction and was not simply a contaminant present with the acetyl-CoA, as the area of the free CoA peak increased when the reaction was allowed to proceed for longer periods of time (data not shown). Based on the observed HPLC peak areas, we estimated that the isolated products consisted of 86% LA-CoA, 7% acetyl-CoA, and 7% free CoA (Fig. 8.5A).

We also tested this procedure for synthesis of HB-CoA, a reagent that is frequently used for carrying out PHA synthase assays and that is normally synthesized chemically (29). The reaction procedure was the same as above, except (*R*)-HB was provided as the CoA acceptor instead of (*R*)-LA. We found that Pct could be used to synthesize HB-CoA from acetyl-CoA and (*R*)-HB (Fig. 8.5B). Based on the observed peak areas, we estimated the products consisted of 88% HB-CoA, 1% acetyl-CoA, and 5% free CoA. The remaining 6% was assigned to the small peak that elutes immediately after HB-CoA, which we were not able to identify, but which we assume contained CoA due to its absorbance at 260 nm.

CoA cycling reaction

While CoA release was observed when LA-CoA was incubated with $PhaC_{Re}$ and $PhaEC_{Av}$, we were not able to generate enough product for further characterization using this type of reaction. We therefore attempted a CoA cycling reaction, which does not require the synthesis of a large quantity of LA-CoA. This type of reaction contains only a catalytic amount of CoA, which is continuously recycled. A reaction was set up using $PhaEC_{Av}$ and LA, as well as a positive control that used HB and a negative control that did not contain synthase. Progress of the reactions was monitored by measuring ATP consumption over time (Fig. 8.6A). We found that the ATP concentration in the positive control decreased more rapidly than in the other reactions, and that all ATP was exhausted by the 20 h time point in the positive control. We also observed that the HB reaction became cloudy by the 4 h time point, presumably due to precipitation of PHB. The LA reaction had a lower ATP concentration than the negative control at all time points, however the rates of ATP disappearance appeared to be similar after the first hour of the

experiment. This suggests that a short initial burst of ATP utilization was responsible for the differences in ATP concentrations between the two reactions throughout the experiment. When visually inspected, the LA reaction appeared slightly more turbid than the negative control, but after centrifugation no pellet was visible in the LA tube (Fig. 8.6B).

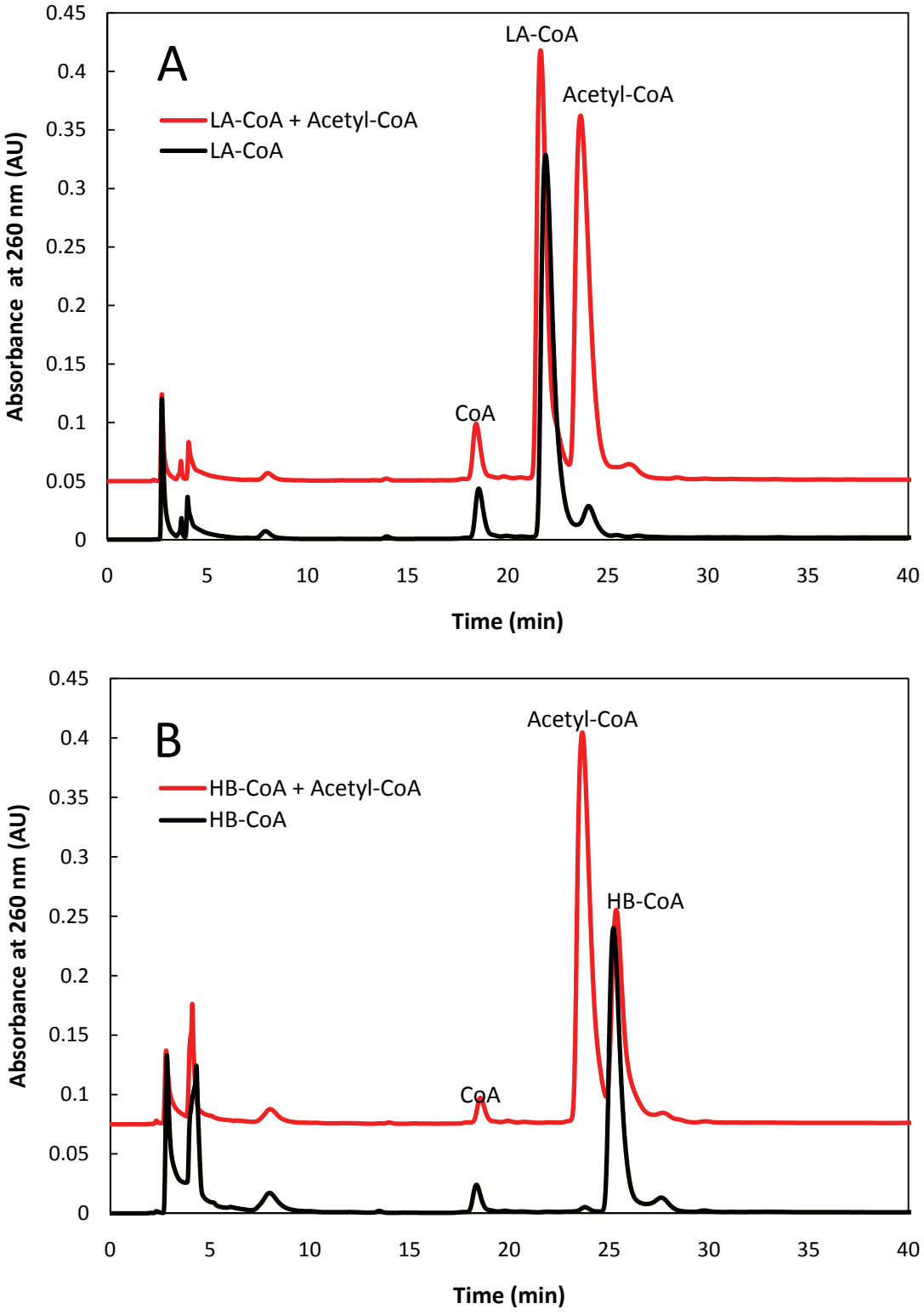

Fig 8.5: HPLC chromatograms of (*R*)-LA-CoA (A) and (*R*)-HB-CoA (B) synthesized enzymatically using Pct. The black traces show the results of the reactions, while the red traces are duplicate samples that have been spiked with acetyl-CoA. The red traces have been shifted up in the y-axis to make peaks easier to differentiate.

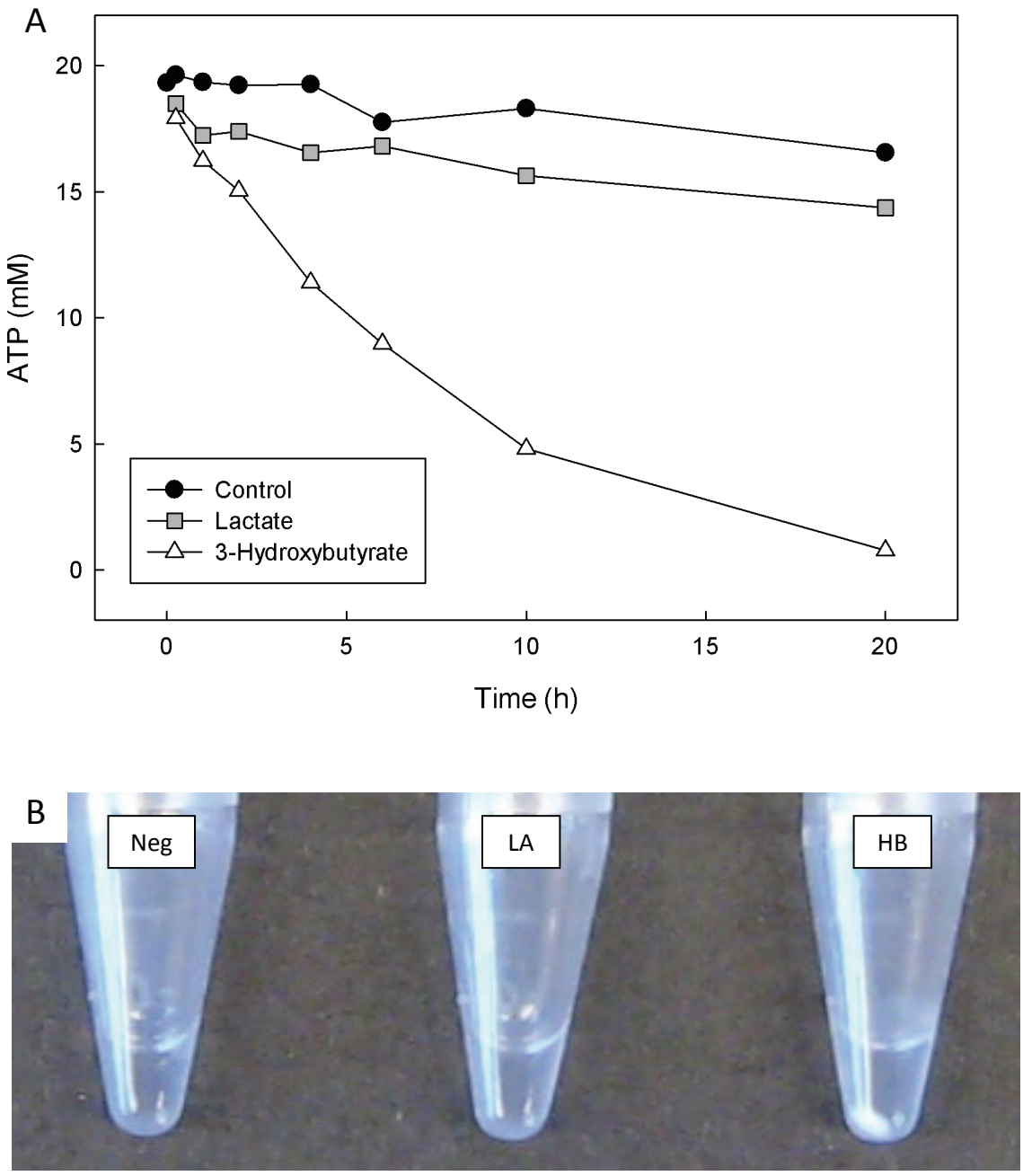

Fig 8.6: ATP concentrations decreased over time in three CoA cycling reactions (A). After 20 h of incubation at 30°C, the samples were centrifuged, and a white pellet was visible in the HB reaction, presumably made up of PHB (B).

Discussion

Previous publications reported that CoA was released from LA-CoA when this substrate was incubated with $PhaEC_{Av}$. Valentin and Steinbüchel used crude lysate from an *E. coli* strain expressing $phaEC_{Av}$ and found that with (*R*)-LA-CoA the activity was 6.6% of the activity with HB-CoA, while (*S*)-LA-CoA showed 1% activity relative to HB-CoA (24). Yuan *et al*. found that (*R*)-LA-CoA yielded 0.04% relative activity with purified $PhaEC_{Av}$, and did not observe any activity using purified $PhaC_{Re}$ (29). In this study, we found that the relative activity of $PhaEC_{Av}$ with LA-CoA was 0.03%, which agrees well with the data of Yuan. In contrast with that report, however, we found that $PhaC_{Re}$ showed 0.3% relative activity with LA-CoA. This difference may be due to the source of $PhaC_{Re}$ used in the two studies. Yuan purified $PhaC_{Re}$ from *E. coli*, while the enzyme used in this study was purified from *R. eutropha*. It has been shown that synthase purified from *R. eutropha* contains short PHB oligomers, even when there is little PHB detectable in the cells (C. Brigham, unpublished data). These oligomers likely prime the synthase, reducing the lag phase and allowing for greater initial activity, as has been shown previously using synthetic enzyme priming agents (9, 26). We believe this priming phenomenon is the reason we observed $PhaC_{Re}$ activity with LA-CoA while Yuan did not.

While the release of CoA indicated that the synthases were catalyzing a reaction, we wanted to isolate enough product to confirm that the LA units were actually being polymerized. This would be difficult in the batch reactions used in the assays because (i) free CoA builds up in the reactions over time and (ii) a large amount of LA-CoA must react to generate a small amount of product (it takes 9.5 mg LA-CoA to make 1 mg PLA). We therefore set up CoA cycling reactions in an attempt to generate more product. The HB control reaction successfully generated PHB, but the results of the LA reaction were unclear. ATP in the LA reaction decreased relative to the negative control in the first hour of the experiment, but after that point little ATP consumption relative to the control was observed (Fig. 8.6A). This finding, along with the CoA release data (Fig. 8.2), suggests that LA-CoA is initially turned over rapidly by the synthase, but after a short time the rate of the reaction decreases dramatically. We believe that short PLA oligomers are initially formed by the synthase, but that these then proceed to "clog" the enzyme and inhibit further polymerization. It is known that polymerization proceeds through an intermediate in which the polymer chain is covalently linked to the synthase (15, 26), so it is reasonable that an intermediate in PLA synthesis could remain tightly bound to the enzyme. Little is currently known about how polymer is released from the synthase (13), so it is difficult to predict why a short PLA chain may remain bound.

Synthesis of LA-CoA from pyruvate and acetyl-CoA in *E. coli* could occur through the reactions of a Ldh and a Pct. We successfully expressed active forms of both of these enzymes in *E. coli* (Fig 8.3, Table 8.3). The strain TR/pCB34 pAGL28 expressed $phaEC_{Av}$ in addition to *ldhA* and *pct*, and therefore should have produced all enzymes necessary for PLA synthesis. When this strain was grown in LB containing 2% glycerol and induced with IPTG, no PLA was detected. (Glycerol was used as the carbon source instead of glucose, because glucose suppresses protein expression from pET vectors.) We planned a number of different experiments to attempt to find conditions that would allow for polymer synthesis, including varying the amount of protein expression and adding HB to the medium to promote P(HB-*co*-LA) production. While planning these experiments, however, reports were published demonstrating PLA synthesis using systems similar to the one we had designed.

Taguchi *et al*. first reported development of an *E. coli* strain that could produce P(HB-*co*-LA) (23). A key finding was that a mutated synthase from *Pseudomonas* sp. 61–3 was able to copolymerize HB-CoA and LA-CoA. The gene encoding this synthase was cloned into a plasmid, along with *pct* from *M. elsdenii* and $phaAB_{Re}$. An *E. coli* strain harboring this plasmid was able to produce P(HB-*co*-LA) with 6mol% LA. Later this group demonstrated that during anaerobic growth the engineered strain could produce copolymer with 47mol% LA, although the cells contained only 2wt% polymer (27). It was also confirmed that all LA units were of the (*R*) form. Incorporation of LA into the polymer chain was shown to lower the T_m and raise the T_g, relative to PHB.

Yang *et al*. were able to create a strain capable of synthesizing both P(HB-*co*-LA) and PLA (28). This group also employed an engineered synthase, this time from *Pseudomonas* sp. MBEL 6-19. The final strain expressed this synthase, along with an engineered *pct* gene from *C. propionicum* and $phaAB_{Re}$. Wild type Pct was found to inhibit growth of *E. coli* and an engineered enzyme with lower specific activity improved cell growth and polymer production. The LA incorporated into the polymer could be increased by lowering the dissolved oxygen and glucose concentrations in fermentations of the engineered strain. It was discovered that as the LA fraction became greater, polymer molecular weight and melt viscosity decreased, while T_g increased. The engineered strain was further improved by altering the chromosome of the *E. coli* harboring the plasmid (11). Genes encoding an acetate kinase (*ackA*), a phosphoenolpyruvate carboxylase (*ppc*), and an acetaldehyde/alcohol dehydrogenase (*adhE*) were deleted. Promoters for *ldhA* and acetyl-CoA synthetase (*acs*) were changed in the chromosome to increase expression of these genes. The result of these mutations was a strain that synthesized

additional lactate and acetyl-CoA, allowing for greater LA-CoA production. The final engineered strain was able to produce 48% of its CDW as P(HB-*co*-LA) with 55mol% LA.

The *in vivo* synthesis of PLA and P(HB-*co*-LA) is a significant step forward for bioplastics research. Addition of LA units to the polymer increases the T_g, although it remains to be seen if this will allow the polymer to be used in new applications. Mechanical testing of these copolymers should provide a better idea of their utility. Currently the molecular weights of polymer with high LA content are low (M_w < 150 kDa), so this may limit how these materials can be processed. The key difference between PHA and PLA is that PLA can be made completely transparent. Work with chemically synthesized copolymers of HB and (*S,S*)-lactide showed that polymer with 47 to 70mol% lactide was completely amorphous, meaning it should therefore be transparent (1). The clarity of the copolymers of (*R*)-HB and (*R*)-LA was not discussed in the published reports, but this property may determine the commercial prospects for this material.

While it is disappointing that other groups synthesized PLA and P(HB-*co*-LA) before we were able to, it is satisfying to know that the strategy we pursued was indeed viable. The work of the Taguchi and Lee groups showed that we would likely have needed to mutate the PHA synthase for successful polymerization of LA-CoA. The finding that more P(HB-*co*-LA) could be made than PLA supports our hypothesis that after polymerizing a few LA-CoA molecules, the synthase is somehow inhibited from adding more LA units to the chain. Copolymerization of HB seems to relieve this inhibition.

I gained important experience expressing and purifying enzymes while working on this project. I also made the significant discovery that the experimental reagent HB-CoA could be easily synthesized from (*R*)-HB and acetyl-CoA using purified Pct. This method could potentially replace the tedious chemical synthesis that was previously used. The CoA containing products of the enzymatic synthesis were rapidly purified using a Sep-Pak column, and 88% purity of the HB-CoA was achieved. If preparative HPLC was used in the purification step, it is likely that the purity could be increased to near 100%.

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Chapter 9
Conclusions and Future Work

Summary of Goals and Achievements

The principal aim of my thesis work was to develop a process to convert palm oil into a useful polyhydroxyalkanoate (PHA) copolymer. In order to achieve this overarching goal, I had to meet the following objectives:

(i) Determine which types of PHAs have properties that make them useful thermoplastics
(ii) Engineer a bacterial strain capable of synthesizing this PHA from palm oil
(iii) Develop a fermentation process for growing the engineered strain to high density
(iv) Design a process for recovery of polymer from bacterial cultures

Analysis of the literature revealed that the polymers normally synthesized by *Ralstonia eutropha*, PHB and P(HB-*co*-HV), are brittle and difficult to process. We identified the copolymer P(HB-*co*-HHx) as a promising material, because of its desirable mechanical properties and because its low melting point makes it easier to process than PHB (10). P(HB-*co*-HHx) is also appealing because intermediates of fatty acid β-oxidation generated during growth on palm oil can be diverted to form HHx-CoA (3).

The first strain investigated for PHA production from palm oil was *Rhodococcus aetherivorans* I24. We discovered that this bacterium grew slowly on plant oil and did not accumulate high levels of PHA (Chapter 2). When grown on glucose, however, it synthesized P(HB-*co*-HV), which encouraged us to clone and characterize the PHA synthase genes from this organism. Expression of the *R. aetherivorans* PHA synthases in *R. eutropha* led to production of P(HB-*co*-HHx) from fatty acids (Chapter 5). When grown on palm oil, however, the PHA produced by these recombinant strains had very low HHx content (<2mol%).

We hypothesized that high intracellular concentrations of HB-CoA in *R. eutropha* could limit HHx incorporation into the PHA. In order to decrease HB-CoA synthesis, we deleted acetoacetyl-CoA reductase genes (*phaB* genes) from the *R. eutropha* genome (Chapter 4). We discovered that deletion of *phaB1* and *phaB3* resulted in a *R. eutropha* strain that produced significantly less PHB than wild type in fructose minimal medium. In palm oil minimal medium and rich medium, only deletion of *phaB1* was

necessary to cause the low PHB production phenotype. The expression patterns of *phaB1*, *phaB2*, and *phaB3* were also investigated, and the activities of the purified enzymes were determined.

The Δ*phaB1,2,3* strain was further engineered to express *phaC2* from *R. aetherivorans*. The resulting strain synthesized PHA with high HHx content, but the amount of PHA accumulated was too low for the strain to be of industrial interest (Chapter 5). PHA production was increased by expression of *phaJ* genes, which encode enoyl-CoA hydratases. High expression of the engineered PHA biosynthetic operon was achieved by cloning the operon into a plasmid and transforming the plasmid into different host strains. The final results of our strain engineering efforts were two strains that accumulated over 65wt% PHA in palm oil fermentations. One of these strains made PHA with 17mol% HHx, while the other made PHA with 30mol% HHx. Plasmid stability in the absence of antibiotics was achieved by deleting *proC* from the genomes of the host strains and cloning this gene into the plasmid. This system ensures that the plasmid is retained in minimal medium without the use of antibiotics.

Studying the growth of bacteria with insoluble plant oil as the carbon source is challenging. We developed a new method for growth of *R. eutropha* on emulsified palm oil, which allowed us to conduct quantitative, reproducible experiments (Chapter 3). We found that the glycoprotein gum arabic (4, 11) was the best emulsifying agent to use in *R. eutropha* cultures. A method for lipid extraction from emulsified oil medium was also developed that allowed us to monitor oil consumption over the course of fermentations. While our emulsified oil method is a useful experimental tool, it cannot be applied at the industrial scale, due to the high cost of the emulsifying agent. We therefore also conducted fermentations with our engineered *R. eutropha* strains that used unemulsified palm oil (Chapter 5).

Despite many reports in the literature describing growth of *R. eutropha* on plant oil, little was known about the oil and fatty acid metabolism of this bacterium. We therefore conducted whole genome microarray experiments to determine relative transcript levels of all *R. eutropha* genes when the organism was grown on different carbon sources (Chapter 6). We identified two operons that were highly expressed during growth on oil that each contained genes encoding all enzymes necessary for fatty acid β-oxidation. Deletion of both operons resulted in strains unable to grow on oil or long chain fatty acids. We also identified a gene encoding a lipase (h16_A1322) that was necessary for efficient growth on unemulsified plant oil.

One of the challenges of industrial PHA production is isolating the polymer from bacterial biomass. We discovered that methyl isobutyl ketone (MIBK) and butyl acetate (BA) are two promising solvents for

extraction of high HHx PHA (Chapter 7). After separating out the residual biomass, PHA can be precipitated from solution by addition of an alkane, such as hexane or heptane. Unused palm oil present at the end of a fermentation remains dissolved in the organic phase after PHA precipitation. We developed an Aspen simulation in which (i) PHA was extracted from a biomass slurry, (ii) the PHA was precipitated and recovered, and (iii) the solvent and precipitant were separated by distillation and recycled. Based on our analysis, butyl acetate/hexane is the most promising solvent/precipitant combination, due to the ease with which these compounds can be separated from water and one another.

Significant efforts were also devoted to producing P(HB-*co*-LA) copolymers. Enzymatic assays indicated that CoA was released when PHA synthases were incubated with LA-CoA. Unfortunately, no PLA could be detected in these experiments. We expressed lactate dehydrogenase and propionate CoA transferase in *E. coli* in order to generate LA-CoA. This strain design is similar to ones that were successfully implemented by other groups (16, 21). Reports published while we worked on this project indicated that it is necessary to mutate PHA synthases in order for them to polymerize LA-CoA. While we were unable to successfully synthesize P(HB-*co*-LA), we did develop a procedure for enzymatic synthesis of the important reagent HB-CoA while working on this project.

Opportunities for Future Work

Strain Engineering

Thermoplastics are generally low price materials. For example, bulk polypropylene typically sells for <$1/lb. While it may be possible to charge a premium for PHAs because of their unique properties, it is still essential to develop an efficient, low cost process for PHA production. An important component of the process is a microbial strain that accumulates a high level of PHA at a high yield. Currently our most promising strain, Re2058/pCB113, accumulates 71wt% PHA with 17mol% HHx when grown on palm oil (Fig. 5.2). We have observed higher PHA accumulation in other strains (although with lower HHx content), indicating that it is possible for *R. eutropha* cells to accommodate more bioplastic. For example, wild type *R. eutropha* H16 can accumulate 80wt% PHB from palm oil (Table 5.3). If the RCDW of the Re2058/pCB113 fermentation in Fig. 5.2A was kept constant at 7 g/L and PHA accumulation was increased from 71wt% to 80wt%, the PHA concentration in the culture would increase from 17 to 28 g/L.

This illustrates the dramatic impact higher PHA accumulation can have on total polymer production in a bacterial fermentation.

There are several strategies that could be employed to attempt to increase PHA production by Re2058/pCB113. One strategy is to increase synthesis of HB-CoA by raising the expression of *phaA* and *phaB* (e.g. by expressing these genes on a plasmid or by changing the promoter in the Re2058 genome). Given that we expended considerable effort to decrease HB-CoA formation in *R. eutropha*, this may initially seem counterintuitive. However, the high level of HHx in the PHA made by Re2058/pCB113 means that the HB content of the polymer could be increased while still yielding a polymer with desirable mechanical properties. If the amount of HHx in the PHA made by Re2058/pCB113 was kept constant and the HB content was increased such that polymer accumulation reached 80wt%, the resulting polymer would still contain 10mol% HHx.

Another approach for increasing PHA accumulation involves sorting and selecting cells based on the amount of PHA they have stored. This can be accomplished by staining the cells with a fluorescent lipophilic dye (e.g. Nile red) and selecting the most highly stained cells via fluorescence activated cell sorting (FACS) (19). In the simplest implementation of this strategy, an oil or fatty acid culture could be sorted, and the portion of the population with the highest signal (e.g. the top 5-10%) could be used to inoculate a new culture. This procedure could be repeated several times to select for high PHA producing cells. In this implementation, cells with abnormally high levels of PHA accumulation would result from random mutations. Alternate approaches could also be used, in which mutants would be actively generated. A chemical mutagen could be included in *R. eutropha* cultures to induce random mutations in all Re2058/pCB113 DNA (i.e. the genome and the plasmid). Another strategy would be to create a library of Re2058 transposon mutants, transform them with pCB113, and sort them. A final possibility would be to mutate the PHA operon in pCB113 by error-prone PCR, transform the mutated plasmids into Re2058, and select high PHA producing cells, which would contain plasmids with beneficial mutations.

One intriguing approach that would almost certainly lead to high PHA accumulation would be to express two different PHA synthases in a *R. eutropha* single strain. For example, pCB113 could be transformed into Re2000. In this case, low HHx PHA would be synthesized by $PhaC1_{Ra}$, while high HHx PHA would be synthesized by $PhaC2_{Ra}$. It is unclear what properties would be exhibited by the resulting PHA mixture. Miscible polymer blends generally have properties intermediate to those of the two pure polymers, but

further studies would be necessary to determine how well high HHx and low HHx PHA blend, and if the blends have practical value as bioplastics.

An issue we identified with the PHA from Re2058/pCB113 and Re2160/pCB113 is that the molecular weight of the polymer produced by these strains is lower than desired. It has been reported that the preferred M_w range of PHA is 500,000 to 700,000 g/mol for most polymer processing techniques (10). We measured that PHA from both Re2058/pCB113 and Re2160/pCB113 had M_w values of ~300,000 g/mol (Chapter 5). Formulating a plan to increase PHA molecular weight is challenging, as the mechanisms that control polymer chain length are not fully understood (8). It has been proposed that the level of PHA synthase expression influences molecular weight, with greater expression leading to shorter PHA chains (14). We could therefore decrease synthase expression levels in our engineered strains and determine the effect this has on PHA accumulation and PHA molecular weight. Another possibility would be to modify the PHA synthase itself. It has been proposed that PHA synthases may have residues outside of their active sites that can terminate growth of polymer chains (18). While this mechanism of chain termination may indeed occur, specific residues responsible for chain termination have never been identified. Therefore a library of mutant $phaC2_{Ra}$ genes would have to be constructed and evaluated to determine if any of the mutant synthases produced PHA with greater molecular weight than the wild type enzyme. A final option for increasing PHA molecular weight would be to delete PHA depolymerase genes (*phaZ* genes) from the Re2058 and Re2160 genomes. It has been observed that the average PHA molecular weight decreases over the course of *R. eutropha* fermentations, even as the total amount of accumulated PHA increases (17). This may be related to the fact that some PHA depolymerases seem to be expressed even during PHA storage conditions (7). Deleting PHA depolymerase genes from the *R. eutropha* genome may therefore prevent or diminish the decrease in PHA molecular weight that occurs during fermentations. It is also possible that *phaZ* deletion strains could accumulate more PHA than strains expressing these genes. While no increase in PHB accumulation was observed in fructose minimal medium flask cultures when *phaZ* genes were deleted from *R. eutropha* (24), the impact of depolymerases on PHA accumulation in high density cultures producing PHA copolymers has never been examined.

While most of the metabolic engineering efforts in the Sinskey lab have focused on PHA synthesis, the productivity of our system could also be improved by speeding the growth rate of *R. eutropha* on palm oil. Microarray experiments allowed us to identify many genes essential for growth of *R. eutropha* on triacylglycerols (Chapter 6). These included a putative lipase gene, genes encoding fatty acid β-oxidation

enzymes, and genes encoding glyoxylate bypass enzymes. It is possible that the reaction catalyzed by one of these enzymes may be the rate limiting step for growth on palm oil. Examination of the data in Chapter 6 suggests it is not one of the β-oxidation operons, as strains in which only one operon was deleted grew as fast as the wild type strain, indicating that lower gene expression did not decrease growth rate. We will therefore construct plasmids that allow for greater expression of the lipase gene and the glyoxylate bypass genes, and determine if these strains grow more quickly than wild type *R. eutropha*.

New Fermentation Strategies and Process Scale Up

While performing unemulsified palm oil fermentations with the strains described in Chapter 5, we discovered that excessive oil had several negative effects on the experiments. *R. eutropha* seemed to grow more slowly in the presence of excess palm oil, although the reason for this growth inhibition is unclear. Another issue is that it is difficult to harvest cells from the fermentation when there is significant residual oil present. After the initial centrifugation, some palm oil is clearly associated with the cell pellet. When the sample is washed with water and hexane this oil is removed, but an interface forms between the organic and aqueous layers after centrifugation. The exact composition of this interface is unknown, but analysis of one set of Re2160/pCB113 fermentations indicated that the dried interface is made up of ~40wt% *R. eutropha* cells (see Chapter 5).

A fermentation strategy should therefore be developed in which the oil in the fermentation is maintained at a concentration high enough that there is sufficient carbon to induce storage of PHA, but low enough that the problems described above are minimized. The most obvious strategy for achieving these goals is to develop a fed batch fermentation protocol. A relatively low concentration of palm oil would be provided at the beginning of a fermentation to allow for initial growth, and additional oil would be added over time for continued PHA production. The oil feed would only begin after sufficient cell growth had occurred to cause oil emulsification. The initial oil concentration and the oil feed rate would be determine empirically, based on the total amount of oil consumed by a given strain in a fermentation. Our current estimate is that 1% palm oil should be used for every 0.1% NH_4Cl, but the exact ratio varies depending on the amount of PHA produced by a given strain. In a more advanced scheme, carbon dioxide in the fermentation exhaust could be measured and used to control the addition of oil. The rate of oil addition would vary with the rate of CO_2 evolution. This control scheme

would have to take into account differences in CO_2 production during the growth and the PHA production phases of a culture.

Another feeding strategy that could decrease the lag phase in non-emulsified oil cultures would be to provide a low concentration of a soluble carbon source at the beginning of a fermentation, along with the palm oil. This would allow for some initial cell growth before substantial TAG breakdown had occurred. A higher biomass concentration earlier in a culture would provide more cells to secrete lipases, and therefore increase the rate of fatty acid release from the oil. A possible soluble carbon source to use in this strategy is a mixture of volatile organic acids (acetate, propionate, butyrate), which can be produced from waste generated by the palm oil industry (22).

Another aspect of *R. eutropha* fermentations that should be considered is the type of nutrient limitation used to induce PHA accumulation. Our lab has typically used nitrogen limitation to stimulate PHA production, but other nutrients can be used as well. The highest level of PHA production reported for *R. eutropha* in the literature was achieved in phosphorous limited cultures (12). As our standard *R. eutropha* minimal medium contains a high concentration of phosphate buffer, P limited cultures must either include a different buffering agent, or be performed in a fermentor that can automatically control the pH of the culture. We performed some preliminary P limited fermentations, but had difficulty obtaining reproducible results. These experiments, however, were performed using strains that did not have the *proC* plasmid stability system, and should therefore be repeated with our latest strains. If phosphorous limited cultures do produce more PHA than nitrogen limited cultures, our minimal medium can be redesigned to take advantage of this fact.

In order to prepare for industrial scale PHA production using the strains described in my thesis, both the density and scale of the cultures must be increased. Until new strains that can accumulate higher percentages of PHA are developed, the easiest way to increase PHA production is to grow more cells in a given volume. In the case of the fermentations described in Chapter 5, this would entail using more nitrogen in the medium. We have found that *R. eutropha* growth is inhibited by NH_4Cl concentrations greater than 0.5%, but that urea can be used to provide higher amounts of nitrogen. We therefore plan to alter the medium so that it contains 0.56% urea (equivalent nitrogen to 1% NH_4Cl), and to grow the culture in the largest fermentor available in the Sinskey lab (4 L). Once conditions have been developed that allow for reproducible 4 L fermentations, we will have a 100 L fermentation performed at the University of Iowa Center for Biocatalysis and Bioprocessing.

After carrying out a 4 L palm oil fermentation, the biomass will be used to test our proposed PHA recovery method. We have typically performed small scale recovery experiments by washing cell pellets with water and hexane, lyophilizing the biomass, and extracting PHA from the dried cells. In an industrial process the biomass would be concentrated, and then PHA would be extracted directly from the wet biomass slurry (refer to Chapter 7 for details). We will test extraction of P(HB-*co*-HHx) from wet biomass with MIBK and butyl acetate. In our lab the solvent extraction would have to be performed in a flask, but we eventually plan to scale up the process further and test PHA recovery using a centrifugal extractor.

Large scale PHA extraction studies will generate material for use in polymer characterization experiments. Thus far we have determined polymer properties that can be measured with small amounts of material (e.g. thermal properties), but further characterization of high HHx P(HB-*co*-HHx) is necessary. Significant quantities of PHA are needed to measure mechanical properties. Each test sample requires gram quantities of polymer, and many samples are necessary to generate statistically significant data. Properties that influence polymer processing will also be measured, such as melt flow rate and melt viscosity. The availability of a large amount of P(HB-*co*-HHx) would also allow us to study blends of P(HB-*co*-HHx) and PHB. As high concentrations of PHB can be made from palm oil with Re2143 (Chapter 4), blending PHB with high HHx polymer may be a way to decrease the total cost of bioplastic.

Studies of *R. eutropha* Metabolism

While the *R. eutropha* strains we have engineered have potential for industrial PHA production, it is likely that a better understanding of *R. eutropha* metabolism would allow for further improvement of our strains, and construction of new strains to meet future needs. One mystery that remains is how *R. eutropha* is able to synthesize (*R*)-HHx-CoA without expression of heterologous *phaJ* genes. It has been shown that PhaB can reduce 3-ketohexanoyl-CoA (5), but strains with all three *phaB* genes deleted can still accumulate P(HB-*co*-HHx) (see Re2135 and Re2136 in Chapter 5). It should be possible to determine experimentally how these strains produce HHx monomers. A transposon library based on Re2135 would first need to be constructed, and then the library could be grown in conditions that stimulate accumulation of PHA with high HHx content (e.g. minimal medium with hexanoate as the carbon source). Cells with low PHA accumulation could be identified by staining colonies on plates with

lipophilic dye, or by FACS analysis of stained cells (19). Some of the low PHA accumulating mutants would likely have transposons inserted in the gene(s) responsible for synthesis of HHx-CoA.

We would also like to increase our understanding of *R. eutropha* fatty acid β-oxidation. We identified two operons in the *R. eutropha* genome encoding the enzymes of fatty acid β-oxidation and found that *R. eutropha* could not grow on long chain fatty acids if both these operons were deleted. The double operon deletion mutant was, however, still able to grow on short chain length, soluble fatty acids (e.g. octanoate). We would like to determine what genes allow for catabolism of these fatty acids. One way to study this would be to conduct microarray experiments using RNA isolated from *R. eutropha* grown on octanoate. Fatty acid catabolism genes other than those identified in the trioleate experiments may be upregulated during growth on octanoate. A second option would be to construct a transposon mutant library based on Re2303 and plate the mutants on minimal medium plates with octanoate as the sole carbon source. If mutants unable to grow under these conditions were identified, it would indicate that a transposon had inserted into a gene essential for octanoate catabolism.

PHA production from volatile organic acids is also a subject our lab has investigated (22). Organic acids such as acetate, propionate, and butyrate can be produced from waste generated at palm oil mills (23). These volatile organic acids represent a low cost feedstock that could be used as a carbon source for PHA production, or that could be used as a supplemental carbon source along with palm oil. Microarray experiments performed with these organic acids as the carbon source could provide insight into how to design new strains to take advantage of this potential feedstock.

Final Thoughts

Our society relies extensively on the fossil resources stored within the Earth. The public most often associates these resources with energy. Coal and natural gas are burned to create electricity, while petroleum is refined into transportation fuels. A less appreciated use of fossil resources is as a feedstock for the chemical industry. Oil and natural gas are used to synthesize a variety of organic compounds, the majority of which are eventually converted into plastics. These plastics are used to manufacture numerous goods and have become an integral part of modern life. As society moves towards a sustainable economy, it is essential that new methods are developed for producing both fuels and chemicals.

Biotechnology can be applied to convert renewable agricultural resources into chemical products. This is already taking place using resources such as corn starch, cane sugar, and palm oil (for examples see (1, 9)). Palm oil is an excellent feedstock for PHA synthesis, which can be produced at high yields in Southeast Asia (20). One concern that must be addressed, however, is how oil palm cultivation influences land use. If tropical rainforests are cleared to establish new oil palm plantations, there are significant carbon dioxide emissions, which will accelerate climate change (2). It is likely that considerable changes will have to be made to the worldwide agricultural system in order to produce both food and biobased chemicals in a sustainable manner. Energy crops such as switchgrass are likely to play an important role once technology has been developed to efficiently convert cellulosic biomass into fermentable sugars (13, 15).

PHAs will become increasingly important as society transitions away from petrochemicals and towards biobased materials. Industrial PHA production already generates fewer greenhouse gas emissions than production of petrochemical plastics (6), and emissions will be reduced as PHA production technology improves and new feedstocks become available. The biodegradability of PHAs means less waste will accumulate in the environment as these materials displace traditional plastics. New processing techniques and additives will expand the applications for which PHAs can be used. The work in this thesis illustrates one possible process for industrial PHA production. I hope that my results will prove useful in the field of PHA research and contribute to the development of a sustainable chemical industry.

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