Metabolic Engineering of *Escherichia coli* for the Synthesis of Defined Polyhydroxyalkanoates from Unrelated Feedstocks

By

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Abstract

The global challenges of energy sustainability and environmental preservation demand solutions that tap into renewable resources and can augment or replace the need for petroleum derived fuels and chemicals. This demand for sustainability is addressed by synthetic biologists through the engineering of microorganisms to synthesize products from renewable feedstocks. Synthetic biology techniques have been applied to the production of transportation fuels, medicines, commodity chemicals and plastics. In the U.S., plastics manufacturing is a 61 billion dollar a year industry. While plastic production makes up a relatively small fraction of the total annual energy consumption compared with the transportation sector, most traditional plastics are derived from petroleum feedstocks. Furthermore, these materials often persist in the environment indefinitely. Recent data from the U.S. Environmental Protection Agency suggest that over 31 million tons of plastic waste is generated annually. Therefore, a renewable and potentially biodegradable source of plastics would be of great value.

This thesis addresses the above challenges by applying synthetic biology to the synthesis of a microbially derived, renewable, biodegradable class of polyesters known as polyhydroxyalkanoates, or PHA. PHA are accumulated naturally in a number of microorganisms as a form of carbon and energy storage. Discovered in the 1920's, this polyester material has drawn commercial interest since the 1980's. Today, PHA are produced at small-scale industrial facilities around the world. However, PHA have faced a number of obstacles in terms of adoption in the commercial market. The two main obstacles are related to cost and utility. First, PHA are currently sold at a significant premium to traditional plastics. For example, polypropylene is sold at about $1.00 per pound compared with $2.25-$2.75 per pound for PHA. Second, PHA can be harvested from the producing organism for direct incorporation into various plastic products but the physical properties of the current generation of PHA prevent their widespread use. To reduce the cost of production and expand the applications of PHA, this thesis explores a number of strategies for the production of novel PHA from inexpensive, renewable feedstocks.

This thesis presents the history of PHA research and development as well as the field of synthetic biology and how it can be applied to renewable plastic production. Next, a review of the existing biosynthetic routes to PHA from unrelated carbon sources is provided along with an update of the diverse

list of monomers known to have been incorporated into PHA since 1995. Given the known routes to PHA, two alternative routes are proposed involving either fatty acid metabolism or polyketide biosynthesis. In the case of the former, a strategy for producing PHA with a defined composition despite the iterative nature of fatty acid metabolism was designed and implemented in a strain of *Escherichia coli*. The success of this strategy hinged on two main findings; the first was the use of a plant thioesterase to produce a pool of free fatty acids with a defined composition while the second was the thorough characterization of *E. coli* β-oxidation to identify key nodes for the prevention of successive rounds of fatty acid breakdown. The resulting strain was capable of producing PHA from glucose as a sole carbon source with a composition that matched the product profile of the plant thioesterase at greater than 17% of the cell dry weight.

In addition to the fatty acid based strategy, as second route to PHA production involving fatty acid biosynthesis was explored. Type I polyketide synthases are a class of multi-functional enzymes that synthesize their products in a predictable, assembly-line fashion. Previous research has demonstrated the possibility of modifying polyketide synthases to produce novel products. In addition, the enzymatic activities inherent to polyketide synthases allow for the generation of compounds appropriate for PHA biosynthesis. Therefore, a strategy was explored for the use of modified polyketide synthases to produce monomers or monomer precursors for the biosynthesis of novel PHA. Two model systems were tested for functionality in both *E. coli* and *Streptomyces* and obstacles to achieving functional monomer synthesis were identified. The need for monomer activation prior to polymerization was also addressed through the construction of a Coenzyme A-ligase selection strain. This platform is useful for the identification of activation enzymes with activity towards a given monomer.

Finally, the possibility of further improvements in PHA biosynthesis from unrelated carbon sources is outlined. This discussion includes the potential for co-expression of PHA granule-associated proteins known as phasins to improve PHA titer. In addition, the effect of alternative carbon sources on PHA production is explored covering both natural substrates (*i.e.,* acetate) and non-natural substrates (*i.e.,* levulinic acid). In summary, this thesis explores routes to tailor-made PHA that begin with inexpensive, renewable substrates in hopes of both reducing the cost of industrial PHA biosynthesis and expanding the applications of this class of biodegradable polyesters.

For Monica

Acknowledgements

I started graduate school at the University of Wisconsin-Madison in August of 2007. However, my fate to pursue a science career was probably sealed in AP Chemistry senior year of high school when Dr. Berlin was trying to fish a racquet ball out of a liquid nitrogen bath. He retrieved the ball with a gloved hand, claimed his finger was frozen and then nonchalantly determined that the best course of action was to smash it with a hammer. The result was shrieking teenagers as -196°C hot dog pieces exploded out of his glove and across the room.

Now, in December of 2012, there are a lot of people that deserve a lot of thanks. With this acknowledgement section I plan to focus on thanking just a few people with the understanding that the rest of you will know who you are – expect that I will be making the rounds to thank you in person. So, in no particular order…

Mom and Dad – In the 5th grade I made a faux-pas in my yearbook such that it read, “role model(s): none.” I don’t know what I was thinking, but I can make it up to you here: Role Model(s): <u>My Mom and Dad</u>. Sorry it took so long.

To the Usreys – I could not have found a better set of in-laws if I had tried. Thank you for encouraging your daughter to date someone younger and for accepting me into your family. Sincerely, D. B.

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To Monica – You rock. I owe you a puppy :)

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Chapter 1: Polyhydroxyalkanoates as a renewable, biodegradable plastic alternative

1.1. Introduction: Challenges in the plastics industry

The year 1907 became a milestone in the history of the plastics industry when Belgian chemist Leo Baekeland invented a process for the synthesis of poly(oxybenzylmethylenglycolanhydride) – the first entirely synthetic plastic commonly known as Bakelite. Since then, plastic materials and resins have become pervasive in modern society. According to the American Chemistry Council, in 2011 the plastics and rubber industry comprised 61 billion dollars in sales with over 100 billion pounds of material produced in the United States alone. While the magnitude of the plastics industry is an economic boon, there are several challenges that must be addressed. Among these challenges are the issues of U.S. energy security and sustainability.

Plastics are traditionally made from finite resources such as petroleum and natural gas. In 2006, the United States consumed 7.2 billion barrels of petroleum and 22 trillion cubic feet of natural gas. Of these totals, 4.6% of petroleum and 1.5% of natural gas was used to make plastics (Source: U.S. Energy

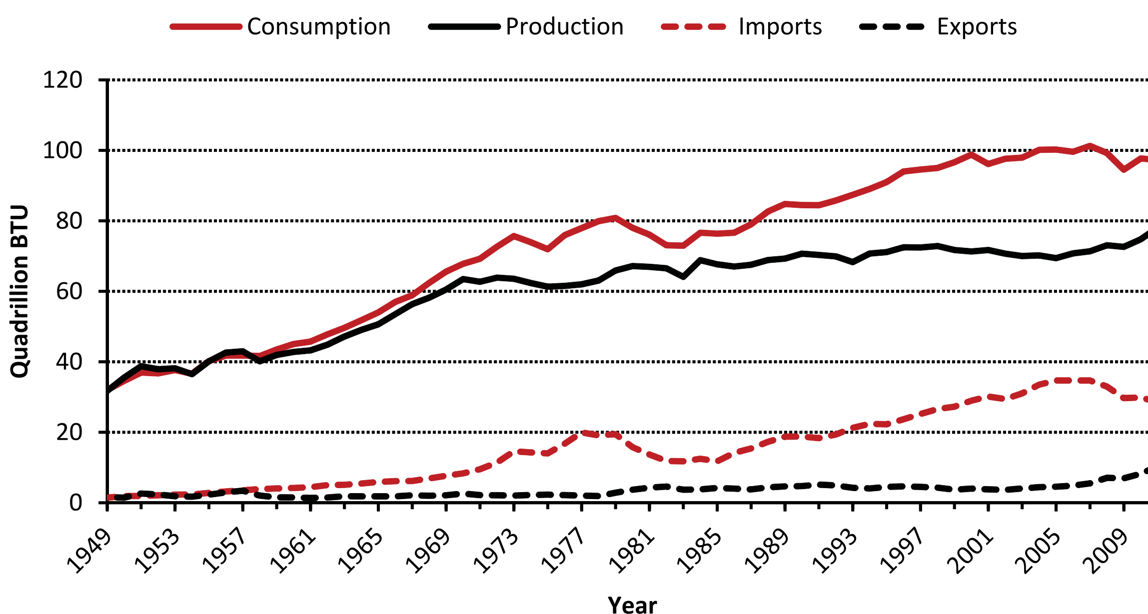

Figure 1.1. U.S. primary energy production, consumption imports and exports (1949-2011). Since 2007, energy imports have declined each year, except for a slight increase in 2010. Most of the imported energy was petroleum. In 2011, net imports (imports minus exports) accounted for 19 percent of all primary energy consumed. Source: U.S. Energy Information Administration Annual Energy review.

Information Administration). In the context of both U.S. and global energy consumption, these numbers may represent only a small fraction of the total. However, with the rising cost and finite availability of the petroleum precursors necessary to produce traditional plastics (Fig. 1.1 and 1.2), it is clear that an alternative, petroleum-independent solution would be of great value. Plastics produced wholly or in part by biological conversion of renewable feedstocks (*e.g.,* sugars) are one potential solution, but there are additional facets to the problem of plastic production beyond energy sustainability and security.

In addition to energy concerns, there is the challenge of managing the disposal of plastic waste. Each year, the U.S. Environmental Protection Agency publishes a report on municipal solid waste generation, recycling, and disposal (EPA, 2011). In 1960, the EPA estimated that less than 1% of the municipal solid waste generated in that year was attributed to plastics. In 2010, for which the most recent data is available, 12.4% of municipal solid waste or 31 million tons consisted of plastic containers, packaging and other plastic goods – an indication of the increasing dependence of the U.S. on plastic. While overall recycling rates have improved substantially over the last three decades (from less than 10% in 1980 to 34% in 2010), recycling rates for plastics remain relatively low (Fig. 1.3). For example, greater than 71% of

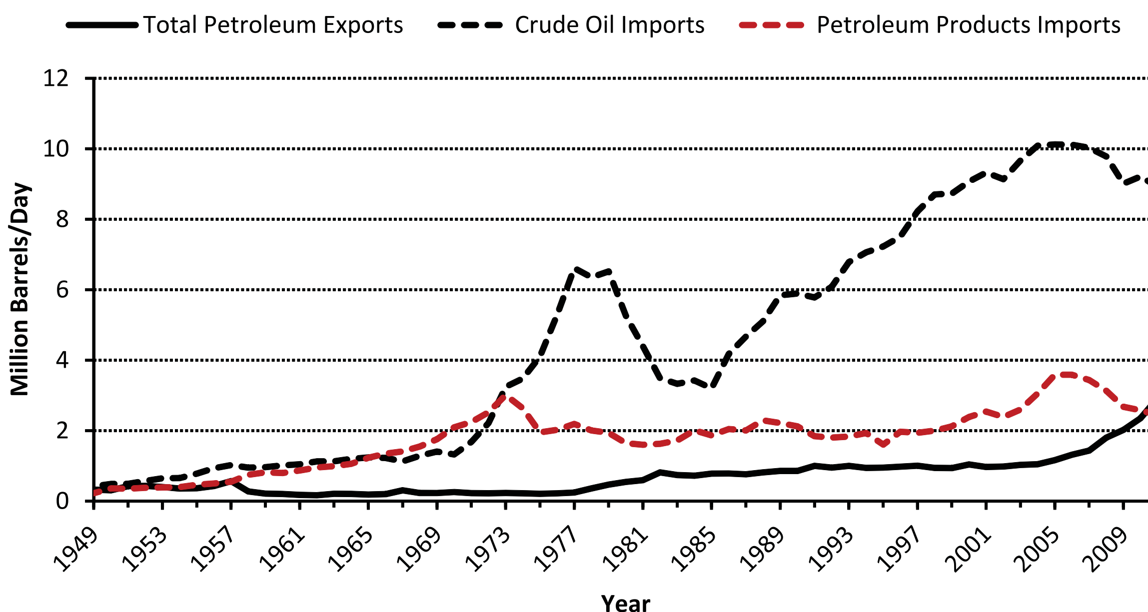

Figure 1.2. U.S. Petroleum Imports and Exports (1949-2011). Crude oil imports reached an all-time high of 10.1 million barrels per day in 2005. In 2011, crude oil imports were 8.9 million barrels/day, 3 percent below the 2010 level, and petroleum product imports were 2.4 million barrels per day. In addition, the U.S. was a net exporter of petroleum products for the first time since 1949. Source: U.S. Energy Information Administration Annual Energy review.

newspapers/mechanical papers are recycled compared with 27.5% of high density polyethylene (HDPE) and 21.0% of polyethylene terephthalate (PET) bottles. Overall recycling rates for all plastic waste was just over 8% in 2010. The challenges of waste management coupled with the need for U.S. energy security demand attention in the near-term and biotechnology offers a number of potential solutions. Specifically, processes for the biological production of a class of renewable, biodegradable plastics known as polyhydroxyalkanoates (PHA).

PHA comprise a class of polyester materials that are synthesized natively by a number of microorganisms. The polyester is accumulated as a means of carbon and energy storage when other nutrients are limiting for growth analogous to the production of fatty tissue in various animal species. Dedicated pathways exist for the conversion of two molecules of acetate to a four carbon hydroxy acid known as 3-hydroxybutyrate (3HB). Polymerization of the 3HB monomers results in an insoluble polyester inclusion inside the cell that can later be depolymerized when the carbon is again required for growth or other metabolic processes. Alternative PHA biosynthesis pathways exist that allow the cell to scavenge additional cellular metabolites for incorporation into the polyester. These metabolites are often aliphatic hydrocarbons such as fatty acids but over 150 unique monomers have been identified as capable of incorporation into PHA. Industrial strains are capable of accumulation of PHA to the extent that at least 80-90% of the dry

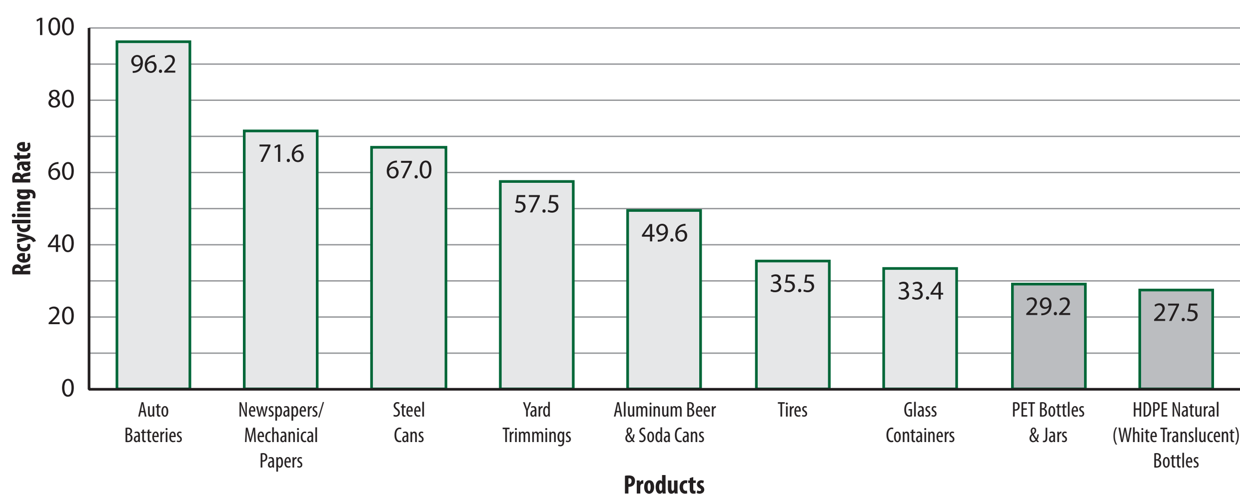

Figure 1.3. Recycling Rates of Selected Products, 2010 not including combustion with energy recovery. Auto batteries and newspapers/mechanical papers saw the highest recycling rates at 96.2% and 71.6% respectively. However, plastic products and specifically, PET bottles and jars and HDPE natural bottles were identified as having less than 30% recycling rates. Source: U.S. Environmental Protection Agency.

cell weight consists of the polyester. Recovery involves cell lysis and separation away from the cell debris at which point the purified PHA can be processed like any other conventional, petroleum derived plastic.

Today, PHA are counted among a number of commercially relevant, bio-derived polymer materials under development (*see* 2.2). It may be that a number of these materials will contribute to addressing the challenges facing the plastics industry in terms of energy independence and sustainability. However, the remainder of this thesis will focus on making a case for the development of PHA as a worthwhile research target with the potential to contribute to obviating the need for plastics derived through petrochemical processes. First, a brief history of PHA is presented to supply context and motivation for contemporary research directions. Then, a rationale is outlined for the research undertaken for this thesis. Finally, Chapter 1 is concluded with a summary of what can be found in the remainder of this thesis.

1.2. A brief history of PHA

An awareness of the history of PHA provides context and motivation for the trends in current scientific research on the topic. The story of PHA begins in the first half of the 20th century before plastic goods were commonplace. Maurice Lemoigne had been studying anaerobic growth of the bacterium *Bacillus megaterium* in the early 1920's at the Pasteur Institute in Lille, France, when he noticed that enzymatic digestion of the fermented bacteria led to a strangely acidic suspension. In 1923, Lemoigne published results postulating that the acidifying agent present was 3HB (Lemoigne, 1923) and in 1927, characterized insoluble inclusions isolated from the cells as a polymer of the acid – poly(3HB) or PHB (Lemoigne, 1927). However, PHB and PHA in general would essentially go unnoticed for years to come.

The rediscovery of PHA came in 1957 and 1960 by microbiologists and biochemists respectively culminating in the first attempt at industrialization in 1982 under the trade name "Biopol". Imperial Chemical Industries, the makers of Biopol, eventually lost interest in production when issues with cost and marketing were encountered and eventually the associated patents were obtained by U.S.-based Metabolix in 2001. In 2006, Metabolix entered into a joint venture with Archer Daniels Midland (ADM) to produce bioplastics via microbial fermentation of plant sugars and oils under the name Mirel. The joint venture, Telles, resulted in the production of 50 thousand tonnes per year of Mirel plastic resin starting in December 2010 at a plant in Clinton, Iowa. However, ADM backed out of the venture in January of 2012 stating,

"uncertainty around projected capital and production costs, combined with the rate of market adoption, led to projected financial returns for ADM that are too uncertain." Metabolix announced in August of 2012 a signed letter of intent to pursue production of Mirel at 10 thousand tonnes per year with Spanish fermentation specialist Antibioticos, although the future of Metabolix and Mirel remains uncertain.

1.3. Rationale for research approach

PHA research has been ongoing for nearly a century and there is a large body of scientific literature on topics ranging from production on wastewater to incorporation of novel monomer units to genome-scale bioinformatics. Still, new studies continue to advance the field and address current research challenges. The work described in this thesis focuses on the production of defined PHA from an unrelated carbon source; "defined" describes the ability to control which monomers are made and incorporated into the final polymer while "unrelated" implies that the monomers can be built from basic metabolites central to host metabolism. The terms "defined" and "unrelated" are important to this work as the production of novel PHA often results in heterogeneous polymers derived from chemically related substrates. While the important methods and motivations are discussed at length in the following chapters, two key ideas central to this thesis are introduced below. The first is the specific subset of PHA investigated throughout this work and particularly in Chapter 3, while the second is the field of synthetic biology which defines the approach inherent to the design of the following experiments.

1.3.1. Medium-chain-length PHA

Today, commercially available PHA such as Mirel are generally limited to homopolymers and copolymers consisting of 3HB (C_4) and 3-hydroxyvalerate (C_5) monomers which contribute to a brittle material. The properties of these plastics can be improved through blending with other materials but in the meantime, PHA are mainly used in niche applications where premium prices are commanded. The first commercially produced PHA was a copolymer of these short-chain monomers as the properties of this PHA lend readily to consumer products such as containers, packaging and films. Efforts have also been made to manufacture longer chain hydroxyalkanoates such as 3-hyrdoxyhexanoate (C_6) and 3-hydroxyoctanoate (C_8) as these polymers are better suited to making products such as molding resins, coatings, films, adhe-

sives, fibers and nonwovens. Furthermore, various co-polymers are available in sufficient quantities for application in tissue engineering and conventional medical devices (Chen and Wu, 2005).

Short-chain PHA are still an important field of study, but the commercial relevance of C_6-C_{14} monomers has shifted the focus of PHA research to include medium-chain-length (mcl)-PHA as an equally important target from both a scientific and commercial standpoint. Until recently, biosynthesis of mcl-PHA involved either directly supplementing the growth media with fatty acids or related aliphatic precursors, or rerouting metabolites from fatty acid synthesis and breakdown (Chen et al., 2000). These pathways generate a multitude of unique PHA that incorporate a variety of functionalities. However, these unique polymers come at a price – specifically the increased costs associated with growth on specialized media. In producing short-chain PHA there is substrate flexibility in that all that is needed is a carbon source which could come from waste oils (Song et al., 2008), cellulose (Keenan et al., 2006) or even CO_2 fixation (van der Meer et al., 2001) – a plasticity that is absent when a structurally related substrate is required. When nearly 50% of the operating cost associated with PHB production on glucose has been due to the price of the media (Van Wegen et al., 1998), decoupling monomer production from the carbon source may prove a more viable alternative to the use of specialty media.

Another major drawback associated with PHA derived from fatty acid synthesis and breakdown products is the lack of control over the final monomer structure and polymer composition. Even when only a single carbon source is provided, more than one monomer chain-length is observed in accordance with the diversity of intermediates produced by fatty acid metabolism (Chen et al., 2000). Consistent with the variety of resulting PHA monomers is the formation of co-polymers with variable composition. It would be unfair to say that no control is afforded when drawing upon these metabolic pathways. However, there still is the necessity to provide more complex and therefore more expensive media components in order to generate products that rarely accumulate as readily as biopolyesters such as PHB.

1.3.2. Synthetic biology

The field of "synthetic biology" has many definitions among its practitioners, but many view it as an extension of recombinant DNA technology in which complex systems are constructed inside a living organism to confer a desired ability. The systems built by synthetic biologists range in function from simple, model circuits used to study biological control (Nandagopal and Elowitz, 2011), to complex biosensors capable of interpreting a cell's environment (Xie et al., 2011), to microbe-based chemical factories capable of producing high-value products (Keasling, 2010b). All of these applications are built using a modular design process in which DNA sequences encoding both structural (*e.g.,* enzyme encoding) and regulatory components are assembled and inserted into model organisms. If a design is successful, the new DNA will confer upon the model organism a new trait or ability.

A growing application of synthetic biology is transplantation of a biological trait from a complex system to a more facile model organism. For example, many natural products have attractive medicinal properties but are made by exotic species that are difficult if not impossible to cultivate. Finding sources of material to perform clinical or pre-clinical studies is therefore challenging and developing processes for commercial production is expensive. The synthetic biology approach begins with the realization that these compounds are made via a metabolic pathway encoded by enzymes in the native organism's genome. These enzymes are proteins that if functionally expressed can perform their chemistry in a different host. Therefore, if all of the essential enzymes needed for producing a target molecule can be expressed in a facile host (*i.e.,* easy to grow, easy to control, easy to scale-up) then the chemical production trait can be exploited for research and/or commercialization. One of the most cited examples of this strategy is the production of artemisinin, an anti-malarial isoprenoid, natively produced in sweet wormwood (*Artemisia annua*) (Westfall et al., 2012). While artemisinin could be produced by extraction from crops of *A. annua*, crop yields and other process variability contributed to costs that were too high for targeted patients in developing nations. Researchers led by Jay Keasling hypothesized that if the genes needed for making artemisinin could be transferred to a common yeast strain, then a low-cost fermentation process could replace the existing extraction process. After several years of research, a strain of *Saccharomyces cerevisiae* was shown to produce gram per liter titers of the drug (Ro et al., 2006; Westfall et al., 2012). With the help

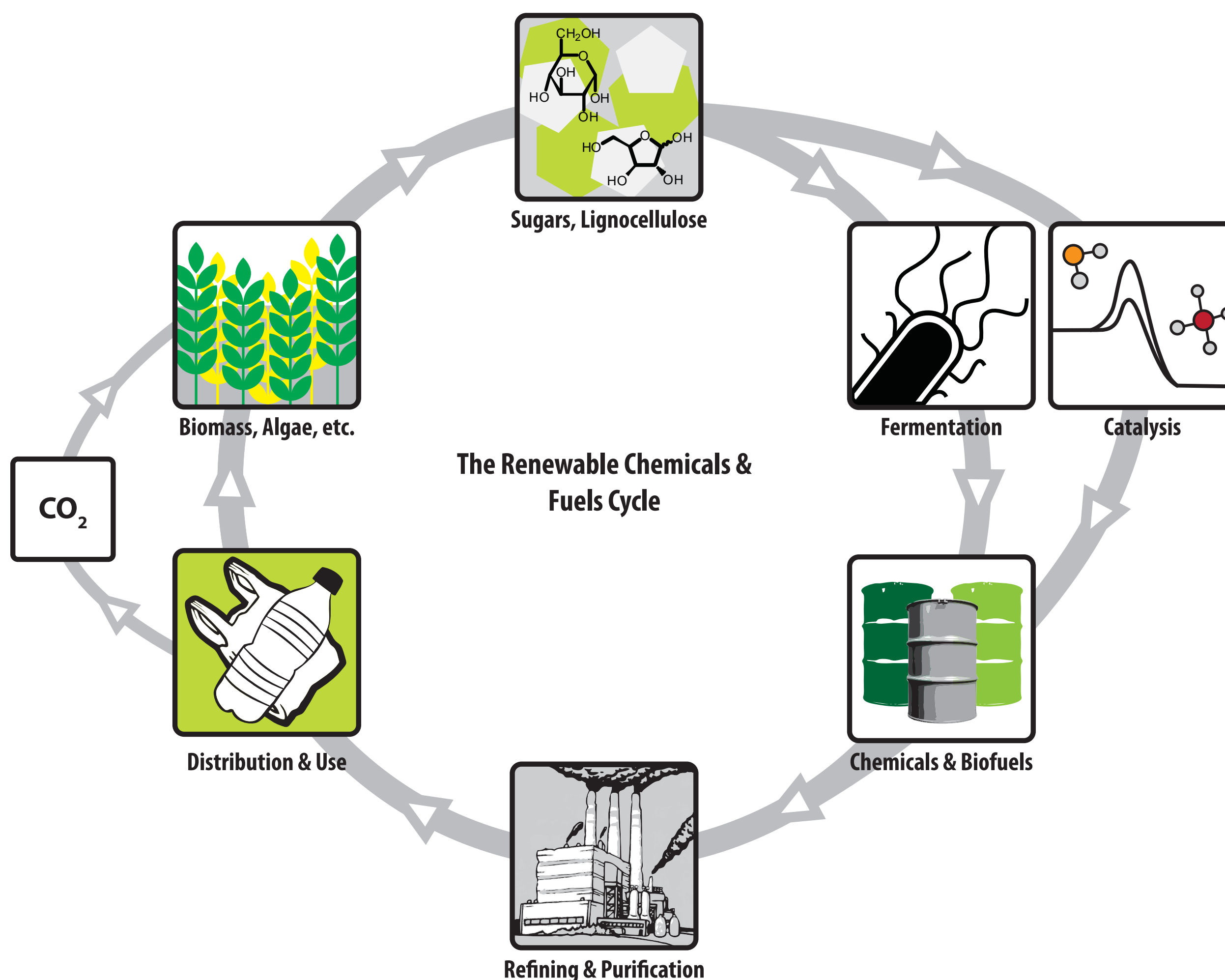

Figure 1.4. The renewable chemicals and fuels cycle. Photosynthesis converts light energy and CO_2 to plant, algal and other forms of biomass. This material can be processed into sugars, lignocellulose or other feedstocks for transformation to various chemicals and fuels via fermentation, traditional catalysis or a combination of the two. Products of catalysis and fermentation are processed for use and depending on the product, can be recycled, composted or otherwise – potentially returning CO_2 to the atmosphere. Synthetic biologists contribute to the cycle through engineering of biomass, biological approaches to process biomass into feedstocks and most notably, engineering of strains for fermentation of these feedstocks into valuable chemicals and fuels.

of Amyris Biotechnologies and the Institute for One World Health, the process has been commercialized by Sanofi-Aventis (Keasling, 2010a).

Synthetic biology approaches have been similarly used to produce natural molecules that have value as fuels, medicines, commodity chemicals, and materials (Keasling, 2010b). Prioritized lists of promising metabolites that could be made via synthetic biology have been assembled (Werpy and Petersen, 2004) and attempts to commercialize biotechnology-based processes are ongoing. More recently, synthetic biol-

ogy has also been used to engineer biochemical pathways for producing non-natural compounds - in other words, molecules not synthesized by any known organism. One example is the engineering of *Escherichia coli* by researchers at Genomatica to overproduce 1,4-butanediol on feedstocks ranging from pentose and hexose sugars to biomass (Yim et al., 2011). This approach combined theoretical modeling to predict genetic manipulations that would enable production with experimental work that tested each prediction. In general, synthetic biologists recognize the necessity for a renewable chemicals and fuels cycle and seek to build and improve upon it (Fig. 1.4).

1.4. Overview of Thesis

The remainder of this thesis explores the application of synthetic biology to the production of defined PHA, especially mcl-PHA from unrelated carbon sources. Chapter 2 comprises a review of known and potential metabolic pathways for the production of PHA starting from inexpensive carbon sources such as glucose. Original research is presented in Chapters 3 and 4 on production of PHA involving fatty acid metabolism and polyketide synthases (PKS) respectively. Chapter 3 focuses on the synthesis of several known strategies for the production of defined mcl-PHA from glucose while Chapter 4 details progress made towards the implementation of modular PKS engineering for PHA monomer production. Finally, Chapter 5 details direction for future research on PHA biosynthesis.

1.4.1. Chapter 2

A major component of PHA research has been to explore the potential of PHA polymerase to incorporate unique monomers with the goal of manipulating the material properties of the resulting polymer. The landscape of PHA monomers is diverse but has not been well documented since the publication of a seminal review in 1995. Chapter 2 of this thesis updates the list of known monomers that have been incorporated into PHA and describes the categories of monomers that are new to the list. A majority of the more than 150 unique monomers were incorporated into PHA by culturing the host strain in the presence of a related substrate. This approach allows for the biosynthesis of novel PHA with the drawback of using expensive, related carbon sources over more cost-effective options such as glucose or glycerol. Discussion of novel monomers thus segues into a review of the known PHA biosynthetic routes that originate from unrelated carbon sources. This approach can be thought of as a three piece puzzle involving monomer

synthesis, activation and polymerization. The emphasis of Chapter 2 is therefore on ways to create PHA with desirable properties by identifying routes to monomers from unrelated, inexpensive carbon sources such as glucose.

1.4.2. Chapter 3

With Chapter 2 providing a foundation for potential approaches to PHA biosynthesis, Chapters 3 and 4 describe original research on the production of defined PHA from unrelated carbon sources using fatty acid and PKS overproduction respectively. Previous work in the Pfleger Lab resulted in a strain of *E. coli* capable of overproduction of medium chain-length free fatty acids with a defined composition (chain-length) via heterologous expression of an acyl-ACP thioesterase. It was hypothesized that a pathway could be engineered for mcl-PHA biosynthesis with a composition matching the fatty acid profile. With fatty acids produced from glucose, defined PHA could be synthesized from an unrelated, inexpensive carbon source. To this end, Chapter 3 describes the approach taken to successfully engineer this strain of *E. coli*. Fatty acid metabolism is an iterative process involving addition or subtraction of two-carbon units to build or disassemble the molecule respectively. In order to achieve conversion of the free fatty acids to PHA without altering the free fatty acid profile, a library of deletions in fatty acid metabolism was assembled and characterized. This library revealed the ability of *E. coli* to complement deletions in fatty acid degradation and informed further strain design. Furthermore, a series of PHA genes were characterized from two *Pseudomonas* species and glucose-based production of mcl-PHA with a defined composition was achieved at up to 17% of the cell dry weight in a bioreactor.

1.4.3. Chapter 4

Given that the strategy discussed in Chapter 3 is limited to PHA derived from fatty acids, an alternative and potentially broader approach to PHA biosynthesis was explored. Chapter 4 outlines a methodology for building a PKS platform for the monomer biosynthesis and progress made toward achieving this goal. PKS are modular enzymes that act in an assembly-line fashion to build complex molecules from basic cellular building blocks. The modular nature of PKS has been compared with Lego® bricks in the sense that PKS parts can be reassembled to produce new products in a predictable fashion. Two PKS enzymes were identified and genetically modified to catalyze the production of PHA monomers. Functional expression of

the constructs in *E. coli* proved to be a key obstacle to PHA biosynthesis and a series of experiments was performed to inform future strain design and construction.

1.4.4. Chapter 5

Finally, Chapter 5 is an aggregate of experiments in support of future work to further advance research into production of defined PHA from unrelated carbon sources. Topics such as pathway tuning for strain optimization and improvement of PHA biosynthesis through co-expression of phasins are discussed. Additional topics include the use of alternatives to glucose such as acetate and levulinic acid, a product of the acid-catalyzed hydrolysis of lignocellulosic biomass. The ability of *E. coli* to produce PHA on various carbon sources is explored. In particular, this chapter elaborates on the potential of levulinic acid as a carbon source for fermentation in both *Pseudomonas putida* and *E. coli*, the former of which is capable of growth on levulinic acid as a sole carbon source. Chapter 5 concludes with a summary of the thesis work and provides direction and advice for future lines of investigation.

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Chapter 2: Synthetic biology strategies for synthesizing PHA from unrelated carbon sources

2.1. Introduction

The commercial potential of renewable, biodegradable plastics has been a driving force for research focused on the biological synthesis of PHA with material properties that match their petroleum-derived counterparts. Since the discovery of PHA in the 1920's, it has been postulated that more than 150 unique monomers have been incorporated into a PHA polymer. Varying the monomer composition impacts the material properties of the polyester and provides handles for further functionalization in specialty applications. The ability to synthesize custom PHA will be critical to the commercial relevance of this class of materials. Therefore, one approach has been to identify substrates for PHA polymerase, usually through feeding studies and subsequent characterization of the resulting material. The broad specificity of PHA polymerases is both impressive in their ability to accept more than 150 hydroxy acids, and appealing from an industrial perspective. Specifically, the opportunity to capitalize on a versatile platform for generating a series of PHA that could supplement and eventually replace traditional plastics has been the perpetual promise of PHA since the first commercial enterprises in the 1980's. In the intervening time, a number of ventures have emerged around the globe [recently reviewed here: (Chen, 2009)] and yet, PHA are not a pervasive material in daily life. Therefore, an important question to address is, "what is the barrier that prevents widespread application of these materials?"

One possible answer to this question centers on the inherent costs associated with PHA production. Simple PHA such as PHB can be made from inexpensive carbon sources like glucose but more complex, and potentially more interesting PHA are often produced by feeding more expensive, structurally related substrates. An approach that is implemented throughout this thesis is to link pathways for unique monomer biosynthesis for tailor-made PHA with the use of inexpensive and renewable feedstocks. This strategy for enhancing PHA production via synthetic biology is based upon the following logic:

1. More than 150 monomers can be incorporated into PHA, providing routes to a wide range of material properties that would be useful in high-value applications.

2. The cost of related feedstocks that are fed to PHA accumulating organisms to make non-traditional PHA precludes the economic viability of these materials.
3. Synthetic biology can and has been used to assemble novel metabolic pathways inside cells for producing high-value molecules relevant to chemical and polymer synthesis.
4. If metabolic pathways that link unrelated feedstocks (*e.g.,* glucose) to high-value PHA monomers are assembled, then PHA production costs could be greatly reduced.

For these reasons, we posit that research should turn towards developing feedstock-independent, synthetic pathways for producing an increased diversity of PHA monomers that when polymerized produce materials capable of competing with traditional, petroleum-derived plastics. To elaborate on this concept, Chapter 2 first describes the current landscape of renewable polymer materials and provides examples of how PHA compares with these other materials. The remainder of this chapter provides a brief summary of the range of monomers that have been incorporated into PHA granules and suggests future research directions that leverage the promising field of synthetic biology to increase the viability of PHA as a petrochemical plastic alternative.

2.2. Renewable polymer materials

Polymers derived from biological materials and/or processes have long been used in commercial products. Given the variety of sources, there are a number of ways these polymers might be classified. From a production standpoint, there are roughly three categories (Figs. 2.1 and 2.2): (i) natural polymers that require processing (i.e., chemical/mechanical) before use, (ii) monomers produced by biological conversion of sugars or other feedstocks that are later polymerized by chemical processes and (iii) biologically derived polymers that are useful without further processing. It is important to note the potentially subtle differences between these three categories. For example, both starch and PHA are biological polymers. Both can be produced in and extracted from plants. However, starch requires chemical modification before it is useful for blending with other polymers, whereas PHA can be potentially used as is. The following section elaborates on these three categories of polymers and concludes with examples of where PHA have succeeded and where other alternatives are preferred.

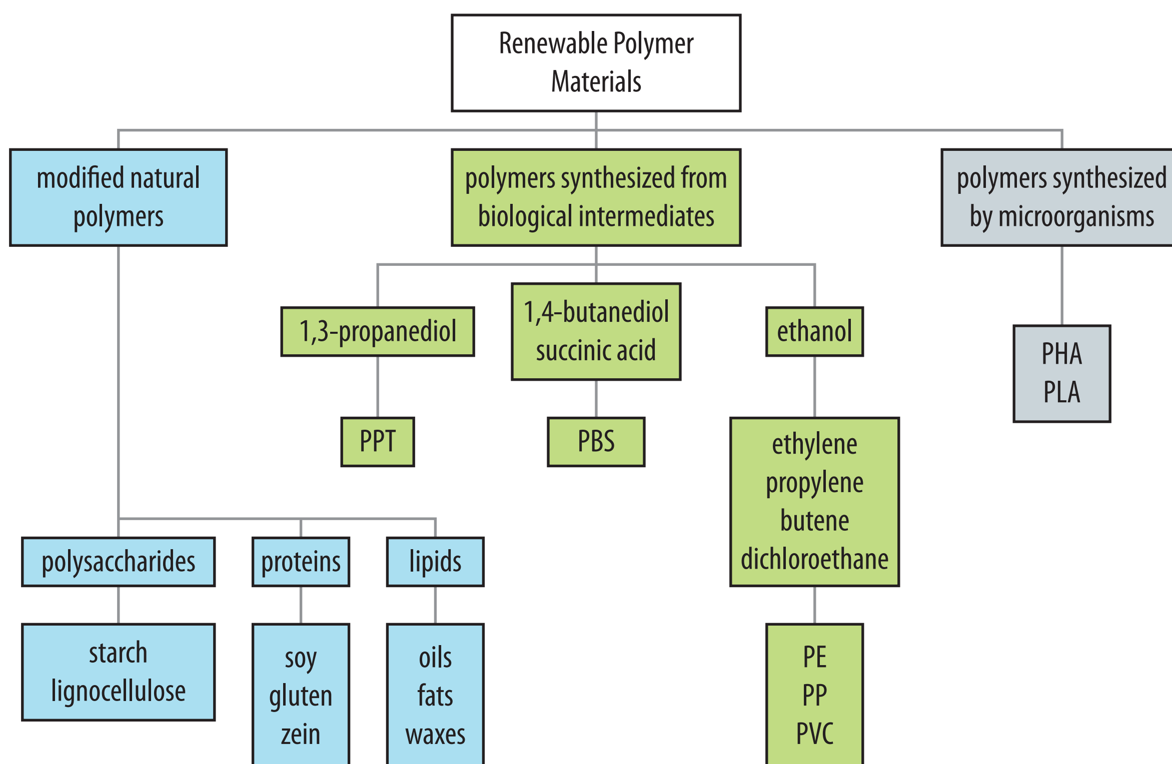

Figure 2.1. Renewable polymer materials organized by origin. PLA could also be grouped under polymers synthesized from biological intermediates. See text for details and abbreviations.

2.2.1. Modified natural polymers

There are three main classes of biological polymers that are currently extracted from biomass on an industrial scale: polysaccharides, proteins and lipids. Of these, polysaccharides are the most relevant to the bioplastics industry. Specifically, starches can be used alone or in combination (*i.e.,* through blending) with other polymeric materials to achieve a renewable, biodegradable product. As of 2008, starch-based products accounted for 170 thousand annual tons of material with production coming from seven different manufacturing firms (Queiroz and Collares-Queiroz, 2009). Starch and mono-, di- and oligosaccharides in general, are often blended with other (biodegradable) polymers as plasticizers (Vieira et al., 2011). A plasticizer is defined by the IUPAC as ‘‘a substance or material incorporated in a material (usually a plastic or elastomer) to increase its flexibility, workability, or distensibility’’ and is used to decrease the glass transition temperature (T_g) of the material. Examples of starch-based products include disposable cups, compostable bags, pen casings, and foamed trays (Satyanarayana et al., 2009).

Proteins and lipids are also relevant materials from a commercial standpoint. While these materials are not generally classified as "bioplastics," they are nonetheless biopolymers. Proteins that come from animals include whey, casein, collagen and gelatin while examples from plants include soy, gluten and zein – the latter of which is a plant storage protein known as a prolamine. Lipid products include oils, fats and waxes. These lipid materials are not necessarily polymers, but are often used in polymer production. For example, 12-hydroxy-9-cis-octadecenoic acid, or ricinoleic acid, is a fatty acid derived from castor oil. Ricinoleic acid can be dehydrated to form a conjugated acid for use in protective coatings or cleaved at the double bond yielding C_{11} and C_7 acids of which the former can be further processed to produce nylon 11 (Naughton, 1974). Furthermore, lipids are often used as substrates for the production of mcl-PHA – a topic which is discussed at length in Chapter 3 of this work.

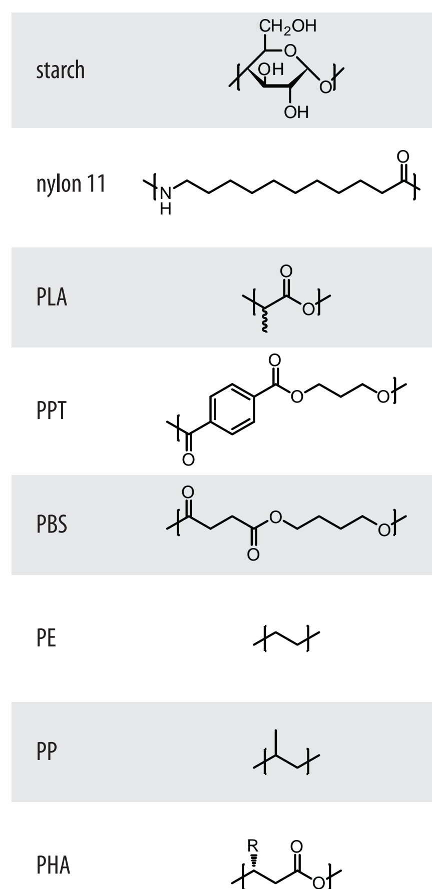

Figure 2.2. Chemical structures of selected polymers discussed in section 2.2. See text for abbreviations.

2.2.2. Polylactic acid

Polylactic acid (PLA) is one of the most successful bio-derived polymers with production in excess of 90 thousand tons as of 2008 (Queiroz and Collares-Queiroz, 2009). PLA is composed of purified lactic acid derived from fermentation of sugars by organisms such as *Lactobacillus* (Wang et al., 2010) and engineered *Enterococcus* (Wee et al., 2008). Depending on the host metabolism, production of both D and L isomers of lactate from pyruvate is possible. The stereochemistry of the final product impacts the material properties of the polymer which is one of the benefits of the use of fermentation over traditional catalytic processes (Mehta et al., 2005). Although PLA is herein categorized as a polymer produced by fermentation of the monomer (lactic acid), recent work has demonstrated the possibility of direct fermentation of PLA homopolymer and copolymers with 3HB (Park et al., 2012; Yang et al., 2010). Since the 1970's, PLA was really only considered as a viable commercial product for biomedical and pharmaceutical applications, but

over time, research advances made by companies such as DuPont and Cargill led to large-scale production of PLA for bulk applications (Sudesh and Iwata, 2008). Because PLA performs similarly to petroleum derived plastics such as polyethylene (PE), polypropylene (PP) and polystyrene (PS) both in terms of processing and material properties, it has found uses is plastic cups and bottles and as component parts in notebook computers and cell phones.

2.2.3. Polypropylene terephthalate

1,3-Propanediol (PDD/PDO) is fermented from glycerol using organisms such as *Clostridium* and *Klebsiella*, and from glucose in the case of engineered strains of *E. coli* (Willke and Vorlop, 2008). PDO is a versatile building block for the production of a variety of polymers. For example, PDO can be polymerized with fossil based dimethylterephthalate (DMT) or purified terephthalic acid (PTA) to produce polypropylene terephthalate (PPT) which has been commercialized under the names Sorona™ and Corterra™ by DuPont Tate & Lyle BioProducts and Shell Chemical Company, respectively (Rincones et al., 2009). Bio-derived PDO has been successful as it is competitive with PDO derived from petroleum when feedstock (sugar) prices are favorable compared with crude oil (Vickers et al., 2012). In addition, PDO produced through fermentation is estimated to consume less non-renewable energy and result in less than half as much CO_2 emissions compared with the petrochemical process. Industrial applications of PDO include adhesives, cosmetics and detergents while polymers such as PPT have applications in fabric and fiber manufacturing.

2.2.4. Polybutylene succinate

Polybutylene succinate (PBS) is a polymer composed of 1,4-butanediol (BDO) and succinic acid – both of which are capable of renewable production via fermentation. As recently as 2011, BDO was produced using traditional catalysis beginning with petroleum-based feedstocks. Starting materials include acetylene and maleic anhydride which are catalytically converted to the final product by the Reppe process and liquid-phase hydrogenation, respectively (Küksal et al., 2002). Today, scientists at Genomatica have found a way to achieve BDO production in *E. coli* at titers of up to 18 g L^{-1} (Yim et al., 2011). This accomplishment was especially impressive as no known enzymatic route to BDO exists in nature.

Succinic acid is an intermediate in the tricarboxylic acid (TCA) cycle and can be a fermentation end-product depending on culture conditions. Several bacterial strains have been studied for succinic acid production including *Actinobacillus succinogenes* and *Corynebacterium glutamicum* (Cheng et al., 2012). Interestingly, succinic acid can be converted to BDO as well as a number of other compounds such as γ-butyrolactone and tetrahydrofuran (Bechthold et al., 2008). As a result, there is a significant demand for succinic acid with worldwide production at 30-50 thousand tons annually and a market expected to be as large as 100 thousand tons by 2015 (Beauprez et al., 2010). When combined with BDO the resulting PBS polymer has desirable thermal and mechanical properties that provide for an effective alternative to traditional plastics such as PE and PP (Xu and Guo, 2010).

2.2.5. Polyethylene and polypropylene

Fermentation of sugarcane (sucrose) to ethanol is one of the most advanced technologies in the field of industrial biotechnology. The U.S. and Brazil are the world's major ethanol producers (Jagger, 2011), although Asia accounts for nearly half of the worlds sugar production followed by Brazil, which makes up an additional third (Guzman and Finch, 2011). Production in the U.S. alone exceeds 15 billion gallons annually with 80-90% used for fuel applications (ICIS, 2011). The bio-ethanol process uses the yeast strain *S. cerevisiae* to achieve final concentrations of 10-12% ethanol under anaerobic batch fermentation conditions. While the resulting ethanol can be used directly as a fuel, a number of subsequent processing steps allow for production of ethylene, butene, propylene and dicholoroethane along with polymers of these compounds.

The production of polymers from ethanol is currently the focus of a number of commercial ventures. The catalytic dehydration of ethanol to ethylene followed by a dimerization process results in 1-butene. Isomerization of 1-butene and metathesis with ethylene results in propylene, while chlorination of ethylene leads to vinyl chloride. Ethylene, propylene and vinyl chloride are all drop-in chemicals for traditional polymer applications. Since late 2010, Braskem has operated a plant in Brazil for the production of HDPE at 200 thousand tonnes per year and is on schedule to begin operating a PP plant with a capacity of up to 50 thousand tonnes per year (Landress, 2012). Further down the road, a joint venture between Dow and Mitsui & Co (Japan) should result in a 250 thousand tonne per year PE plant to be operational in

2015. A final venture between Belgium and Brazil-based companies is exploring the use of vinyl chloride from ethanol for a renewable polyvinylchloride (PVC) plant.

2.2.6. Polyhydroxyalkanoates

PHA are a naturally occurring class of biopolyesters common to eubacteria and archaea – especially soil bacteria such as pseudomonads and bacilli. PHA are unique among fermentation derived polymers and plastics because the host organism is responsible for both monomer synthesis and polymerization *in vivo*. Moreover, PHA are a class or polyesters rather than having a single molecular makeup as is the case for many of the monomers/polymers described above. The most basic requirement for PHA production is a hydroxyacyl intermediate which allows for variation in the position of the hydroxyl moiety, the chain-length of the carbon backbone and the chemical make-up of the side chain in general. This monomer diversity allows for the biosynthesis of plastics with a range of chemical and mechanical properties and the result is a renewable, usually biodegradable polyester that can be harvested directly from the cell for use in traditional plastic applications.

Depending on the type of PHA produced, the polyester is suitable for incorporation into products such as molding resins, coatings, films, adhesives, fibers and nonwovens. Additionally, PHA create excellent barriers to water vapor, oxygen and other gases and are hydrolytically and UV stable (Imam et al., 1998). Several attempts at commercialization of PHA have been made dating back to an unsuccessful venture by Imperial Chemical Industries in the 1980's. A number of companies around the world are currently invested in industrial PHA processes. In the U.S., Metabolix maintains a portfolio of over 700 PHA patents and is the primary industrial presence. In January of 2012, a partnership between Metabolix and ADM ended along with the operation of the sole production plant. Meanwhile, PHA is being commercialized around the globe. For example, Tianjin GreenBio Materials Co. has the largest PHA production facility in the world located in Binhai District, Tianjin, China with a capacity of 10,000 tons per year.

2.2.7 PHA: Successes and setbacks

While PHA have struggled to gain a commercial foothold, other renewable plastics such as PLA and PBS have has some success due to their lower cost. In addition, companies such as Coca-Cola have

Table 2.1. Prices for a selection of common polymers derived from both traditional (petrochemical) and alternative (biological) routes. Pricing reflects most up to date data available from ICIS Chemical Business.

Compound	Price ($/lb)
PVC	0.45
PET	0.59-0.85
PP	0.74
LDPE	0.77
PLA	0.85-1.25
PS	1.00
Starch-based biodegradable plastics	1.50-2.20
BASF Ecoflex	2.00
PLA/PBS	2.00-2.50
PHA (Mirel)	2.25-2.75

Table 2.2. Prices for a selection of common feedstocks associated with polymer synthesis. Pricing reflects most up to date data available from ICIS Chemical Business.

Compound	Price ($/lb)
glucose	0.20
stearic	0.30
glycerin (low grade)	0.40
ethanol	0.42
naptha	0.42
coconut oil (~45% lauric acid)	0.50
oleic	0.60
glycerin (pharmaceutical grade)	0.65
butanediol	0.98
propionate	1.00

sought alternatives for their plastic bottles which are manufactured from renewably sourced terepthalic acid and monoethylene glycol with plant-derived precursors such as paraxylene and ethylene, respectively (Harracksingh, 2012). While many non-PHA renewable plastics are being developed (Harracksingh, 2012; Sudesh and Iwata, 2008), PHA biochemistry offers flexibility to pursue a wide range of material properties from the same platform.

The most promising, near-term commercial applications of PHA are in the medical, biomaterials and pharmaceutical industries where premium prices are easily commanded or in other niche markets where there is a demand for biodegradability. For example, Metabolix, has had commercial success in incorporating Mirel™ (PHA) into biodegradable gardening containers and premium eco-friendly beach toys. On the other hand, the potential of PHA as a replacement for non-biodegradable, petroleum derived plastics has yet to be realized due to the premium (~$0.75/lb) over comparable, renewably sourced polymers and even larger premiums compared to traditional plastics (Table 2.1). Metabolix's Mirel™ is marketed at $2.25-$2.75/lb, whereas polypropylene, a plastic with comparable material properties, is available at $0.75/lb. Oil price volatility and increasing demand for sustainable alternatives have continued to motivate researchers to improve PHA production processes despite past obstacles to commercialization.

2.3. Diversity of PHA monomers

In 1995, the review "Diversity of Bacterial Polyhydroxyalkanoic Acids," detailed the known monomer constituents of this class of polyester materials (Steinbüchel and Valentin, 1995). At the time of publication, a striking 91 unique monomers had been characterized as substrates for a PHA polymerase. Since then, it has been postulated that more than 150 unique PHA monomers have been described in the literature (Steinbuchel and Lutke-Eversloh, 2003). Confirming this claim, we have identified an additional 64 monomers that have been described in the PHA literature bringing the current total to 155 unique monomers (Figs. 2.3, 2.4 and Appendix 2). Of the 64 unique monomers described since the 1995 review, many are closely related to prior classes of known PHA monomers, but a handful of new functional groups were successfully incorporated. These include dimethyl substituted carbons, terminal methyl- and fluorophenoxy, thiophenoxy, oxo (keto) and acetylthioester groups (Fig. 2.5). Additionally, an expanded number of 4-hydroxy and methyl substituted PHA monomers have been added to the list of unique monomers.

Of the 155 unique monomers that have been incorporated into PHA, a relatively small subset has been produced from an unrelated feedstock (carbon source). Here, a related feedstock is defined as any

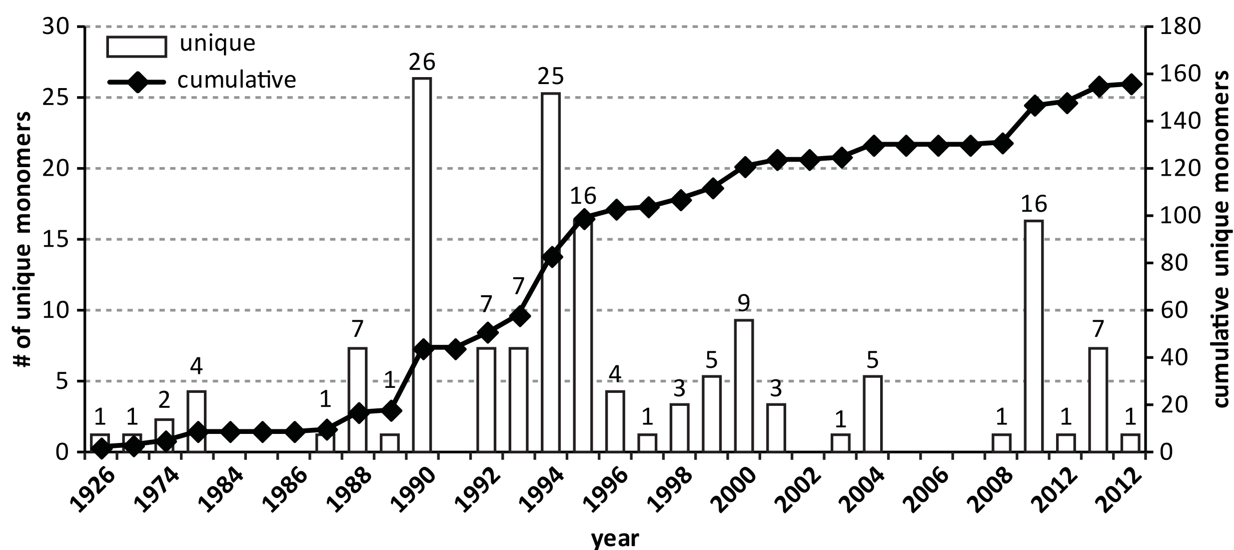

Figure 2.3. Unique PHA monomers discovered since 1926. Bars indicate number of unique PHA monomers discovered in a given year while points indicate the cumulative total number of unique monomers. Note that the horizontal axis is non-linear for 1926-1983 and linear from 1983-2012.

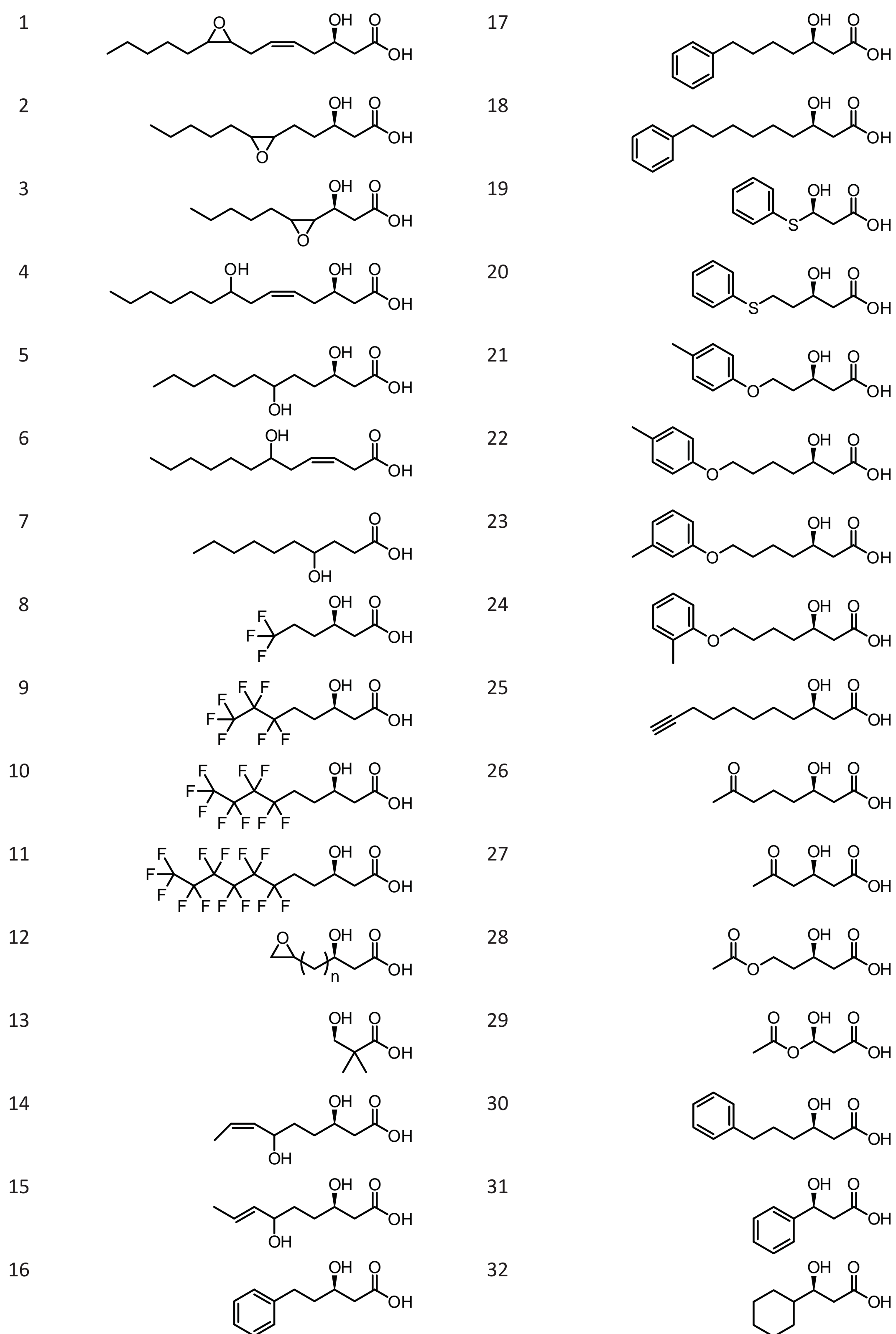

Figure 2.4. Structures of 64 unique monomers discovered since 1995. See Appendix 1 for names and references.

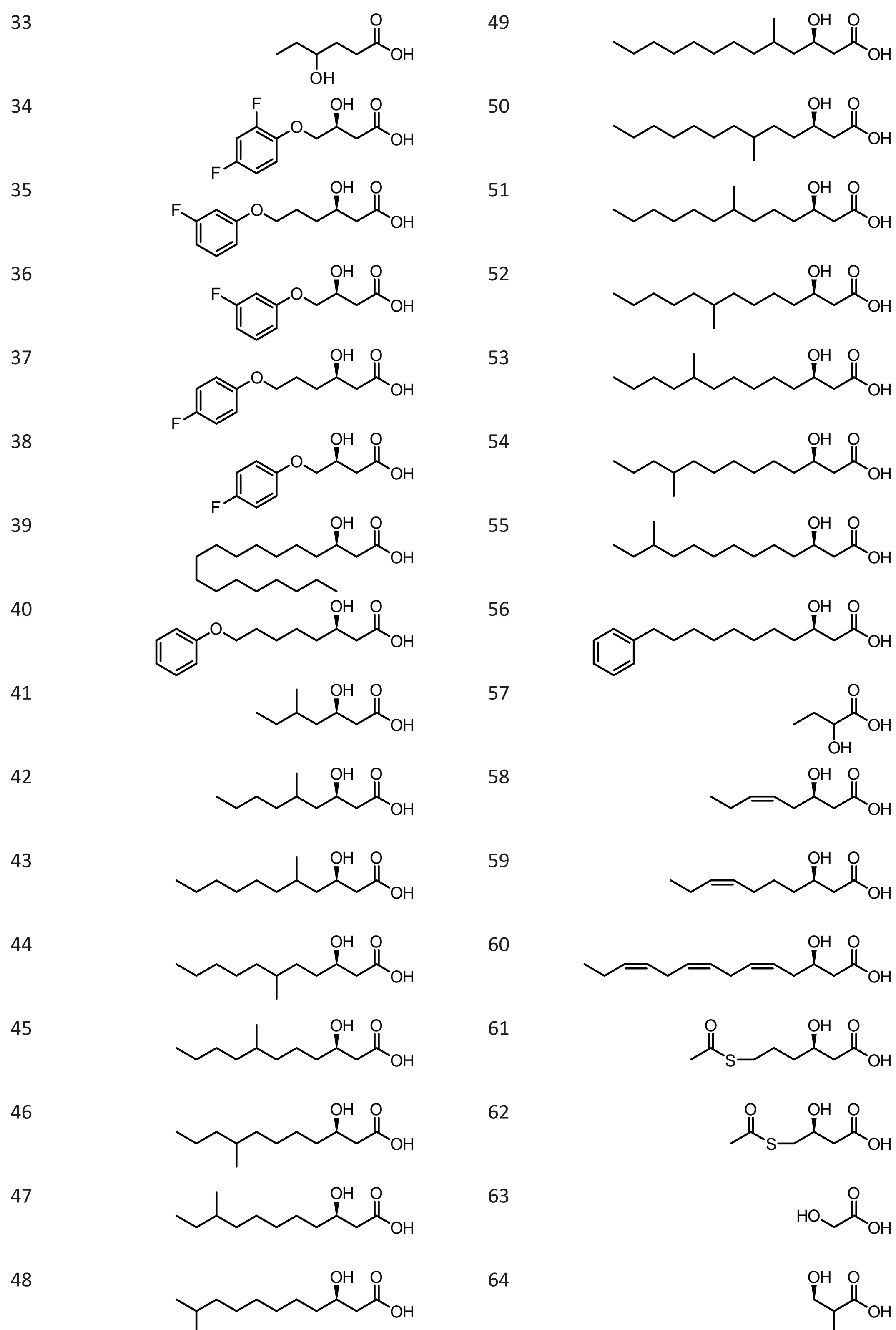

Figure 2.4. (continued)

starting material that is structurally related to the resulting monomer and especially the resulting side-chain/pendant group. In many cases feeding of exotic feedstocks will be the only feasible route to producing the desired PHA monomer. However, the costs associated with synthesizing, purifying, and feeding complex precursors to a PHA producer will significantly limit economic viability – especially in situations where traditional chemistry can be used to make similar polyesters (Table 2.2). Conversely, there are examples where existing biochemical pathways can be metabolically engineered to produce desirable monomers without the need of feeding related substrates. For example, feeding of lipids to PHA accumulating bacteria is one route to produce mcl-PHA (*see* 2.5.4). These compounds could also be produced by engineering fatty acid metabolism to synthesize the lipids *de novo.* Considering the value of plant oils and the corresponding demand for their use in biodiesel and oleochemical production, it will be more economical to use an unrelated feedstock.

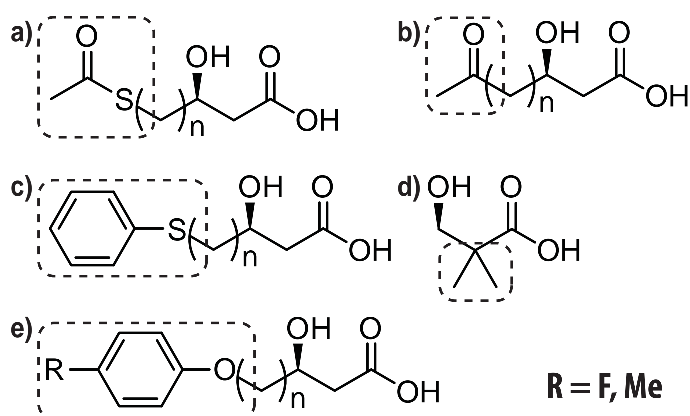

Figure 2.5. New categories of unique PHA monomers described since 1995 including (a) acetylthioester (b) oxo (keto) (c) thiophenoxy (d) dimethyl substituted carbons and (e) terminal methyl- and fluorophenoxy groups where R = F, Me (para-substitution shown as an example). See Fig. 2.4 for monomer structures.

A successful industrial process will produce PHA at or near theoretical yields and identifying an optimal feedstock is non-trivial. For example, the stoichiometric carbon yields of C_{12} mcl-PHA on glucose, acetate and C_{12} fatty acids are 1/2, 1/6 and 1 respectively. Given current prices, the cost of C_{12} mcl-PHA per pound based on maximum theoretical carbon yield is \$0.33 for glucose, \$0.67 for acetate and \$0.46 for coconut oil assuming a composition of 100% lauric acid (Eq. 2.1).

$$\frac{\$}{\text{lb feedstock}} \times \frac{\text{MW feedstock}}{\text{MW 3HD}} \times \frac{\text{mol}_C\ \text{feedstock}}{\text{mol}_C\ \text{3HD}} = \frac{\$}{\text{lb 3HD}} \qquad \textbf{Eq. 2.1}$$

where,

$$\frac{\text{mol}_C\ \text{feedstock}}{\text{mol}_C\ \text{3HD}} = \text{stoichiometric ratio of feedstock to 3HD on a carbon basis}$$

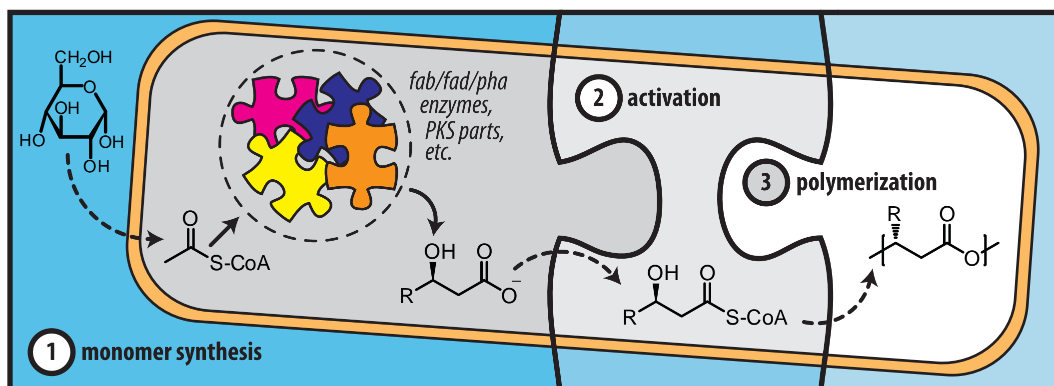

Figure 2.6. PHA biosynthesis viewed as a three piece puzzle. The first piece consists of feeding an unrelated carbon source such as glucose to a cellular host possessing the necessary parts (metabolic pathways) for the conversion of cellular building blocks to hydroxyalkanoic acid monomers. The second piece is the activation of these monomers with CoA followed by polymerization by a PHA synthase in the third and final piece of the puzzle.

While this price estimate does not take into account carbon lost during metabolism or factors such as the ability of the cellular host to grow on and tolerate the aforementioned substrates, these numbers argue that a careful analysis of the economics of feedstock choice is critical. Furthermore, this type of analysis may indicate circumstances in which the use of a structurally related feedstock may be economically favorable for PHA biosynthesis. When considering mcl-PHA production involving *de novo* fatty acid biosynthesis (*see* Chapter 3), it is important to not that theoretical yields of fatty acids on glucose have yet to be achieved (Lennen and Pfleger, 2012). However, the US Department of Agriculture estimates the 2012/13 world coconut oil supply at 6 million metric tons indicating that commercial-scale production of mcl-PHA from coconut oil may not be viable. Thus, depending on the scenario, adopting a synthetic biology approach for constructing and engineering pathways to novel PHA monomers could lead to the development of processes for manufacturing higher-value PHA at low cost.

2.4. Synthetic biology for PHA economy

The synthetic biology approach to making a PHA from an unrelated carbon source can be viewed as a three piece puzzle (Fig. 2.6). First, one must identify a (ideally flexible) metabolic pathway that leads to a defined composition of organic acids. Second, the resulting molecules must be activated via coenzyme A (CoA) ligation and modified (where necessary) to exhibit a hydroxyl group with the required chirality. Third, the monomers must be presented to a PHA synthase capable of catalyzing polymerization. When suitable side-chains (*e.g.,* a terminal alkene) are present, a fourth, post-synthesis step involving chemical

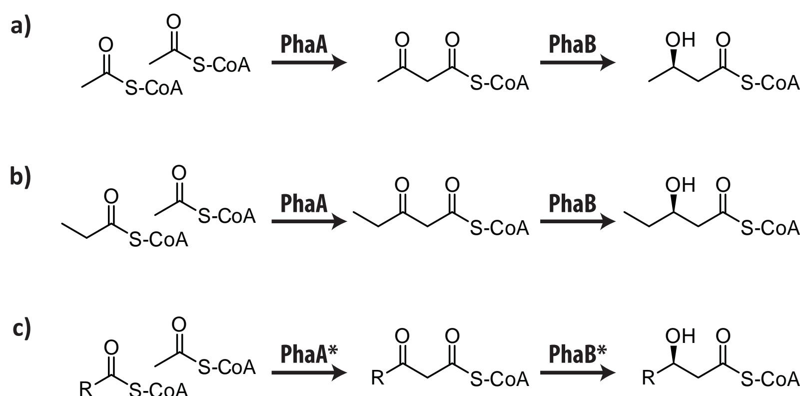

Figure 2.7. Dedicated pathways for scl-PHA biosynthesis. The pathways for (a) the biosynthesis of PHB involves the condensation of two acetyl-CoA by β-ketothiolase (PhaA) and formation of the hydroxyl group by acetoacetyl-CoA reductase (PhaB). Alternatively this metabolic pathway can lead to the formation of (b) hydroxyvalerate monomers or (c) provide hypothetical route to a number of longer chain-length monomers if variants of PhaA and PhaB can be identified or engineered.

functionalization is conceivable (in addition to blending PHA with other polymers) to achieve the desired material properties and process economics. The remainder of the review will examine each of these steps in more detail.

2.5. Monomer synthesis

2.5.1. PHB: Context for monomers from unrelated feedstocks

To appreciate the elegance of native PHA biosynthesis routes and contextualize routes to PHA from unrelated feedstocks, it is important to begin with PHB. The archetypical and arguably most studied PHA monomer is 3HB, a four carbon hydroxycarboxylic acid. PHB is a polyester of 3HB that is readily accumulated by a number of organisms as a form of carbon and energy storage. PHB synthesis is conferred by the *phaABC* genes (Fig. 2.7) best studied in the bacteria *Cupriavidus necator* (a.k.a. *Ralstonia eutropha*) (Madison and Huisman, 1999). The genes *phaA* and *phaB* encode β-ketothiolase (EC 2.3.1.9) and acetoacetyl-CoA reductase (EC 1.1.1.36) for the condensation of two acetyl-CoA units to acetoacetyl-CoA and subsequent reduction of the newly formed β-keto group to 3-hydroxybutyryl-CoA. The short-chain-length (scl) specific PHA polymerase (*phaC*, no EC number), often referred to as PHB polymerase, incorporates the monomer units into the PHB polymer.

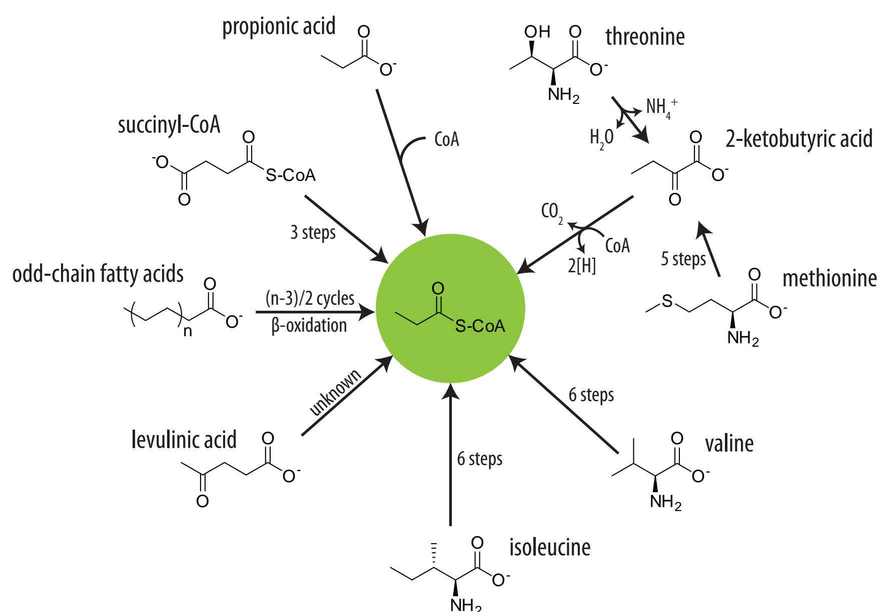

Figure 2.8. Known biosynthetic routes to propionyl-CoA.

The PHB pathway is dedicated to PHA biosynthesis (*i.e.,* not used to make other metabolites) and affords tight control of the resulting monomer composition. One exception is that when propionate is introduced as a feedstock it is possible to produce 3-hydroxyvalerate (3HV), a five carbon hydroxycarboxylic acid monomer. 3HV is produced analogous to 3HB with the key difference being the replacement of one of the two acetyl-CoA units with propionyl-CoA in the initial condensation step carried out by the β-ketothiolase. Many organisms are capable of synthesizing a co-polymer of 3HB and 3HV (3HB-co-3HV) when propionate is present. While PHB is brittle (glass transition temperature (T_g) = 4 °C, melting temperature (T_m) = 175 °C), incorporation of 3HV yields a copolymer with a slightly lower T_g and T_m depending on the fraction of 3HV incorporated (*e.g.,* for mol% 3HV = 20; $T_g \approx 2$ °C, $T_m \approx 171$ °C). Because poly(3HB-co-3HV) has properties that lend readily to consumer products such as containers, packaging and films, it was the first commercially produced polymer. However, as mentioned, processes for making poly(3HB-co-3HV) have traditionally involved an external supply of propionic acid.

2.5.2. 3HV from unrelated feedstocks

The case of 3HV is one example of a focused research effort to identify biosynthetic pathways initiating from an unrelated feedstock (Fig. 2.8). Propionyl-CoA, the activated form of propionic acid can

be derived from the amino acids valine, threonine, methionine and isoleucine (Steinbuchel and Lutke-Eversloh, 2003). While valine, isoleucine and methionine catabolism to propionyl-CoA consists of a number of intermediate steps, the threonine pathway passes through a single intermediate, α-ketobutyrate, yielding one molecule each of propionyl-CoA, CO_2 and ammonia (Slater et al., 1999). Plant species such as *Arabidopsis* (Rebeille et al., 2006) and bacteria such as *Pseudomonas* (Inoue et al., 2003) are additionally capable of methionine catabolism to α-ketobutyrate. Eschenlauer *et al.,* demonstrated that the addition of 1 mM valine or threonine to glucose minimal media resulted in PHA consisting of up to 4% 3HV (Eschenlauer et al., 1996). More recently, an engineered strain of *E. coli* was shown to be capable of producing either (R)- or (S)-3-hydroxyvaleric acid via threonine catabolism with titers as high as 0.50 g L^{-1} and 0.31 g L^{-1}, respectively (Tseng et al., 2010).

There also exists the possibility to feed alternative, low cost carbon sources for the production of PHA monomers. For example, levulinic acid is readily derived from lignocellulosic biomass via acid treatment and is a promising feedstock for producing chemicals (Alonso et al., 2010). Bacteria such as *P. putida* and *C. necator* are capable of PHA incorporating 3HB, 3HV and 4-hydroxyvaleric acid (4HV) monomers into PHA when fed levulinic acid (Jaremko and Yu, 2011; Martin and Prather, 2009). A proposed pathway for levulinic acid catabolism that includes 4- and 3-hydroxyvaleryl-CoA is based on the PHA composition observed in these cells.

Two other known routes to endogenous production of propionyl-CoA include odd chain-length fatty acid β-oxidation and metabolism of TCA cycle intermediates. In the case of the odd chain-length fatty acids, β-oxidation results in (n-3)/2 molecules of acetyl-CoA and one molecule of propionyl-CoA where n is the chain-length. It is unusual for bacteria to produce odd-chain fatty acids endogenously and therefore, an exogenous supply (feeding) is required such that the propionyl-CoA generated can be considered to be derived from a related carbon source. As for TCA cycle intermediates, it is possible to convert succinyl-CoA to propionyl-CoA via methylmalonyl-CoA. This strategy has been used to engineer a strain of *Salmonella enterica* to accumulate poly(3HB-co-3HV) on glycerol as a sole carbon source (Aldor et al., 2002).

2.5.3. 3-hydroxypropionic and 4-hydroxybutyric acids

3-hydroxypropionate (3HP) and 4-hydroxybutyrate (4HB) have also been targeted as monomers for PHA biosynthesis. Strategies for obtaining these monomers have involved the introduction of PDO for 3HP or BDO for 4HB as a co-feedstock for the production of copolymers with 3HB (Meng et al., 2012). The incorporation of 3HP and 4HB monomer units has been shown to change the material properties of the resulting polyester to be much more elastic (for P(3HP): Tg ≈ 78 °C, Tm ≈ -21 °C; for P(4HP): Tg ≈ 61 °C, Tm ≈ -47 °C) (Meng et al., 2012; Zhou et al., 2011). Metabolic pathways exist for the production of PDO from glycerol, which itself is a relatively inexpensive feedstock (Table 2.2) (Biebl et al., 1999; Mu et al., 2006). Alternatively, BDO can be derived from succinic acid or α-ketoglutarate (not to be confused with α-ketobutyrate), which are both intermediates in the TCA cycle – an integral part of central metabolism (Yim et al., 2011). Efforts have been made to overproduce succinic acid via fermentation for use as an alternative to the route from petrochemically derived maleic acid or maleic anhydride [reviewed here: (Beauprez et al., 2010)]. Succinic acid could serve as an intermediate for BDO production, however, an intermediate step in this metabolism is 4HB, obviating the need to produce BDO as the precursor.

Alternatively, γ-butyrolactone (GBL) feeding has also resulted in the incorporation of 4HB in PHA accumulating strains as 4HB is the acidified form of GBL (Vigneswari et al., 2009). Molecules containing GBL function as quorum sensing molecules in a number of bacteria (Miller and Bassler, 2001). While GBL has not been a target for overproduction, 3-hydroxy-GBL and 3,4-dihydroxybutyric acid have been produced in *E. coli* from glucose as a sole carbon source (Dhamankar et al., 2011).

2.5.4. Medium-chain-length (mcl)-PHA biosynthesis

PHA are classified based on the carbon chain-length of the monomers that make up the polymer itself. PHA consisting of 3-5 carbon monomers such as 3HB and 3HV are considered scl-PHA, while PHA incorporating monomers with a chain-length ≥ 6 carbons are considered mcl-PHA. Most mcl-PHA are derived from fatty acid metabolism whereby intermediates of either fatty acid biosynthesis or β-oxidation can be scavenged and incorporated into a polyester (Fig. 2.9). These pathways work in iterative cycles. Therefore, the resulting PHA is a heteropolymer consisting of monomers ranging from 6 to 14 carbons in length. Several enzymes have been shown to link fatty acid metabolism to mcl-PHA biosynthesis including

enoyl-CoA hydratase (PhaJ, EC 4.2.1.119), (R)-3-hydrocyacyl-acyl-carrier protein (ACP) thioesterase (PhaG, EC 3.1.2.-), and mcl-PHA polymerases (PhaC1), which tend to have a broad specificity for monomers between 6 and 14 carbons. Recently, an acyl-CoA ligase from *P. putida* (PP_0763) was shown to act on (R)-

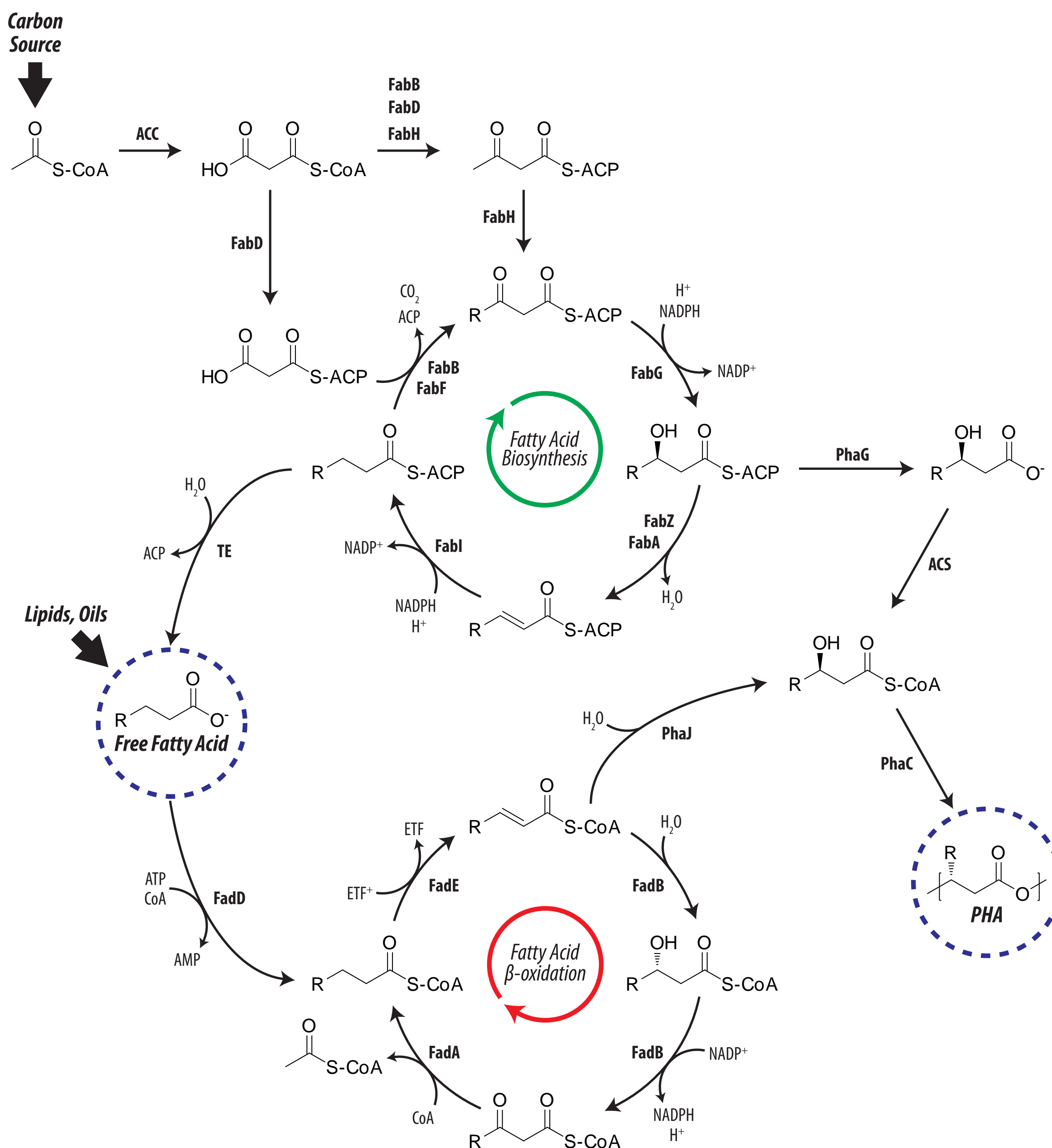

Figure 2.9. Routes to mcl-PHA monomer biosynthesis via fatty acid biosynthesis and β-oxidation starting from the central metabolite acetyl-CoA. Key enzymes are bolded to indicate steps that convert intermediates of fatty acid metabolism to hydroxyalkanoates. ACP = acyl-carrier protein. Block arrows represent multiple enzymatic reactions.

3-hydrocyacids derived from fatty acid biosynthesis via (R)-3-hydrocyacyl-ACP thioesterase (Wang et al., 2012a). By co-expressing a thioesterase, CoA-ligase and an mcl-PHA polymerase, up to 400 mg L^{-1} mcl-PHA heteropolymer could be synthesized from glucose as a sole carbon source.

While the focus of this chapter is on PHA derived from unrelated carbon sources, much PHA research has employed a number of feeding strategies that allow for the accumulation of mcl-PHA from substrates such as alkanes, acyl-alcohols and fatty acids. Most bacteria can grow on fatty acids as a sole carbon source, although manipulation of the regulatory pathways may be required depending on the chain-length (*see* Chapter 3). Certain bacteria, such as *Pseudomonas oleovorans* are additionally capable of utilizing aliphatic alkanes as a carbon source via conversion to fatty acids (Van Beilen et al., 1994). Alkanes are first metabolized to the n-alcohol by alkane hydroxylase and then to the aldehyde by alcohol dehydrogenase before finally being converted to the fatty acid by aldehyde dehydrogenase. The resulting fatty acids can be activated to the CoA form and are then substrates for β-oxidation. *P. oleovorans* is capable of PHA biosynthesis from alkanes (Lageveen et al., 1988) and it is possible to confer this ability to organisms such as *E. coli* via heterologous expression (Eggink et al., 1987).

2.5.5. Manipulation of β-oxidation for mcl-PHA homopolymer

Early attempts at increasing the production of PHA via β-oxidation intermediates involved single gene deletions – usually in (S)-specific enoyl-CoA hydratase (FadB) – or by addition of inhibitory molecules such as acrylic acid (Fiedler et al., 2000), salicylic acid (Choi et al., 2009), 2-bromooctanoic acid and 4-pentenoic acid (Lee et al., 2001) which inhibit β-oxidation enzymes. Functional inactivation of the transcriptional regulator of β-oxidation (FadR) has also been implemented to increase flux through this pathway (Rhie and Dennis, 1995). Recently, a number of labs have focused on the production of mcl-PHA homopolymers in both *P. putida* and *E. coli*. These have generally been feeding strategies wherein a single chain-length fatty acid has been fed to a β-oxidation impaired strain expressing PHA biosynthesis genes. In *Pseudomonas*, homopolymer synthesis was achieved through inactivation of six genes involved in native fatty acid degradation and one gene associated with native PHA metabolism (Liu et al., 2011). When supplied with either C_{10} or C_{14} fatty acids, C_{10} or C_{14} homopolymer was observed. Additional work in *Pseudomonas* demonstrated production of C_4-C_9 homopolymers when the corresponding fatty acid was

supplied exogenously (Wang et al., 2011). A similar approach was implemented in *E. coli* expressing a broad activity polymerase whereby PHA homopolymer was synthesized from fatty acids with a chain-length of 4 to 14 carbons (Tappel et al., 2012).

2.5.6. Combining fatty acid biosynthesis and beta-oxidation to produce mcl-PHA

Approaches to producing mcl-PHA from unrelated carbon sources have also been explored. A popular strategy is to use native or heterologously expressed thioesterases to generate a pool of free fatty acid for incorporation into a heterogeneous polyester. Two thioesterases from *E. coli*, the broad specificity thioesterase II (TesB) (Chung et al., 2009) and a multifunctional thioesterase (TesA) (Qiu et al., 2005) have been targeted for PHA biosynthesis as well as a plant thioesterase from the California Bay Laurel (BTE/FatB) (Rehm and Steinbuchel, 2001). Chapter 3 of this thesis describes in detail a strategy where β-oxidation manipulation and thioesterase based approaches were combined in a single strain for the production of a PHA with a composition matching the fatty acid profile generated by the thioesterase (Agnew et al., 2012). This strategy offers an opportunity to produce a PHA with a defined composition simply by tuning the specificity of the thioesterase (Yuan et al., 1995) or by identifying an existing thioesterase with a desirable profile (Cantu et al., 2010).

2.5.7. Megasynthase engineering

While fatty acid metabolism produces acyl-chains with the essential hydroxyl group needed for polymerization, the pathways result in a limited set of side chains (*i.e.,* saturated, mono-unsaturated, or modified by addition of a methyl-branch or cyclopropane ring) when incorporated into PHA. An attractive possibility for producing a wider range of monomers lies in exploiting PKS which use many of the same chemistries as fatty acid biosynthesis to produce highly substituted acyl-chains. PKS are large, modular enzymes that catalyze the assembly of complex natural products through consecutive condensation and reduction reactions (Sattely et al., 2008; Weissman and Leadlay, 2005). The molecular machines responsible for type I polyketide biosynthesis, often called megasynthases, function in a modular fashion similar to assembly lines seen in classical manufacturing processes (Sattely et al., 2008). A polyketide's carbon backbone is assembled and modified in a stepwise fashion. At each stage, a different enzymatic domain operates on a growing acyl-chain, performing monomer addition, reduction, and chemical modification

until the final product is released. The order of domains, (*i.e.,* the sequence of the protein) dictates the structure of the final compound. Therefore, the ability to fine-tune these modifications through manipulation at the enzyme level has long been a research and drug discovery goal (Sherman, 2005). As such, efforts to understand the logic behind polyketide biosynthesis and to engineer novel polyketides have progressed greatly in the past decade (Wong and Khosla, 2012).

Given this better understanding, we hypothesize that megasynthase engineering offers a flexible path to PHA monomers and Chapter 4 of this thesis describes progress made towards this goal. A majority of the "newly discovered" monomers as well as many of the originally described 91 monomers could be made via a PKS strategy. PKS incorporate a broad range of chemical building blocks both as starter and extender units (Chan et al., 2006; Chan et al., 2009; Moore and Hertweck, 2002). In our proposed strategy, the starter unit determines the structure of the PHA side-chain terminus. Known PKS starter units possess hydroxyl, amino, (hydroxy and amino substituted) phenyl, (hydroxy substituted) hexyl, pyrrole and branched carbon substituents. Incorporation of reactive groups into the PHA side chains via PKS starter units would enable subsequent functionalization of the polymer. Further diversity can be introduced in the form of methyl, hydroxyl and amino moieties at the α-carbon of each PKS extender unit. Lastly, the PKS reductive cycle manipulates the β-carbon of each acyl-chain generating keto, hydroxyl (including the correct stereochemistry needed for polymerization by a PHA synthase), enoyl, and methylene variants. While many monomers could be produced by a PKS strategy, each megasynthase will synthesize a defined molecule based on the domain architecture of the enzyme. This means that the troubles encountered in producing a homopolymer via the iterative fatty acid pathway can be avoided.

2.6. Conversion of organic acids to PHA monomers

2.6.1. Generation of hydroxyacids with required stereospecificity

Stereospecificity is critical to PHA polymerization. PHA synthases requires the (R) stereoisomer in the case of a 3-hydroxyalkanoate. While most PHA monomers acquire the correct stereochemistry after activation to the CoA thioester, there is also the possibility to achieve this modification prior to CoA activation. Conveniently, ketoreductases used by fatty acid biosynthesis and many PKS pathways generate (R)-hydroxyacyl-ACP intermediates that are substrates for hydroxyacyl-ACP thioesterases such as PhaG.

Thioesterase cleavage generates the corresponding free acid that can be activated for polymerization by CoA ligation. In contrast, β-oxidation intermediates (which are already activated as CoA thioesters) are substrates for ketoacyl-CoA reductase (FabG) and enoyl-CoA hydratase (PhaJ). These reactions incorporate the correct (R) stereochemistry. Alternatively, the native PHB pathway enzymes (PhaA, PhaB) could be mutated to permit elongation (with acetyl-CoA) and ketoreduction (of the ketone at the beta position) of organic acids missing the requisite hydroxyl group.

2.6.2. CoA activation

A monomer unit cannot be incorporated unless covalently linked to a CoA. Novel PHA biosynthesis pathways will require this additional step to generate thioesters from organic acids (*e.g.,* PKS release their products via hydrolysis). Conveniently, there are several enzymes capable of activating acid monomers for polymerization. CoA transferases can move CoA from acetyl-CoA (or another thioester donor) to a desired organic acid. Examples are found in short chain fatty acid catabolic pathways (*e.g.,* AtoDA). Alternatively, CoA ligases such as AlkK use ATP to activate organic acids prior to formation of the thioester bond with free CoA (Satoh et al., 2005). These enzymes are classified based on the substrate they act upon, however most show relaxed specificity by acting on additional substrates – although usually with reduced activity (Satoh et al., 2005). Substrate flexibility has also been engineered through the use of rational design to expand the substrate range for CoA ligases (Wu et al., 2007). Alternatively, it may be possible to cleave acyl-chains from PKS ACP domains using CoA instead of water. This has been shown via site directed mutagenesis of a Rat type II thioesterase which conferred acyltransferase capabilities and resulted in CoA-products (Witkowski et al., 1994).

2.7. Polymerization

The final step in PHA biosynthesis involves PHA polymerase (PhaC) catalyzing the formation of an ester bond between the terminal carboxylic acid group from one monomer with the (usually β) hydroxyl group from a second monomer (Fig. 2.6). PHA polymerases have activity on a broad range of substrates and can be classified in one of several ways including substrate specificity, the architecture of the enzyme and the nucleotide/amino acid sequence. In terms of activity for a given monomer, PHA polymerases are generally biased toward either scl-PHA or mcl-PHA. The scl-PHA polymerases tend to be specific for

the polymerization of 3HB, although they have activity towards 3 and 5 carbon monomers as well. The mcl-PHA polymerases, on the other hand, have a broader activity and catalyze the polymerization of 6-14 carbon monomers. Polymerization of longer chain-length monomers including C_{16} and C_{18} has also been demonstrated (Lee et al., 1995; Singh and Mallick, 2008).

There is an abundance of PHA synthase gene sequences and substrate specificity data in the literature (Rehm, 2003; Rehm, 2007; Rehm and Steinbuchel, 1999). Bioinformatic and structural comparisons of these enzymes will likely lead to structure-function relationships that can be used to identify synthases for novel PHA monomers. Alternatively, random mutagenesis and rational re-design approaches have been used improve the kinetics of polymerases (Nomura and Taguchi, 2007). Due to the difficulty associated with the crystallization of these proteins, techniques such as error-prone PCR, gene shuffling, and saturation of key amino acids have been employed. For example, site-directed mutagenesis of the PHA synthase (*phaC*) from *C. necator* was demonstrated to increase the substrate tolerance of the enzyme (Tsuge et al., 2004). Similarly, engineering of mcl-PHA polymerase PhaC1 from *Pseudomonas sp.* 61-3 conferred the enzyme with the ability to polymerize both scl- and mcl-PHA (Takase et al., 2003). More recently, truncations of two PhaC from *C. necator* and *Pseudomonas aeruginosa* and subsequent recombination to form hybrid polymerases resulted in altered product specificity (Wang et al., 2012b). Even with engineered polymerases, co-feeding of substrates is sometimes necessary to incorporate particular monomers into PHA granules. For example, in an attempt to incorporate monomers with fluorinated side-chains, feeding a single, fluorinated precursor resulted in no polymer accumulation (Kim et al., 1996). Upon co-feeding of nonanoic acid, various fluorinated monomers were successfully incorporated into a polymer.

A final consideration is the extent of polymerization. The material properties of plastics, PHA included, are often dependent on the molecular weight of the polymer. In this regard, PHA have the advantage of high molecular weights and low dispersities that lend to commericial applications. When produced from glucose, PHB is characterized by weight-average molecular weights (MW) of 500-1,000 kDa and melting temperatures of 170-180°C (Madison and Huisman, 1999). Recent studies have also demonstrated production of PHB with MW values exceeding 6,000 kDa and dispersities less than 2 (Hiroe et al., 2012).

2.8. Conclusions

In summary, the scientific framework is in place for the construction of metabolically engineered pathways for synthesizing custom PHA monomers from unrelated carbon sources. While a large number of monomers have been successfully incorporated into PHA granules, the target price of commercial PHA limits the economic viability of these materials when derived from feedstocks more expensive than glucose or glycerol. Synthetic biology has been used to construct metabolic pathways for producing a wide range of commodity chemicals including unnatural molecules. We hypothesize that synthetic biology can be similarly used to engineer metabolic pathways from renewable feedstocks to PHA monomers. If successfully incorporated, novel monomers will have a significant impact on the material properties of the resulting polyester. We anticipate that this approach will increase the industrial relevance and commercial viability of PHA in the polymer marketplace.

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Chapter 3: Engineering *E. coli* for production of C_{12}-C_{14} PHA from glucose

3.1. Introduction

The overarching theme of this thesis is the challenge of engineering metabolic pathways to produce PHA with a desired monomer composition from an unrelated and renewable source. Whereas Chapter 4 focuses on a PKS platform for monomer production, Chapter 3 explores a better characterized and proven route to PHA with the aforementioned qualities. This chapter describes the synthesis of a number of ideas previously explored in the literature. Namely, these ideas encompass expression of an acyl-ACP thioesterase for production of free fatty acids, conversion of free fatty acids to mcl-PHA and manipulation of β-oxidation to preserve the composition of the fatty acid pool for a defined polymer product. The novelty of the work presented in this chapter is the thorough characterization of *E. coli* β-oxidation under conditions of free fatty acid metabolism and PHA biosynthesis. It is important to note that this work was made possible in part by the previous efforts made in the Pfleger lab revolving around the overproduction of free fatty acids (FFA) in *E. coli* (Lennen et al., 2010; Youngquist et al., 2012).

The desire to produce mcl-PHA with a defined composition is partly motivated by the observation that the structural properties of PHA can be engineered with varying degrees of crystallinity and elasticity by changing the identity and/or percentage of co-monomers (Khanna and Srivastava, 2005). This class of PHA consisting of fatty acids containing six or more carbons is an attractive polymer desired for novel applications in medical devices, cosmetics, and tissue engineering (Chen and Wu, 2005). As described previously, many PHA accumulating organisms are capable of using fatty acids as a substrate for PHA biosynthesis. This strategy requires either a low-cost source of fatty acids to be fed exogenously or a means for producing the required fatty acids endogenously. For this reason, current PHA research is focused on engineering metabolic pathways to produce monomers from unrelated carbon sources such as glucose (Li et al., 2010; Theodorou et al., 2012).

In fatty acid biosynthesis, an iterative cycle of two carbon elongations in conjunction with a series of reductions yields long-chain fatty acids (≥16 carbons). Intermediates exist as ACP bound thioesters and can be substrates for mcl-PHA biosynthesis. It was recently shown that heterologous expression of *P. putida phaG, phaC* and a predicted acyl-CoA ligase led to accumulation of 400 mg L^{-1} mcl-PHA in *E. coli*

when grown on glucose as a sole carbon source (Wang et al., 2012). In this instance PhaG functions as an (R)-3-hydroxyacyl-ACP thioesterase to produce free (R)-3-hydroxyalkanoic acids which are ligated to CoA and polymerized by PhaC. Due to the loose substrate specificity of the PhaG thioesterase and the iterative nature of fatty acid metabolism, the resulting mcl-PHA is a heteropolymer consisting of C_6-C_{14} monomer units.

Alternatively, mcl-PHA can be produced by exogenous feeding of FFA or other lipid mixtures (palm oil, soybean oil, etc.). Here, enzymes such as PhaJ act on β-oxidation intermediates to generate a heterogeneous pool of 3-hydroxyacyl-CoA thioesters, substrates for PhaC polymerization, thereby assembling a heterogeneous polymer comprised of a range of chain-lengths equal to or smaller than the fatty acids fed. While mcl-PHA heteropolymers are likely to have utility, a homopolymer of a desired chain length will enable precise control of polymer properties. Recently, both *P. putida* KT2442 and *E. coli* were engineered to accumulate mcl-PHA homopolymer when fed specific chain-length fatty acids (Liu et al., 2011; Tappel et al., 2012; Wang et al., 2011). In the case of *Pseudomonas*, six gene knockouts in β-oxidation and one in PHA biosynthesis were required to produce pure C_{10} and C_{14} PHA from C_{10} and C_{14} fatty acids respectively and a 16% C_{10}-co-84% C_{12} mcl-PHA from C_{12} (Liu et al., 2011). Using a similar fatty acid feeding strategy, a second study involving β-oxidation impaired *Pseudomonas* produced a series of C_4-C_9 PHA homopolymers including C_5 and C_7 based polyesters (Wang et al., 2011). In *E. coli*, deletions in β-oxidation combined with constitutive expression of short-chain fatty acid metabolism and overexpression of mcl-PHA biosynthesis genes allowed for production of homogeneous PHA ranging from 4-14 carbons when cultured on the corresponding fatty acids (Tappel et al., 2012).

A third approach to producing mcl-PHA combines fatty acid biosynthesis and β-oxidation in a hybrid metabolic pathway. Acyl-ACP thioesterases are used to produce FFA of a desired length and β-oxidation converts FFA to PHA monomers. Many thioesterases have been employed for the production of mcl-PHA including TesB (Chung et al., 2009), TesA (Qiu et al., 2005), and the California Bay Laurel (*Umbellularia californica*) thioesterase (BTE) (Rehm and Steinbuchel, 2001). Expression of BTE in *E. coli* generates a large pool of C_{12} and C_{14} FFA by depleting the pool of long-chain acyl-ACP which regulates the early steps in fatty acid biosynthesis. Co-expression of BTE and a PHA polymerase in *E. coli* LS1298 (Δ*fadB*) or RS3097 (Δ*fadR*) was shown to produce C_{10} PHA from 3-6% cell dry weight (CDW) when grown on Lysogeny Broth (LB)

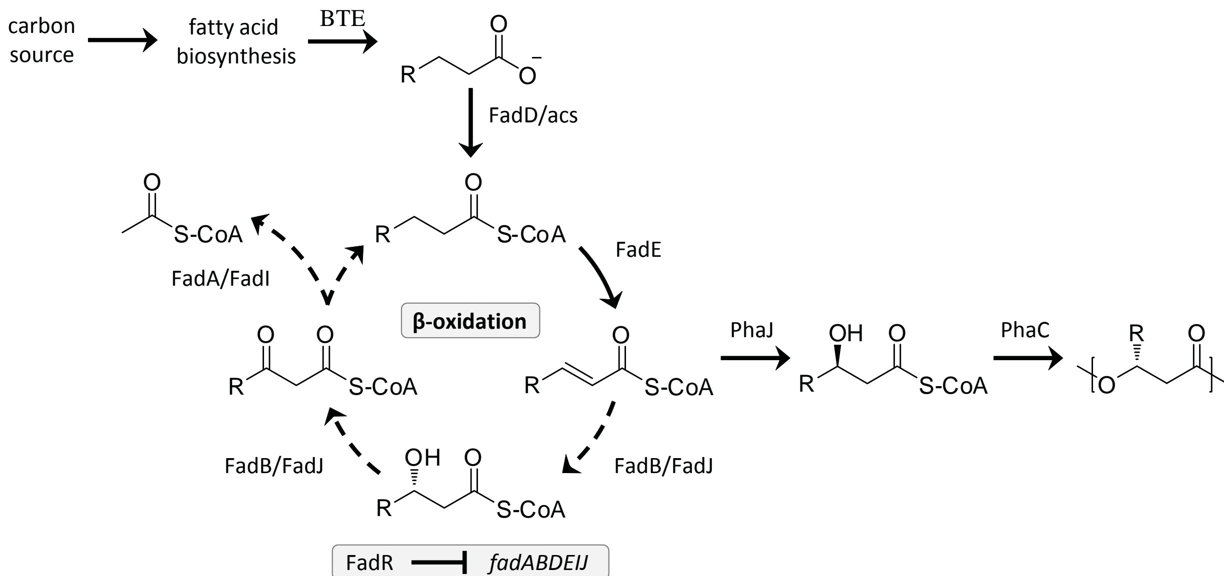

Figure 3.1. Metabolic pathway for mcl-PHA biosynthesis in *E. coli.* A carbon source (*i.e.,* glucose) is catabolized to acetyl-CoA which enters fatty acid biosynthesis for production of fatty acyl-ACPs. C12 and C14 acyl-ACPs are substrates for the thioesterase, BTE, which catalyzes free fatty acids formation. An acyl-CoA ligase (e.g., FadD) activates the free fatty acids for degradation via a partially intact β-oxidation cycle generating substrates for PhaJ to produce mcl-PHA monomers for polymerization by PhaC. The resulting monomer composition is identical to that of the FFA pool generated by the thioesterase.

supplemented with gluconate and acrylic acid (an inhibitor of β-oxidation) (Rehm and Steinbuchel, 2001). However, it is unclear if other monomer units were present in this study.

Here, we demonstrate a metabolic pathway for converting glucose into mcl-PHA composed primarily of 3-hydroxydodecanoate monomers (Fig. 3.1). A panel of mutant *E. coli* strains was characterized to determine the impact of β-oxidation enzymes on fatty acid consumption and mcl-PHA synthesis. Additionally, two PHA synthases (PhaC) and four enoyl-coA hydratases (PhaJ) for producing mcl-PHA were characterized in *E. coli*, identifying a promising combination for making mcl-PHA. The impact of different modes of regulating acyl-CoA synthetases on PHA titer was examined. Finally, a strain of *E. coli* was engineered to produce mcl-PHA with composition matching the product profile of the thioesterase. This strategy involved constructing a strain of *E. coli* in which key genes in fatty acid β-oxidation were deleted in conjunction with the overexpression of BTE, *phaJ3* and *phaC2* from *P. aeruginosa* PAO1 and PP_0763 from *P. putida* KT2440. The resulting strain produced over 17% CDW mcl-PHA when grown in minimal glucose-based media.

3.2. Results

3.2.1. Effect of fad deletions on dodecanoic acid catabolism

β-oxidation of fatty acids occurs in three stages. First, FFA are imported across the outer membrane via FadL and activated as CoA thioesters by FadD in the inner membrane. The acyl-CoA thioesters are a key regulatory signal which abrogates the DNA binding ability of FadR. In the absence of acyl-CoAs FadR represses expression of enzymes involved in β-oxidation. Once activated, acyl-CoAs are catabolized to acetyl-CoA via an iterative pathway comprised of four enzymatic reactions (Fig. 3.1) involving acyl-CoA dehydrogenase (FadE), enoyl-CoA hydratase (FadB), (3*S*)-hydroxyacyl-CoA dehydrogenase (FadB), and ketoacyl-CoA thiolase (FadA). Three additional *fad* genes – *fadK*, *fadI* and *fadJ* have strong sequence homology to *fadD*, *fadA* and *fadB*, respectively and have been shown to be critical for anaerobic beta-oxidation (Campbell et al., 2003). Each cycle ends when FadA (or FadI) cleaves a ketoacyl-CoA to generate an acetyl-CoA and an FA-CoA reduced in length by two carbons that is the substrate for the next round. Finally, *E. coli* possesses additional β-oxidation capacity in the *ato* genes which are responsible for processing short-chain FFAs.

The metabolic engineering strategy conceived for producing mcl-PHA from endogenously synthesized fatty acids requires the disruption of β-oxidation such that (R)-3-hydroxyacyl-CoA thioesters can be polymerized but not catabolized to acetyl-CoA. Therefore, it was necessary to assess the ability of strains harboring various deletions in β-oxidation (*fad*) genes to catabolize dodecanoic acid after 24 and 48 h of shake flask cultivation (Fig. 3.2a). The base strain, K12 MG1655 Δ*araBAD*, was not observed to completely catabolize all of the dodecanoic acid until 48 h, while a *fadR* mutant was able to consume all of the dodecanoic acid within 24 h. A *fadB* deletion, which based on previous reports was expected to greatly impair dodecanoic acid catabolism under aerobic conditions, consumed 20% of the dodecanoic acid. To completely block dodecanoic acid consumption over the course of 48 h, a double knockout, Δ*fadB,* Δ*fadJ* strain was required. Similarly, a Δ*fadA* strain consumed ~20% of the dodecanoic acid while a Δ*fadA*, Δ*fadI* double mutant demonstrated negligible dodecanoic acid consumption. The performance of other *fad* strains and the effect of a *fadR* deletion combined with these strains, which generally improved the rate of dodecanoic acid metabolism, is shown in Fig. 3.2a.

To determine if metabolism of exogenously fed dodecanoic acid correlated with metabolism of endogenously produced FFAs, β-oxidation deletion strains were transformed with pTrc99a-BTE and grown for 48 h on LB supplemented with glucose (Fig. 3.2b). Final fatty acid concentrations and especially saturated dodecanoic acid concentrations correlated with exogenous consumption data (Fig. 3.2a). Specifically, strains capable of complete consumption of exogenous dodecanoic acid after 48 h accumulated little to no endogenous dodecanoic acid while strains that were the most impaired in exogenous C_{12} consumption yielded the largest concentrations of endogenous C_{12} FFA. While FFA uptake has been well studied

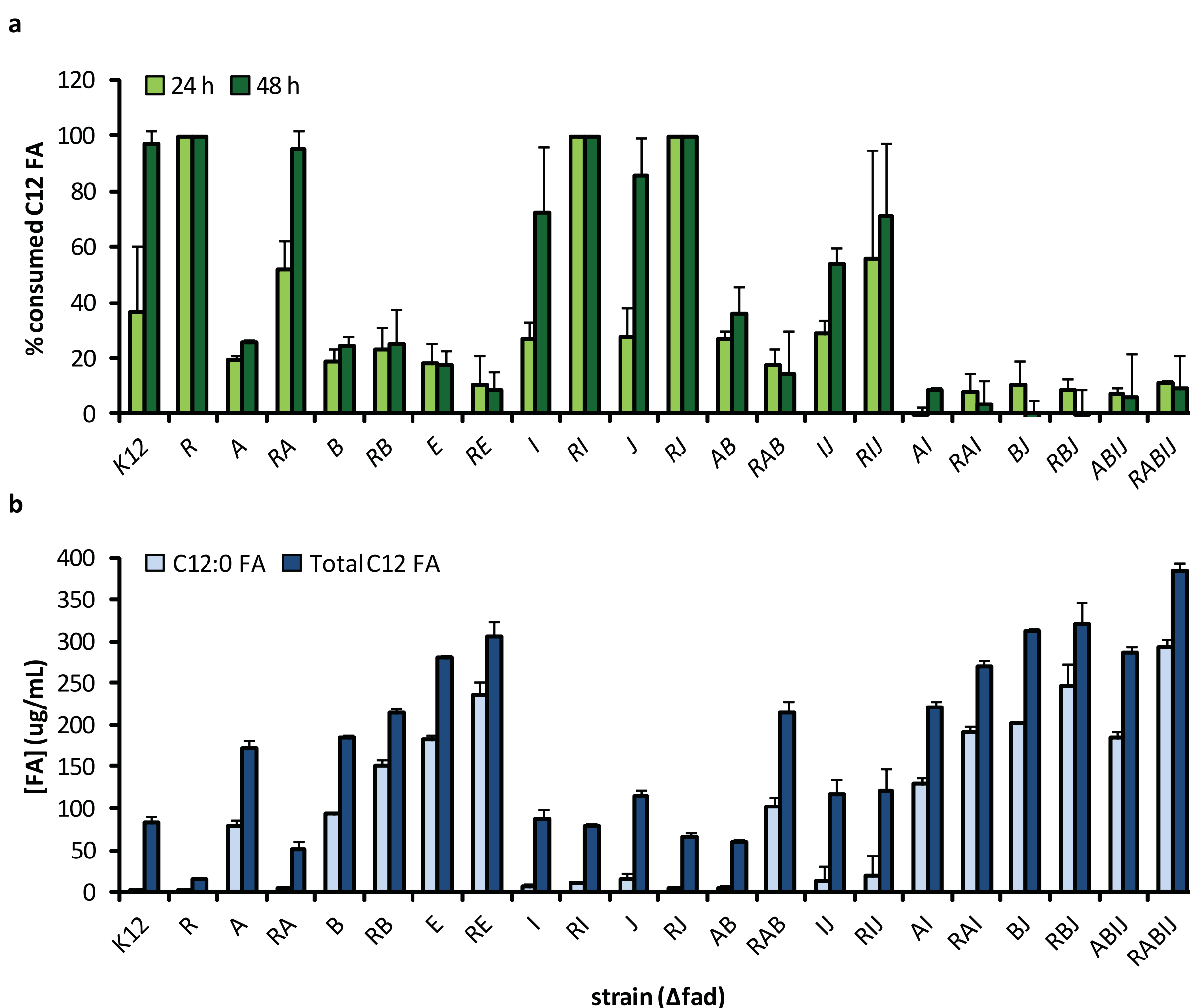

Figure 3.2. Metabolism of dodecanoic acid by a library of β-oxidation knock-out strains of *E. coli* with specific fad deletion(s) indicated on the horizontal axis.. (A) Metabolism of exogenously fed dodecanoic acid after 24 and 48 h of shake flask cultivation as a percent of the initial fatty acid concentration. (B) Metabolism of endogenously synthesized fatty acids in strains with plasmid-based expression of BTE after 48 h of cultivation. Data for both saturated (C12:0) and total C12 (including unsaturated and hydroxy) species are presented.

(DiRusso and Black, 2004), the mechanism of FFA secretion is poorly understood. It should be noted that the data presented in Fig. 3.2b does not distinguish rates of FFA secretion and reuptake from catabolism of intracellular FFA.

3.2.2. Effect of fadD regulation on dodecanoic acid catabolism

The proposed mcl-PHA pathway requires the activation of free fatty acids and oxidation by FadE to yield enoyl-CoA thioesters. These genes could be upregulated by increasing the rate of acyl-CoA synthesis (*e.g.* replacing P_{fadD} with a stronger promoter), removing repression via FadR, or both. Therefore, a *fadD* overexpression strain was constructed by replacing the native *fadD* promoter with the strong, isopropyl β-D-thiogalactopyranoside (IPTG) inducible *trc* promoter (Brosius et al., 1985). Dodecanoic acid consumption in this strain was compared with the base strain, Δ*fadR* and Φ(P_{trc}-*fadD*) Δ*fadR* combination strains (Fig. 3.3). Interestingly, the Δ*fadR* strain completely consumed the dodecanoic acid after 8 h while complete consumption was not observed for the Φ(P_{trc}-*fadD*) overexpression strain until 24 h. Surprisingly, a Φ(P_{trc}-*fadD*) Δ*fadR* combination strain consumed dodecanoic acid at a rate in between the Φ(P_{trc}-*fadD*) overexpression and Δ*fadR* strains. Deletion of *fadR* may provide the additional benefit of upregulating *fadE* expression which is required to produce enoyl-CoA thioesters in this mcl-PHA biosynthesis strategy.

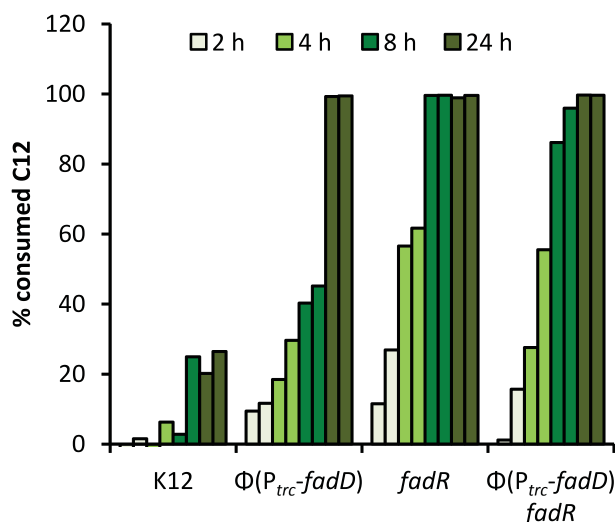

Figure 3.3. Comparison of the effect of a *fadR* deletion with *fadD* overexpression via a chromosomal fusion of the *trc* promoter (Φ(P*trc-fadD*)) on exogenous dodecanoic acid metabolism in *E. coli* over a 24 h period. Data is presented as a percent of the initial fatty acid concentration. Data shown for two replicates.

3.2.3. Production of mcl-PHA in fad strains in the presence of exogenous dodecanoic acid

Two PHA biosynthetic enzymes are required to confer *E. coli* with the ability to synthesize mcl-PHA from enoyl-CoA thioesters, a PHA polymerase (PhaC) and an (R)-specific enoyl-CoA hydratase (PhaJ). *P. aeruginosa* DSM1707 *phaJ1-4* have been previously characterized in *E. coli* LS5218 (Tsuge et al., 2003). Here, genes from *P. aeruginosa* PAO1 were selected based on

Table 3.1. GC/MS analysis of the composition of mcl-PHA produced in *E. coli* LS5218 expressing combinations of two *phaC* and four *phaJ* from *P. aeruginosa* PAO1 after culturing in the presence of exogenous dodecanoic acid.

Relevant Genotype	Cell Dry Weight (g L^{-1})	PHA Content (wt. %)	PHA Composition (wt. %)			
			C_6	C_8	C_{10}	C_{12}
phaC1 phaJ1	1.0	0.3	8.4	90.7	0.0	0.9
phaC1 phaJ2	1.2	4.4	4.8	49.6	28.9	16.8
phaC1 phaJ3	1.4	10.8	3.9	43.5	33.0	19.6
phaC1 phaJ4	1.0	2.8	5.2	52.3	25.6	16.9
phaC1	1.1	0.6	4.7	65.1	22.0	8.3
phaC2 phaJ1	1.0	2.2	34.0	54.8	6.7	4.5
phaC2 phaJ2	1.1	13.9	11.1	35.9	28.8	24.2
phaC2 phaJ3	1.1	19.1	8.2	32.3	32.2	27.3
phaC2 phaJ4	0.9	9.4	9.6	35.0	29.3	26.1
phaC2	1.1	1.8	6.9	48.5	26.7	17.9

Note: C6: 3-hydroxyhexanoate; C8: 3-hydroxyoctanoate; C10: 3-hydroxydecanoate; C12: 3-hydroxydodecanoate.

sequence identity with DSM1707 and the ability of this strain to accumulate mcl-PHA. Individual *phaJ* and *phaC* clones were co-expressed from plasmids pMSB-6 and pBAD33-C280* respectively in LS5218 grown in the presence of exogenous dodecanoic acid as a sole carbon source. All *phaJ-phaC* combinations yielded mcl-PHA identified as methyl esters of 3-hydroxyacyl-chains after processing (Table 3.1). The observed acyl-chains ranged in length from C_6 to C_{14} corresponding to mcl-PHA monomers (C_6-C_{12}) and components of lipid A (C_{14}). The combination of *phaJ3* and *phaC2* was selected based on the ability to produce mcl-PHA containing C_{12} monomer units at yields greater than other combinations tested (Table 3.1).

P. aeruginosa phaC2 was cloned downstream of *phaJ3* into pMSB-6 yielding pDA-JC and the plasmid was transformed into a selection of *fad* deletions strains for mcl-PHA production. Table 3.2 shows the ability of a Δ*fadR*, Δ*fadRB*, Δ*fadRBJ* and Δ*fadRABIJ* strains to accumulate mcl-PHA as well as the monomer composition of the resulting polymer. Most notably, Δ*fadR* and Δ*fadRB* strains both produced mcl-PHA with a heterogeneous monomer composition, although the fraction of C_{12} monomers in the Δ*fadRB* strain was greatly increased over that of the Δ*fadR* strain. The Δ*fadRBJ* and Δ*fadRABIJ* strains were both capable of producing mcl-PHA homopolymer consisting entirely of C_{12} monomers with the yield of PHA in the

Table 3.2. GC/MS analysis of the composition of mcl-PHA produced in a series of *E. coli* β-oxidation deletion strains containing plasmid pDA-JC after culturing in the presence of exogenous dodecanoic acid.

Relevant Genotype	Cell Dry Weight (g L^{-1})	PHA Content (wt. %)	PHA Composition (wt. %)			
			C_6	C_8	C_{10}	C_{12}
Δ*fadR*	0.97±.09	1.71±.18	4.0	30.2	34	31.8
Δ*fadRB*	0.96±.08	0.39±.13	n.d.	8.3	42.4	49.3
Δ*fadRBJ*	1.10±.19	0.38±.15	n.d.	n.d.	n.d.	100.0
Δ*fadRABIJ*	0.93±.02	0.75±.03	n.d.	n.d.	n.d.	100.0

Note: C6: 3-hydroxyhexanoate; C8: 3-hydroxyoctanoate; C10: 3-hydroxydecanoate; C12: 3-hydroxydodecanoate.

Δ*fadRABIJ* strain slightly improved over that of the Δ*fadRBJ* strain. This result was consistent with the relative rates of dodecanoic acid catabolism and the endogenous production data (Fig. 3.2).

3.2.4. Accumulation of mcl-PHA in a ΔfadRABIJ strain with endogenous dodecanoic acid production

Expression of the California Bay Laurel (*Umbellularia californica*) thioesterase (BTE) in *E. coli* results in the accumulation of FFAs composed predominantly (≥80%) of saturated C_{12} and unsaturated $C_{12:1}$ species with the remainder comprised mainly of C_{14} and unsaturated $C_{14:1}$ FFAs (Voelker and Davies, 1994). A codon optimized version of BTE (Lennen et al., 2010) was integrated into the chromosome of *E. coli* K-12 MG1655 Δ*araBAD* Δ*fadR* Δ*fadIJ* into the *fadBA* locus, resulting in a Δ*fadRABIJ* strain with one copy of the Φ(P_{trc}-BTE) cassette. This strain (SA01) when transformed with pDA-JC and grown in MOPS minimal media supplemented with 1% glucose accumulated mcl-PHA at a % CDW on par with a Δ*fadRABIJ* strain cultured with exogenous dodecanoic acid (Fig. 3.4). A significant amount of residual

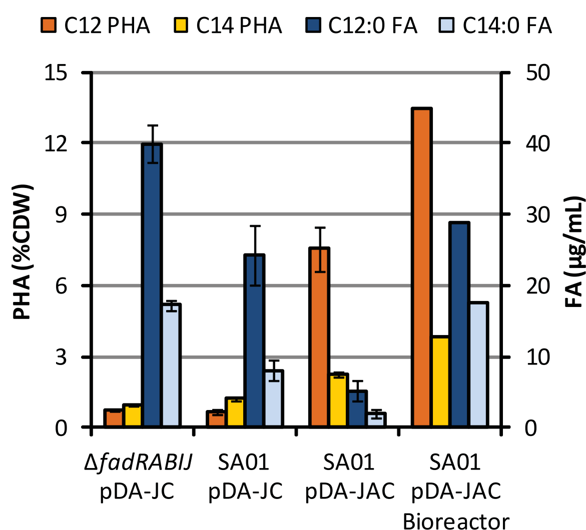

Figure 3.4. Production of mcl-PHA and FA in *E. coli* in the presence of exogenous or endogenous dodecanoic acid. Strain Δ*fadRABIJ* was cultured in the presence of dodecanoic acid while SA01 strains expressing BTE were capable of endogenous FA production on glucose minimal media.

dodecanoic and tetradecanoic acid was also observed indicating that there is room for further pathway optimization.

3.2.5. Effect of overexpression of PP_0763 on mcl-PHA accumulation in a ΔfadRABIJ strain with endogenous dodecanoic acid production

Given the presence of excess FFA, it was hypothesized that the rate of fatty acyl-CoA production was not balanced with FFA synthesis. Therefore, the predicted acyl-CoA ligase, PP_0763 from *P. putida* KT2440 was cloned between *phaJ3* and *phaC2* in pDA-JC resulting in pDA-JAC. Strain SA01 was transformed with pDA-JAC which resulted in the production of 9.8% CDW mcl-PHA, a 10-fold increase compared to the same strain without PP_0763 (Fig. 3.4). When cultured in a 1 L bioreactor, mcl-PHA accumulation

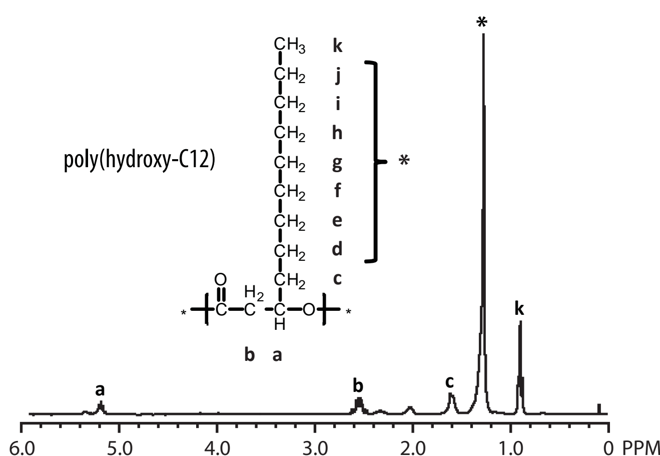

Figure 3.5. 1H NMR of purified C_{12} mcl-PHA

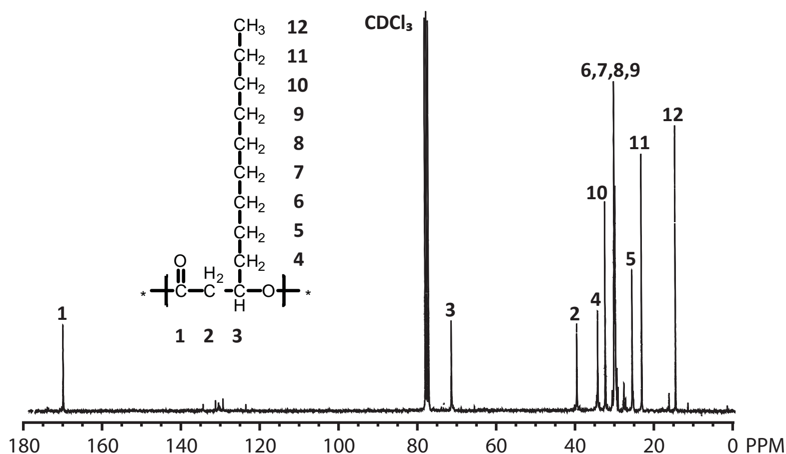

Figure 3.6. 13C NMR of purified C_{12} mcl-PHA

increased to 17.3% CDW after 96 h. The identity of the purified product was confirmed to be predominantly polyhydroxydodecanoate by ^{1}H and ^{13}C NMR (Figs. 3.5 and 3.6).

3.3. Discussion

3.3.1. Effect of fad deletions on dodecanoic acid metabolism

Previous work has demonstrated that the ability to use fatty acids ≥ C_{12} as a sole carbon source is lost in the case of deletions in *fadB* (Dirusso, 1990), however, a *fadB(A) phaC*$^{+}$ strain was still capable of aerobic production of mcl-PHA heteropolymer indicating that *E. coli* can complement *fadB* activity (Langenbach et al., 1997; Prieto et al., 1999; Qi et al., 1997; Ren et al., 2000; Snell et al., 2002). Moreover, a *fadA* insertion mutant was capable of aerobic growth on oleic acid ($C_{18:1}$) as a sole carbon source after extended incubation (< 5 days) on solid media (Campbell et al., 2003), further indicating that additional β-oxidation activity is present. The data indicate both *E. coli* Δ*fadA* and Δ*fadB* mutants are capable of dodecanoic acid metabolism after 24 h, although with reduced capability compared to WT. Conversely, *E. coli* Δ*fadR* Δ*fadA* catabolized dodecanoic acid more efficiently than WT with nearly complete consumption of the dodecanoic acid after 48 h. As *fadR* is a negative regulator for *fadIJ*, it is likely that *fadIJ* is capable of complementing *fadBA* and restoring β-oxidation activity to that of WT. However, a Δ*fadR* Δ*fadB* strain did not show increased dodecanoic acid catabolism over the 48 h period. Therefore, *fadJ* may not be able to complement a *fadB* deletion as effectively as in the case of *fadI* with *fadA*.

Deletions of *fadI* or *fadJ* had a minor negative effect on dodecanoic acid metabolism compared to WT which is expected if *fadBA* function as the major contributors to aerobic β-oxidation. Similarly, Δ*fadR* Δ*fadI* and Δ*fadR* Δ*fadJ* strains were comparable to a Δ*fadR* strain. An unexpected result was the reduced rate of dodecanoic acid consumption in both a Δ*fadBA* and Δ*fadIJ* double knockout compared to WT. These data indicate that functional expression of *fadBA* is not essential for dodecanoic acid metabolism under the conditions tested. It is important to note that dodecanoic acid metabolism was still active in a Δ*fadIJ* strain which agrees with previous work that demonstrated both aerobic and anaerobic growth for a Δ*fadIJ* (*yfcYX*) strain on oleic acid (Campbell et al., 2003).

Based on the behavior of the aforementioned deletions, it was anticipated that a Δ*fadA* Δ*fadI* or Δ*fadB* Δ*fadJ* strain would be incapable of dodecanoic acid metabolism. This result was confirmed for these strains, a Δ*fadBA* Δ*fadIJ* strain and for each of the strains when combined with a *fadR* deletion. These results agree with the observation that an *E. coli* Δ*fadB* Δ*fadJ* strain expressing the *phaC* polymerase from *Pseudomonas oleovorans* from a plasmid was incapable of mcl-PHA accumulation (Snell et al., 2002). Recent work in an *E. coli* LS5218 Δ*fadB* Δ*fadJ* strain for PHA homopolymer production on exogenously fed fatty acids corroborates these results as well (Tappel et al., 2012).

3.3.2. Comparison of fadD overexpression and fadR deletion on dodecanoic acid metabolism

The ability of a *fadR* deletion to improve the initial rate of C_{12} metabolism led to the question of whether overexpression of *fadD* would result in a similar phenotype. Therefore, a chromosomal *trc* promoter fusion with *fadD,* Φ(P_{trc}-*fadD*) was tested both individually and in combination with a Δ*fadR* strain. Over a 24 h period, the Φ(P_{trc}-*fadD*) strain was capable of improved C_{12} consumption compared with WT but was not as efficient as a Δ*fadR* or Φ(P_{trc}-*fadD*) Δ*fadR* combination strain. Overexpression of *fadD* increases the cytoplasmic acyl-CoA pool faster than in WT resulting in faster de-repression of all β-oxidation genes regulated by *fadR*, while in a Δ*fadR* strain, there is no repression of β-oxidation genes allowing for faster initial turnover of exogenous fatty acids.

3.3.3. Effect of soluble vs. membrane associated CoA-ligases

Although mcl-PHA production in strain SA01 expressing pDA-JC was achieved with a defined composition from a non-fatty acid feedstock, a large amount of endogenously produced FFA remained in the culture broth. Therefore, it was hypothesized that the limiting step in PHA biosynthesis was CoA ligation. Or put another way, intracellular FFAs were hypothesized to be leaving the cell at a faster rate than FadD ligation with CoA, the product of which (acyl-CoA) is not exportable. Two models of the CoA synthetase reaction can be envisioned (DiRusso and Black, 2004). First, cytoplasmic FFA, freshly produced by BTE, could be directly bound by a cytosolic FadD and converted to CoA thioesters. Alternatively, cytoplasmic FFA could begin to traverse the inner cell membrane, periplasm, and outer membrane and be re-imported for FadD activation. The import of extracellular fatty acids across the outer membrane is facilitated by FadL. Once across the outer membrane, FFA traverse the periplasm and intercalate into the inner membrane.

FFA then bind to the FadD active site and become phosphorylated from an ATP donor. The final CoA ligation, disassociation of FadD from the inner membrane and association of the fatty acyl-CoA with the cytoplasm likely takes place in one concerted event. If the rate of re-import is inferior to continued export (which would be down the concentration gradient) dodecanoic acid could accumulate extracellularly as was observed in the BTE expressing strains. To address the issue of fatty acid accumulation, a predicted soluble CoA-ligase encoded by *P. putida* gene PP_0763 (*acs*) was identified for co-expression as this enzyme has been shown to be an effective medium-chain-length acyl-CoA ligase when heterologously expressed in *E. coli* (Wang et al., 2012). Co-expressing *acs* with PHA biosynthesis genes in SA01 resulted in a 5-fold increase in mcl-PHA accumulation in shake flasks and almost a 12-fold increase in C_{12} content. This data supports the conclusion that balancing FFA production and CoA activation will be critical to maximizing mcl-PHA yields.

3.3.4. Bioreactor scale-up of mcl-PHA production from glucose

The described PHA production strategy is the first to produce a defined mcl-PHA from an unrelated carbon source. The highest mcl-PHA production (17.3% CDW) was achieved by cultivating strain SA01 pDA-JAC in a 1 L bioreactor using a fed-batch strategy. For comparison, prior studies achieved ~6% CDW of an undefined mcl-PHA in *E. coli* when grown on gluconate (Rehm and Steinbuchel, 2001) and 11.6% CDW of undefined heteropolymer in *E. coli* grown on glucose (Wang et al., 2012). Finally, recent work in both *P. putida* and *E. coli* demonstrated production of mcl-PHA homopolymer in the case of feeding exogenous fatty acids (Liu et al., 2011; Tappel et al., 2012). In *putida*, an 85% C_{12}-co-15% C_{10} PHA was produced at 9% CDW while a Δ*fadR* Δ*fadB* strain of *E. coli* was capable of making 28.6% CDW C_{12} homopolymer. Based on maximum theoretical yield calculations, *E. coli* is capable of producing 0.38 g (R)-3-hydroxydodecanoic acid per g glucose fed. Thus, further optimization of the described pathway for mcl-PHA biosynthesis should lead to additional improvements in the yield on glucose as a sole carbon source. For example, improvements in PHA biosynthesis could be achieved through expression of alternative polymerases or hydratases with a higher activity for C_{12} units. Besides *fadJ* (*yfcX*), there exist at least five additional genes with homology to *fadB* on the *E. coli* chromosome (Park and Lee, 2004). When these genes were overexpressed in *E. coli* Δ*fadB* in the presence of a PHA polymerase and LB + 0.2% decanoic acid (C_{10}), a 1.3- to 2.0-fold improvement in PHA accumulation (% CDW) was achieved over an empty vector control. Along

with *fadJ*, overexpression of *ydbU*, *paaF* and *paaG* resulted in the greatest improvement. By contrast, no PHA accumulation was detected in *E. coli fadB*$^+$ under the same conditions. Therefore, these gene products may have a role in both C_{12} metabolism and PHA biosynthesis in *E. coli* and overexpression of these genes in addition to or in place of *phaJ* could improve PHA accumulation.

3.4. Conclusions

A scheme was presented for the production of mcl-PHA homopolymer from a non-fatty acid related carbon source at up to 13.5% CDW. Examination of a series of β-oxidation deletion strains provided an understanding of knockouts required to completely inhibit iterative degradation of both exogenously fed and endogenously produced fatty acids. Specifically, disruption of both the aerobic and anaerobic pathways (*i.e., fadBA* or *fadIJ*) proved essential for the proposed mcl-PHA biosynthesis pathway. Co-expression of *phaJ3* and *phaC2* from *P. aeruginosa* PAO1 in *E. coli* Δ*fadRABIJ* yielded polyhydroxydodecanoate in the presence of dodecanoic acid feeding. When the plant acyl-ACP thioesterase, BTE, was expressed in this strain, PHA comprised primarily of hydroxydodecanoate monomers was observed. Finally, expression of an additional, soluble CoA-ligase improved production 5-fold resulting in the highest reported production of mcl-PHA homopolymer for a scheme involving a thioesterase.

It is anticipated that this strategy can be generalized to produce a variety of mcl-PHA homo- and heteropolymers where the resulting monomer composition can be tailored based on the known fatty acid production profile of a particular acyl-ACP thioesterase. If integrated with pathways for converting renewable substrates to acetyl-CoA, processes for synthesizing designer mcl-PHA can be developed. The use of inexpensive feedstocks will ultimately allow renewable, biodegradable PHA to compete on a cost-basis with analogous, petroleum derived plastics.

3.5. Material and methods

3.5.1. Bacterial strains, reagents, media, and growth conditions

All strains used in this study are listed in Appendix 3. *E. coli* DH5α was used to construct and propagate plasmids. *E. coli* K-12 MG1655 Δ*araBAD* was used as the base strain for studying β-oxidation and PHA production. Chemicals and reagents were purchased from Fisher Scientific (Pittsburgh, PA) unless

otherwise specified. Enzymes used for cloning were purchased from New England Biolabs (Ipswich, MA). Oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA) and sequences are listed in Appendix 4. For all growth experiments, single colonies were used to inoculate 5 mL starter cultures that were grown overnight prior to inoculation of experimental cultures. All growth experiments were performed at 37°C in a rotary shaker (250 rpm). Where necessary, cultures were supplemented with 100 µg mL^{-1} ampicillin and/or 34 µg mL^{-1} chloramphenicol.

For dodecanoic acid catabolism experiments (Figs. 3.2a and 3.3), each strain was cultured in 25 mL of LB to an optical density at 600 nm (OD_{600}) of 1.0. Cultures were centrifuged (1,000 x *g* for 20 min) and resuspended in 50 mL of M9 minimal media supplemented with 0.25 g L^{-1} sodium dodecanoate from a 5 g L^{-1} sodium dodecanoate aqueous stock solution. This amount was chosen because higher levels impaired growth of *E. coli* MG1655 Δ*araBAD* (data not shown). Under these conditions, soluble dodecanoic acid existed in equilibrium with a solid precipitate. After transfer, cultures were incubated at 37 °C with shaking and 2.5 mL culture samples were taken at 24 and 48 h for fatty acid methyl ester (FAME) analysis. In the case of *fadD* overexpression constructs, 1 mM IPTG was added at an OD_{600} of 0.02 and again after resuspension in minimal media.

For dodecanoic acid production experiments (Fig. 3.2b), each strain was inoculated to OD_{600} of 0.05 in 5 mL of LB + 0.4% (D)-glucose and induced with 1mM IPTG at an OD_{600} of 0.2. After induction, cultures were incubated for 48 h at 37 °C with shaking at which point, cultures were harvested for PHA and FAME analysis.

For shake flask experiments summarized by Table 3.1, 35 mL of LB was inoculated to OD_{600} of 0.05 and incubated with shaking until cultures reached OD_{600} of 1.0. Cultures were centrifuged (1,000 x *g* for 20 min) and the cell pellet resuspended in 50 mL M9 minimal media supplemented with 2.5 g L^{-1} dodecanoic acid and inducer(s) (1 mM IPTG; 0.2% (L)-arabinose). Cultures were harvested at 96 h for PHA and FAME analysis.

For PHA production experiments detailed in Table 3.2 and Fig. 3.4, 50 mL of MOPS + 1% (D)-glucose was inoculated to OD_{600} of 0.05 and induced with 1mM IPTG at an OD_{600} of 0.2. After induction, cultures were incubated for 96 h at 37 °C with shaking at which point, cultures were harvested for PHA and

FAME analysis. For strains lacking chromosomal expression of BTE, 0.25 g L^{-1} sodium dodecanoate from a 5 g L^{-1} sodium dodecanoate aqueous stock solution was added at the time of induction.

Bioreactor experiments were performed in a 3 L stirred bioreactor (Applikon Biotechnology, Inc., Schiedam, Netherlands) using a 1.0 L working volume. Temperature was maintained at 37 °C using an electric heat blanket and temperature, pH, and dissolved oxygen (DO_2) were monitored using specific probes. Vessel pH was maintained at 7.00 ± 0.05 by addition of 1M NaOH or 1M HCl solutions. Agitation was provided by a single impeller with the stirrer speed set to 700 rpm. Stirrer speed was occasionally increased to ensure the DO_2 content did not decrease below 40% saturation in order to maintain an aerobic environment (Becker et al., 1997; Tseng et al., 1996). Air inflow was maintained at 1.5 L min^{-1}.

Bioreactor experiments were inoculated at an OD_{600} of 0.05 with a culture of strain SA01 harboring plasmid pDA-JAC grown to an OD_{600} of ≥ 2.5 in MOPS minimal media supplemented with 1% glucose. Induction with 1 mM IPTG occurred when the OD_{600} of the bioreactor reached 0.2. The reactor was operated in batch mode with one addition of 10 g of glucose (50 mL of a 20% (w/v) glucose solution) at 24 h post-induction. The OD_{600} of the culture was monitored periodically and 15 mL of culture taken every 24 h for FAME and PHA analysis. The contents of the bioreactor were harvested at 96 h post-induction for PHA and FAME analysis.

3.5.2. Plasmid Construction

All plasmids used in this study are listed in Appendix 3. Plasmid pBAD33-C280* (Lee et al., 2007) was constructed by PCR amplification of plasmid pBAD33 with primers C280*-F/R (Appendix 4) (Guzman et al., 1995). The PCR product was treated with *Dpn I* and *Xho I* digestion and circularized by ligation with T4 DNA ligase. Genomic DNA was isolated from *P. putida* KT2440 and *P. aeruginosa* PAO1 with a Wizard® Genomic DNA Purification Kit (Promega). PHA genes *phaJ1-4* and *phaC1-2* were amplified by PCR from a *P. aeruginosa* PAO1 genomic DNA template with the respective phaC and phaJ primers (Appendix 4). PP_0763 was amplified by PCR from a *P. putida* KT2440 genomic DNA template with primers acs-F/R (Appendix 4). All constructs were confirmed by DNA sequence analysis. Annotated sequence files for relevant constructs were deposited in GenBank.

3.5.3. Chromosome engineering

Chromosomal gene deletions were created in *E. coli* K12 MG1655 Δ*araBAD* by P1 transduction (Thomason et al., 2007) using phage lysates generated from members of the KEIO collection (Baba et al., 2006). Deletions of *fadBA* and *fadIJ* were generated as described previously using pKD13 as template (Datsenko and Wanner, 2000). Chromosomal integration of a Φ(P_{trc}-BTE) expression cassette (a fusion of the IPTG inducible *trc* promoter with BTE) was constructed as described previously (Youngquist et al., 2012). Briefly, an insertion template was generated by PCR amplification of a fragment comprising $lacI^{Q}$-P_{trc}-BTE-FRT-Cm^{R}-FRT from plasmid pBTE-int. Primers contained 40 base pairs of sequence homology to regions of the *E. coli* chromosome flanking the *fadBA* locus (Appendix 4) to guide λ red mediated recombination. To construct the *fadD* promoter replacement, Φ(P_{trc}-*fadD*), the region consisting of *$lacI^{Q}$*-P_{trc}-*fadD* was PCR amplified off of plasmid pTrc-*fadD*. A region of pKD13 comprising the kanamycin resistance cassette flanked by FRT sites was PCR amplified separately. The two PCR products were stitched together in a third PCR, generating a linear DNA that was integrated onto the chromosome of *E. coli* DY330 via λ red mediated recombination. For each mutant strain, resistance markers were removed by inducing FLP recombinase encoded on plasmid pCP20 which was subsequently cured by growth at a non-permissive temperature (Datsenko and Wanner, 2000). All chromosomal mutations were verified by colony PCR.

3.5.4. Fatty acid and PHA extraction and characterization

FAME analysis was performed on 2.5 mL of culture or supernatant as described previously (Lennen et al., 2010). For PHA analysis, cells were harvested by centrifugation (3000 x *g* for 25 min), washed with 25 mL 1X phosphate buffered saline (PBS), and lyophilized overnight. PHA content was analyzed by GC/MS based on the method of Kato *et al.* (Kato et al., 1996). PHA was converted to the corresponding monomer-esters by combining 2 mL of chloroform and 2 mL of 3% H_2SO_4 in methanol (v/v) with 10 mg of lyophilized cells in a 10 mL disposable glass centrifuge tube. 50 µL of 10 mg mL^{-1} pentadecanoic acid in ethanol was added as an internal standard. The mixture was heated at 105 °C in a heat block for 24 hours followed by addition of 5 mL of 100 mg mL^{-1} $NaHCO_3$ in water. The mixture was vortexed and centrifuged (1,000 x *g* for 10 min) and the aqueous layer was removed by aspiration. The organic (chloroform) phase (1 µL) was analyzed using a Shimadzu GCMS QP2010S gas chromatograph mass spectrometer equipped with an AOC-

20i auto-injector and a Restek Rxi®-5ms column (catalog #13423). The temperature program used was as follows: 60°C hold for 1 minute, ramp from 60°C to 230°C at 10°C per minute and a final hold at 230°C for 10 minutes. The MS was operated in scanning mode between 35 and 500 m/z.

3.5.5. PHA purification and nuclear magnetic-resonance spectroscopy

PHA was extracted for analysis by nuclear magnetic-resonance (NMR) as described previously (Jiang et al., 2006) and modified based on communications with Chris Nomura (State University of New York). Briefly, lyophilized cells were washed with methanol to remove fatty acids and other impurities followed by a second lyophilization step. The material was extracted with 120 mL refluxing chloroform in a Soxhlet apparatus followed by evaporation of the chloroform to recover the purified PHA. 10-15 mg of product was dissolved in 1 mL deuterated chloroform and analyzed at room temperature on a Bruker AC-300 spectrometer for ^{1}H NMR and on a Varian Mercury-300 spectrometer for ^{13}C NMR.

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Chapter 4: A PKS platform for the transformation of basic cellular building blocks into diverse PHA with a defined composition

4.1. Introduction

Opportunities for improving the commercial appeal of PHA can be roughly partitioned into two categories. The first involves reducing the cost of PHA production while the second relates to adding value to the PHA product itself. A survey of the PHA literature illustrates that both of these strategies have been examined. Reducing the cost of PHA production can be accomplished by using inexpensive feedstocks such as glucose, biomass or various waste-streams. Alternatively, methods for recovering the final product and for purifying the material away from the cellular debris is an energy intensive process that can involve large amounts of solvent or high-capital cost separation equipment. For the production of PHA from an unrelated feedstock, reducing the cost of the starting material is a problem left to the manufacturer. While a number of methods have been developed to efficiently recover PHA from a biological system, major advances in PHA process technology may not be realized until PHA are in greater demand as an alternative to inexpensive, petroleum derived plastic counterparts. Therefore, many researchers have explored the production of PHA with more desirable material properties for use in niche applications where a premium price is more readily commanded.

Due to the natural ability of PHA polymerase to polymerize diverse organic acids, many studies have focused on the feeding of specific carbon sources to achieve a polyester product consisting of monomers with a chemical structure related to the starting material. This approach allows for the incorporation of a number of functional groups including halogen, amino, alkene and ester moieties. PHA monomers with these unique functionalities result in polymers with varied chemical and mechanical properties. For example, a terminal alkene can provide a handle for chemical functionalization or cross-linking while monomers with longer side-chains or increased branching result in materials with lower glass transition and melting temperatures. Furthermore, a number of studies have focused on controlling the precise composition of the polymer. Specifically, creating PHA with a homogenous composition which can be challenging in a cellular environment where the monomers are often readily modified or broken down into other molecules for use elsewhere in the cell.

While PHA with unique chemical compositions may be more attractive, the feedstocks associated with their biosynthesis can be much more expensive than simple substrates such as glucose. Therefore, the opportunity exists to identify routes to more desirable PHA – especially those with a diverse chemical make-up – that originate with an inexpensive, unrelated feedstock. The key to this approach lies in identifying a platform that is capable of producing a wide range of chemical structures while still affording control over the specific composition of the monomer pool available for PHA biosynthesis.

It is worth noting at this point that metabolic engineering strategies have identified a number of specific pathways for producing unique PHA monomers that begin with unrelated carbon sources. Here, instead, we have sought to identify a more universal approach. The advantage of this strategy is that one need not identify a new approach each time a new type of monomer becomes desirable to synthesize. Rather, the existing platform could be tuned by simply rearranging or adding to an existing set of basic catalytic building blocks. Thus, the goal was to identify a tunable platform for transforming basic cellular building blocks into diverse PHA with a defined composition. The platform that best fits this description is a set of enzymes known as type I PKS.

The use of type I PKS was inspired by 3HB production using a modified type I fatty acid synthase (FAS) (Fig. 4.1). In *E. coli*, fatty acid biosynthesis is a carried out a by a set of distinct enzymatic domains that function iteratively to load building blocks onto a protein scaffold, condense these building to elongate the growing acyl-chain, and perform chemical modifications to achieve the desired product. In other organisms, these enzymatic activities are collected into one multi-functional megasynthase. By rendering non-functional a single activity associated with this megasynthase, it was possible to change the product of the FAS from a long-chain fatty acid to the four carbon 3HB.

The key to the success of the type I FAS strategy for PHA production was the iterative nature of this megasynthase. Without the use of just one of the five core catalytic domains, the intermediate product, 3HB stalls on the FAS and is released by the proof-reading (thioesterase) TE domain. No further iterations of the elongation/modification process are possible, so only 3HB can be made with this approach.

Type I PKS are structurally and functionally related to the type I FAS megasynthases (Fig. 4.2), yet produce a wide range of chemical products. Analogous to an industrial assembly line, starting materials

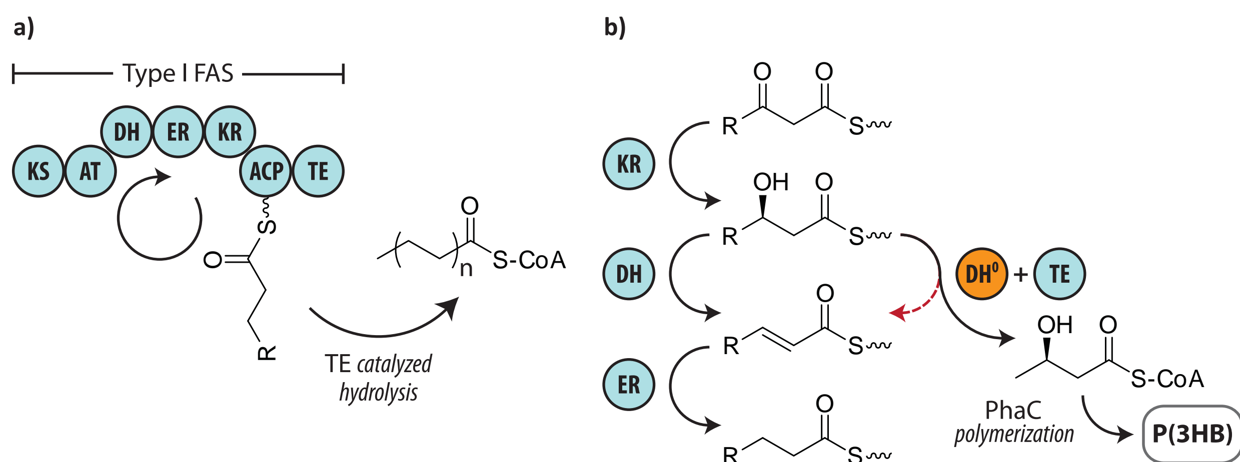

Figure 4.1. Architecture and catalysis of a wild type and modified rat type I FAS. (a) The wild type FAS normally catalyzes the biosynthesis of a 16 carbon fatty acyl-CoA (palmitate) with (b) the KR, DH and ER domains functioning together to completely reduce the β-keto group resulting in a saturated acyl chain. If the DH domain is inactivated, the FAS is incapable of further reducing the β-hydroxy group resulting in cleavage of 3HB by the proof-reading TE domain.

are loaded onto one end of a type I PKS and pass through various catalytic "weigh-stations" which perform the extension and modification steps. Acyltransferase (AT) and ketosynthase (KS) domains are involved in elongation, while ketoreductase (KR), dehydratase (DH) and enoylreductase (ER) domains are responsible for modification. The clusters of catalytic domains known as modules are only responsible for one round of activity. In contrast to the FAS, this architecture allows for a variety of functional groups to be incorporated into the final product. Furthermore, research has demonstrated the possibility to manipulate the building blocks that compose a given PKS as one would recombine LEGO® bricks (Fig. 4.3). Prior efforts to manipulate PKS have yielded design criteria that provide a strong foundation for engineering these megasynthases to produce diverse PHA with defined composition from unrelated feedstocks. This chapter outlines the efforts undertaken to build a proof-of-principle strain for PHA production centered on the use of a modified type I PKS.

4.2. Results and discussion

4.2.1. Strategy for PHA production

Production of PHA via a PKS strategy involves three basic steps. First, a specially-designed PKS converts organic building blocks (*e.g.,* acetyl-CoA, malonyl-CoA, propionyl-CoA) derived from an unrelated

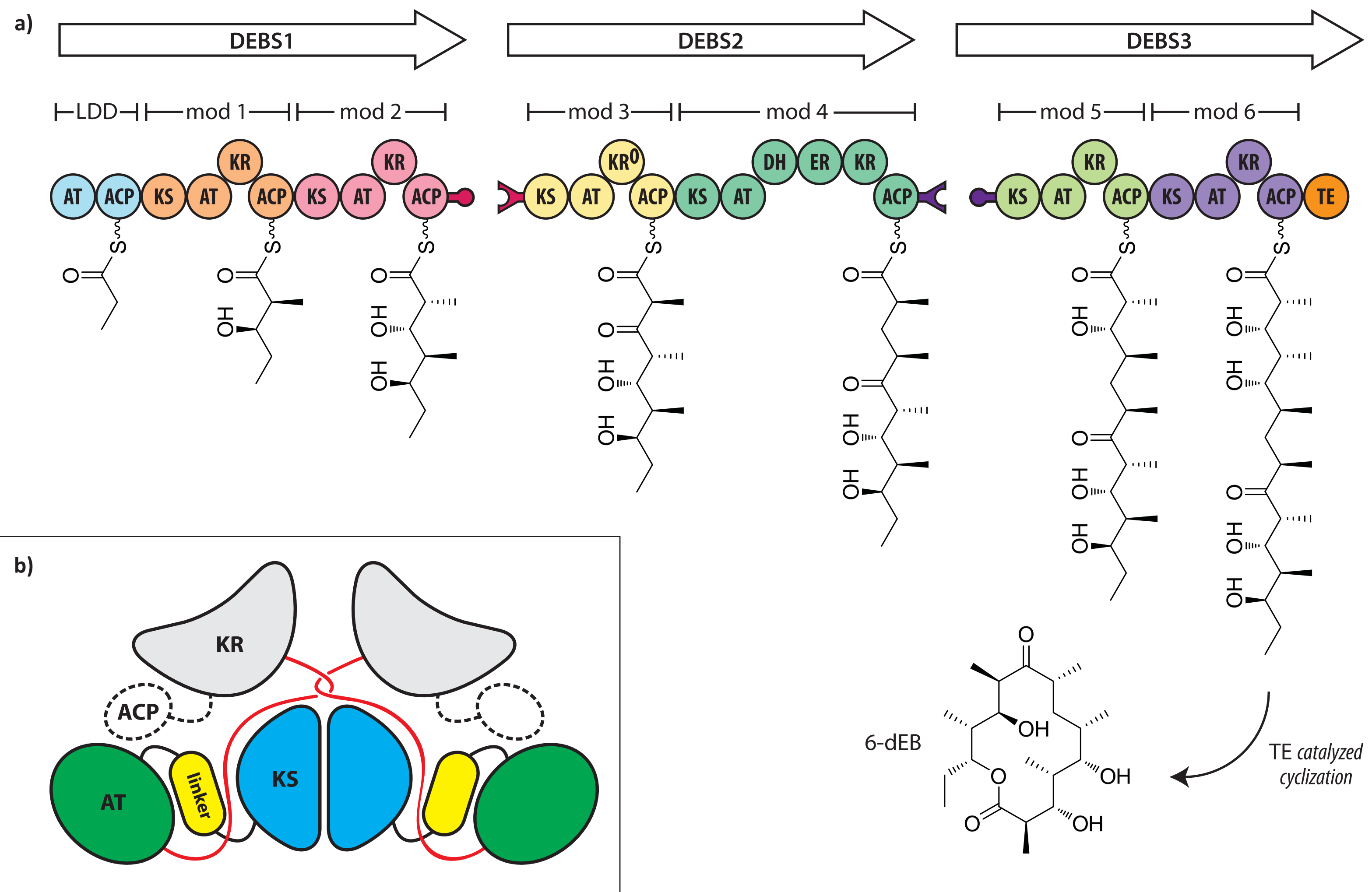

Figure 4.2. Schematic of the DEBS type I PKS showing (a) the transciption units (DEBS1, 2, 3), modular organization, domain organization and catalysis that results in the formation of the erythromycin precursor, 6-dEB. While the architecture of a type I PKS is often represented in a linear fashion to highlight the assembly line nature of the megasynthase, a more representative depiction (b) features the homodimeric and intertwined nature of the modules (*i.e.,* DEBS module 1).

feedstock (*e.g.,* glucose) to PHA monomers (*e.g.,* acyl-CoA) or monomer precursors (*e.g.,* organic acids). Second, the precursors must be modified where necessary and activated for polymerization. Third and finally, the activated monomers are polymerized to form the final PHA product. The latter two steps are relatively trivial in comparison with identifying and engineering an appropriate monomer synthase in step one. The promiscuity of PHA polymerizing enzymes has already been described along with techniques for improving the activity of a PHA polymerase towards a given substrate (*see* 2.7.). Similarly, activating enzymes, known as CoA ligases, are often capable of accepting a broad range of substrates. Strategies for identifying an appropriate CoA ligase are described in further detail later on in the chapter.

4.2.2. Identification of PKS building blocks

Over a thousand PKS gene clusters have been cloned and sequenced, however, less than 10% of these clusters have been characterized beyond identification of the primary DNA sequence (Wong and Khosla, 2012). Of the characterized PKS described in the literature, the 6-deoxyerythronolide B (6-dEB) synthase or DEBS is arguably the most extensively studied type I PKS (Fig. 4.2). Erythromycin, the product of DEBS (following glycosylation) was discovered at Eli Lily in the early 1950's, but the genetic identity of the DEBS cluster was not determined until the early 1990's (Cortes et al., 1990; Tuan et al., 1990). The DEBS PKS is native to *Saccharopolyspora erythraea* and is somewhat unique in its ability to be functionally expressed in *E. coli* (Pfeifer et al., 2001). The DEBS PKS natively has one loading module and six extension modules and produces a 'heptaketide' twenty-one carbon macrolide ring that is the precursor to the antibiotic erythromycin. By truncating DEBS (leaving only the loading module, the first two extension modules and the terminal TE domain), a functional 'triketide' synthase was engineered (Fig. 4.3b). The product of this truncated PKS is a nine carbon "mini-lactone." Further truncation by way of removal of the second extension module led to a functional "diketide synthase" (DKS) capable of producing the six carbon diketide, 3-hydroxy-2-methylvaleric acid (Figs. 4.3c and 4.4a).

The product of the DEBS DKS, if activated to the CoA form, is a demonstrated substrate for incorporation into PHA (Satoh et al., 1992). Therefore, the DEBS DKS was identified as a potential candidate for use as a PHA monomer synthase. However, there is no precedent for the use of engineered PKS to produce PHA. As a more basic proof-of-concept system was desired, a PKS capable of synthesizing a well-

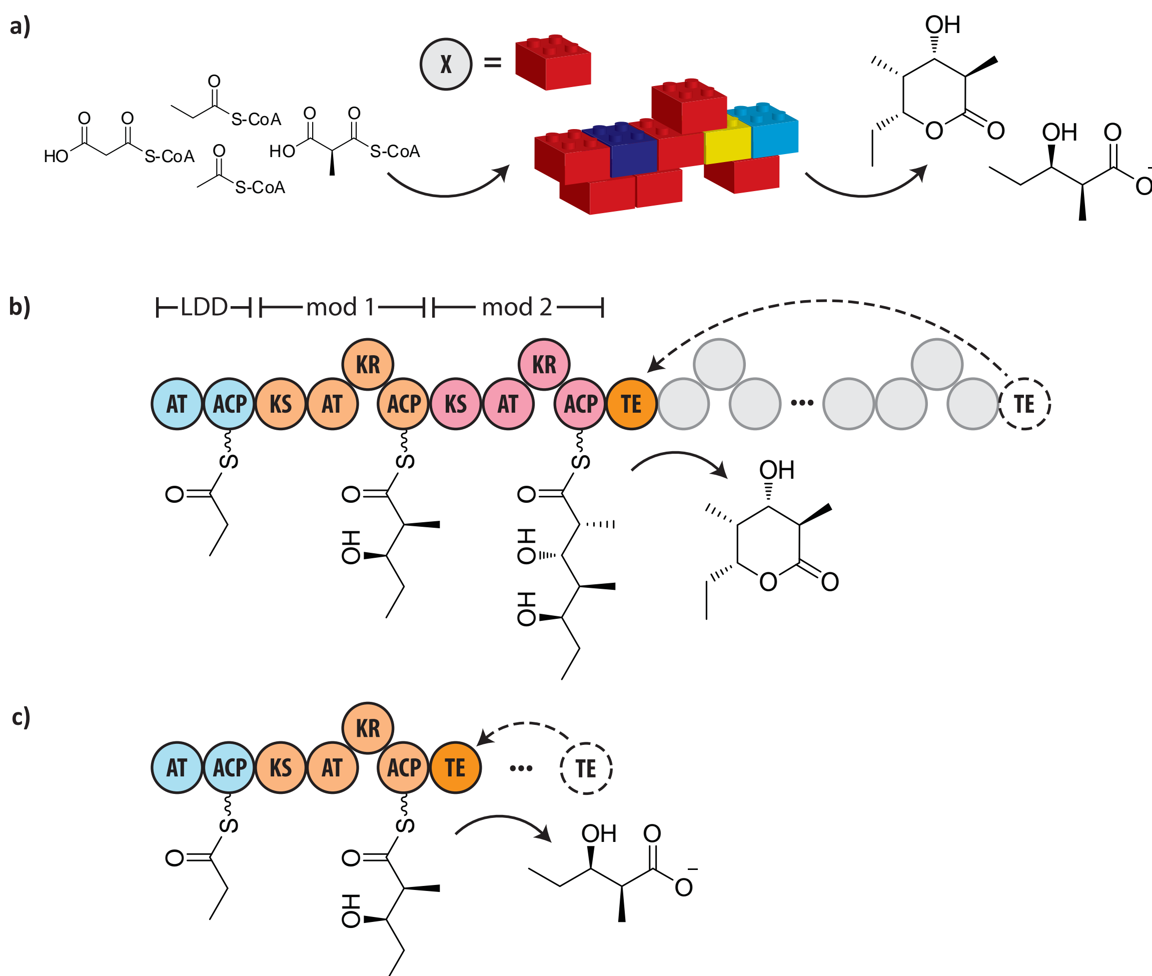

Figure 4.3. "Lego-ization" of type I PKS. Given the assembly-line architecture of PKS, it is conceivable to (a) feed PKS substrates to a hybrid, synthetic PKS to generate a desired product. Demonstrated hybrid PKS included (b) the DEBS triketide synthase in which the TE domain is fused to the terminal ACP of module 2 and (c) the DEBS diketide synthases in which the TE is fused to module 1.

characterized PHA monomer, namely 3HB, was sought. In order to synthesize 3HB, an appropriate PKS would consist of a loading domain specific for acetyl-CoA and an extension module that uses malonyl-CoA. Additionally, this module should contain a functional KR domain and no additional reductive domains (*i.e.,* DH, ER) to generate a β-hydroxyl group with the appropriate stereochemistry. Due to the architecture and assembly-line function of PKS, knowing the structure of the polyketide product allows for the elucidation of the hypothetical product of a DKS analogue. A survey of the literature revealed that the PKS antibiotic niddamycin has a chemical structure indicative of a PKS with the aforementioned requirements. Thus, the predicted product of a niddamycin DKS is 3HB (Fig. 4.4b,c).

4.2.3. Construction of the DEBS and niddamycin DKS

Attempts to obtain the original constructs for the DEBS DKS were unsuccessful so genomic DNA from *S. erythraea* and *Streptomyces calestis* (the native producer of niddamycin) was obtained for use as a template for the construction of DEBS and niddamycin DKS. Construction of the DEBS DKS as described previously involved fusion of the ACP domain of module one (ACP1) with the terminal ACP-TE didomain associated with module 6 (ACP6). Amino acid sequence alignment of the amino acid sequence ACP1 with ACP6 allowed for the identification of a site where an artificial Pst I restriction site could be incorporated to allow for a seamless fusion while conserving the amino acid sequence. An analogous strategy was employed in the case of the niddamycin DKS although the niddamycin PKS contains only five modules so the ACP1 was fused with ACP5. It is important to note that all ACP require activation to the *holo* form via covalent addition of a phosphopantetheine (PPT) group at a conserved serine residue (Fig. 4.5) (Walsh et al., 1997). In both the DEBS and niddamycin ACP fusions, this serine was N-terminal and proximal to the fusion site. As a result, the residue was contributed by the ACP from the first extension module. Both constructs were cloned into the medium copy vector pTrc99a in front of the IPTG inducible *trc* promoter and a consensus ribosome binding site (RBS) (AGGAGG).

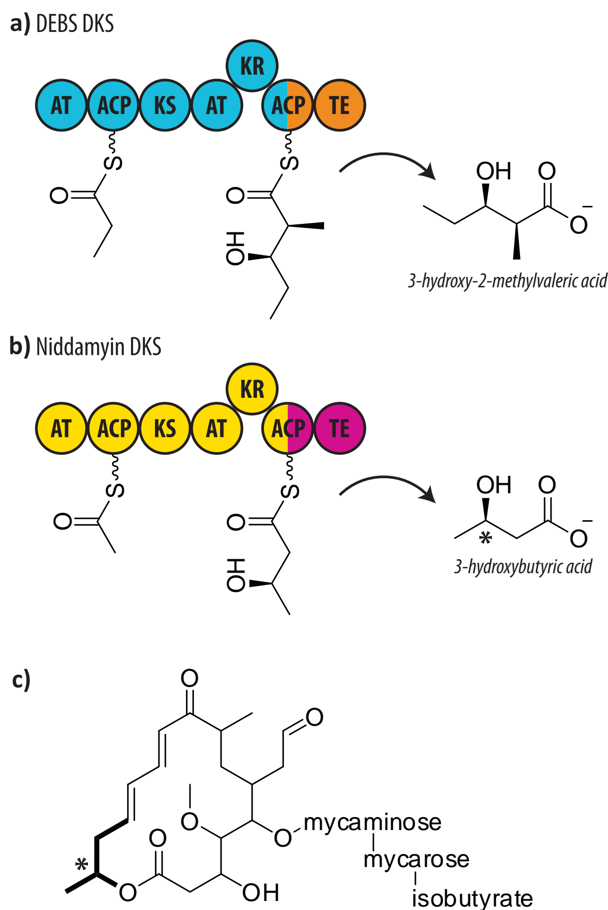

Figure 4.4. Hybrid DKS for (a) DEBS module 1 + TE which produces 3-hydroxy-2-methylvaleric acid and (b) niddamycin module 1 + TE which theoretically produces 3HB based on (c) the structure of niddamycin.

4.2.4. Identification and cloning of a CoA ligase and PHA polymerase

The second step of the PKS based PHA production strategy required the identification of a suitable CoA ligase for the activation of either 3HB or the DEBS diketide 3-hydroxy-3-methylvaleric acid. While no CoA ligases annotated as specific for 3HB were found, a number of these enzymes have been characterized to be active on a broad range of substrates. Therefore, two CoA ligases were selected. The first was the acetoacetic acid CoA ligase from *S. erythraea*, AcsA, while the second was a PHA granule associated CoA ligase, Acs1, from *P. putida* GPo1. The former enzyme was chosen based on the similarity of the chemical structure of acetoacetic acid to 3HB. The latter enzyme was selected based on previous work that characterized the activity of this CoA ligase on a range of 3-hydroxalkanoic acids (Ruth et al., 2008).

The third and final step for PHA production involves the polymerization of the activated monomers. The scl-PHA polymerase PhaC from *C. necator* is an effective and specific catalyst for the polymerization of 3HB. $PhaC_{Cne}$ also has activity on other scl-hydroxyalkanoates such as 3HV and was implicated in the formation of PHA incorporating 3-hydroxy-2-methylbutyric acid and 3-hydroxy-2-methylvaleric acid (Satoh et al., 1992). The latter two PHA monomers were synthesized by polyphosphate accumulating bacteria in a wastewater treatment process. Therefore, $PhaC_{Cne}$ was selected as the primary candidate for the final polymerization step. $PhaC_{Cne}$ and either Acs1 or AcsA were cloned as an artificial operon into the low copy plasmid pBTrcK under the IPTG inducible *trc* promoter and individual consensus RBS. Plasmids pBTrcK and pTrc99a were chosen due to their compatible origins of replication and uniform induction systems.

4.2.5. Strain construction and initial analysis

E. coli was selected as a host for PHA production due to the genetic tractability of the organism and its track-record as an effective host for both PHA production and functional expression of the DEBS PKS and its derivatives. In particular, *E. coli* BAP1, a derivative of *E. coli* BL21 (DE3) is particularly suited to PKS expression (Pfeifer et al., 2001). BAP1 expresses the 4'-phosphopantetheinyl transferase gene from *Bacillus subtilits*, *sfp*, which is capable of activating a number of PKS associated ACP domains including those associated with DEBS (Lee et al., 2009). In addition, a deletion in the *prp* operon renders BAP1 incapable of native propionate metabolism. This is beneficial as propionate is used in the formation of DEBS PKS starter and extender substrates.

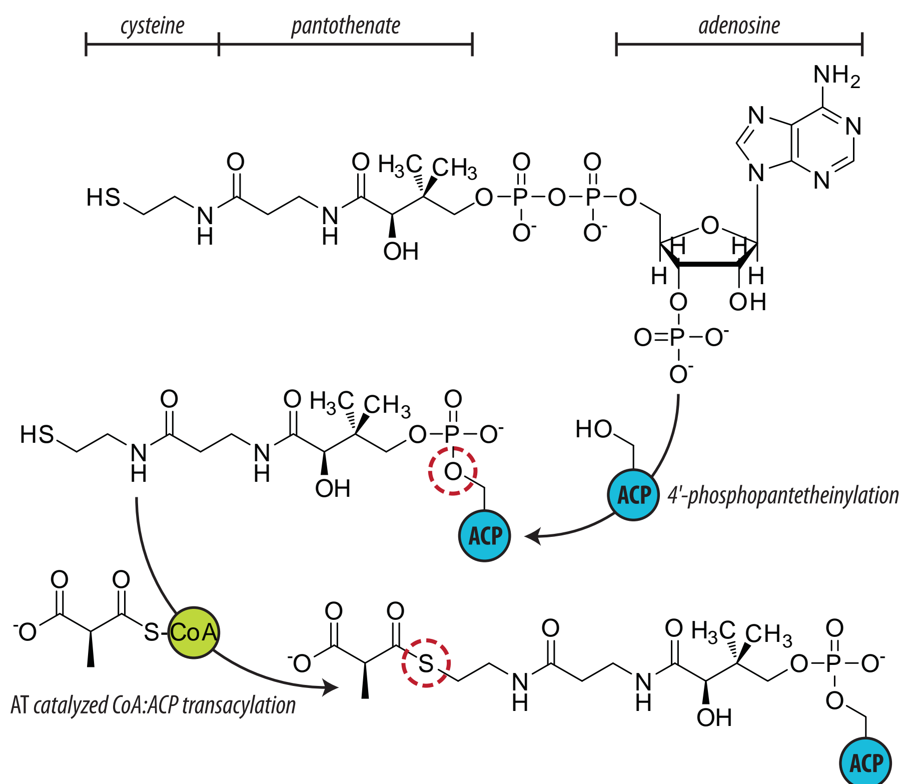

Figure 4.5. Chemical structure and mechanism of 4'-phosphopantetheinylation. An *apo*-ACP is activated at a conserved serine residue with the phosphopantetheine group. The resulting *holo*-ACP can now accept PKS substrates such as methylmalonyl-CoA through formation of a thioester bond.

E. coli BAP1 was transformed with plasmids containing either the DEBS or niddamycin DKS and a CoA-ligase and PHA polymerase. The resulting strains were predicted to have the ability to convert glucose (or any other usable carbon source) to PHA composed of either 3HB or 3-hydroxy-2-methylvaleric acid. Cultures of each strain were analyzed for the presence of both free hydroxyalkanoic acids and PHA polyester. In initial experiments, neither the free acids nor the polyester were observed. Therefore, it was necessary to dissect the engineered pathway and investigate which components were defective.

4.2.6. Analysis of DKS expression in E. coli

Given the lack of free hydroxy acids, we hypothesized that the PKS were not being functionally expressed. Therefore, we attempted to measure the PKS DNA, mRNA, and protein levels in the cell. Plasmid constructs were isolated from culture and sequenced. The resulting data indicated that no mutations were present in either the gene or the regulatory elements on the plasmid. To measure PKS mRNA levels,

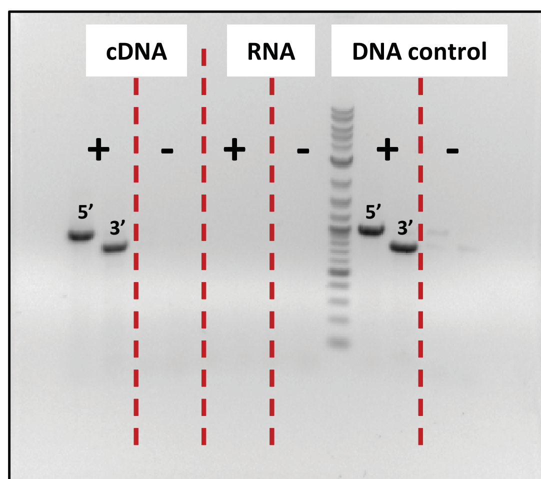

Figure 4.6. Standard agarose gel analysis of RT-PCR products for niddamycin DKS expression. From left to right: PCR amplification of DKS cDNA, DKS mRNA and plasmid pNidDKSv2. Plus (+) and minus (-) indicate presence of reverse transcriptase in cDNA synthesis step.

total RNA from both DEBS DKS and niddamycin DKS expressing strains was collected and analyzed via reverse transcription (RT)-PCR. PCR primers were designed to probe both 5' and 3' ends of the DKS transcripts. The resulting data confirmed that full-length mRNA was successfully transcribed for both the DEBS DKS and niddamycin DKS constructs (Fig. 4.6).

Given that transcription was not the problem; the next step was test for translation. Due to the predicted size of the DKS proteins (289 kDa for niddamycin DKS, 241 kDa for DEBS DKS) we pursued alternative approaches to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) for visualization of full-length protein. One method for probing translation involves coupling expression of the gene of interest with expression of a detectable marker such as a fluorescent protein or antibiotic resistance gene (Mendez-

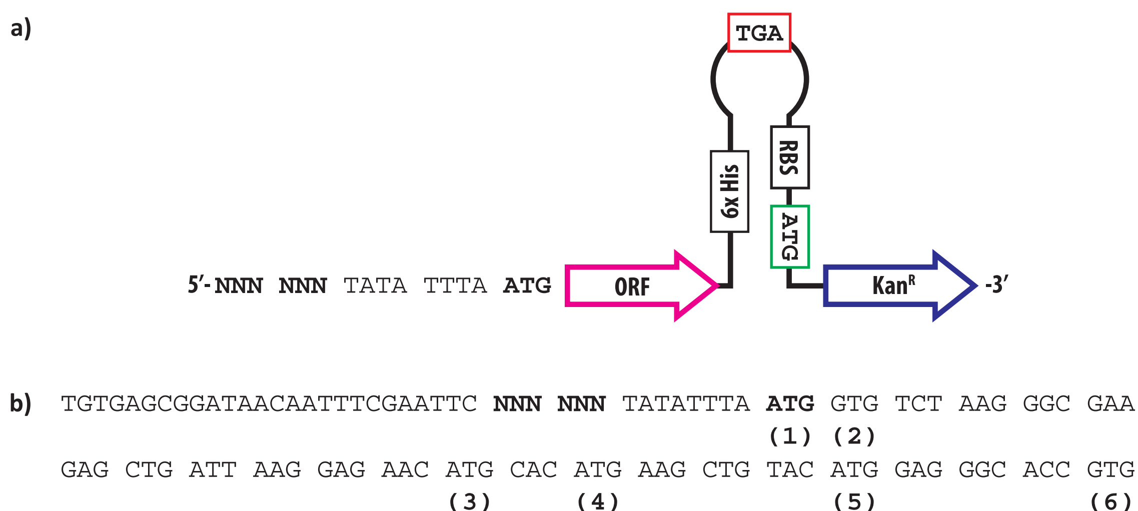

Figure 4.7. Schematic of the translational coupling system (Mendez-Perez et al., 2012). (a) From 5' to 3', the degenerate RBS (in the case of the RFP library), eight nucleotide spacer, start codon, ORF, hairpin structure containing the His-tag and stop codon for the ORF and RBS and start codon for the coupled kanamycin resistance marker. (b) A sample sequence is shown for the case of degenerate RFP coupling experiment with potential start codons labelled 1-6.

Perez et al., 2012). This method was successfully demonstrated and used by a Daniel Mendez-Perez in the Pfleger lab to show which domains of a megasynthase were not expressing in *E. coli*. In this system, plasmid pLIC-KM encodes an mRNA cassette that ties translation of a kanamycin resistance gene (*aphII*) to a target open reading frame (ORF) (Fig. 4.7a). Transcription of the cassette results in the formation of a strong RNA hairpin that sequesters the RBS and start codon of the downstream *aphII* gene. This hairpin is disrupted revealing the *aphII* translation signals only if a ribosome successfully traverses the length of the upstream ORF. In practice, cell growth in the presence of kanamycin can be measured as a proxy for successful translation of the target ORF.

Translation of the niddamycin DKS was examined using the pLIC-KM coupling cassette. The full-length DKS construct was divided into three regions comprised of the loading domain (region A), module 1 + ½ ACP (region B) and ½ ACP + TE (region C). Each of these regions was individually cloned into pLIC-KM with an artificial start codon and consensus RBS. Strains of *E. coli* BAP1 transformed with translation coupling constructs were grown in the presence of 0-200 μg mL^{-1} kanamycin (Fig. 4.8). The resulting

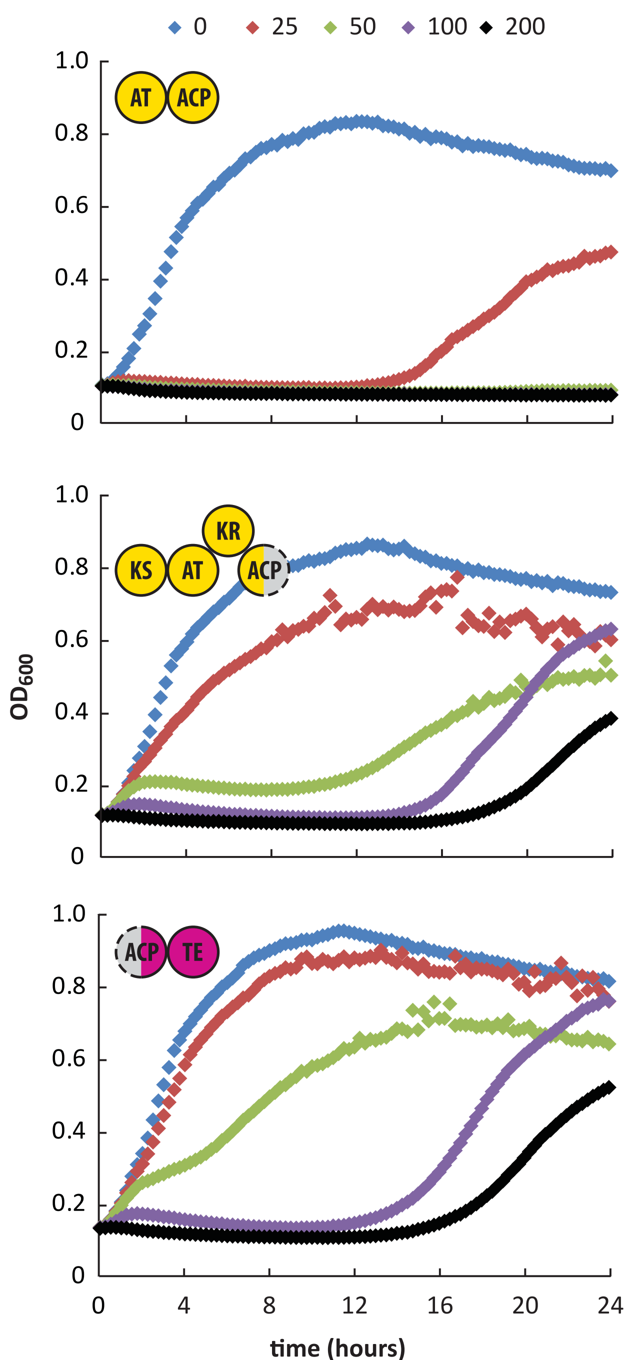

Figure 4.8. Growth curves for strains harboring the DKS translational coupling constructs in the presence of 0-200 ug/mL kanamycin. The region of the DKS tested is depicted in the upper left corner of the respective plot.

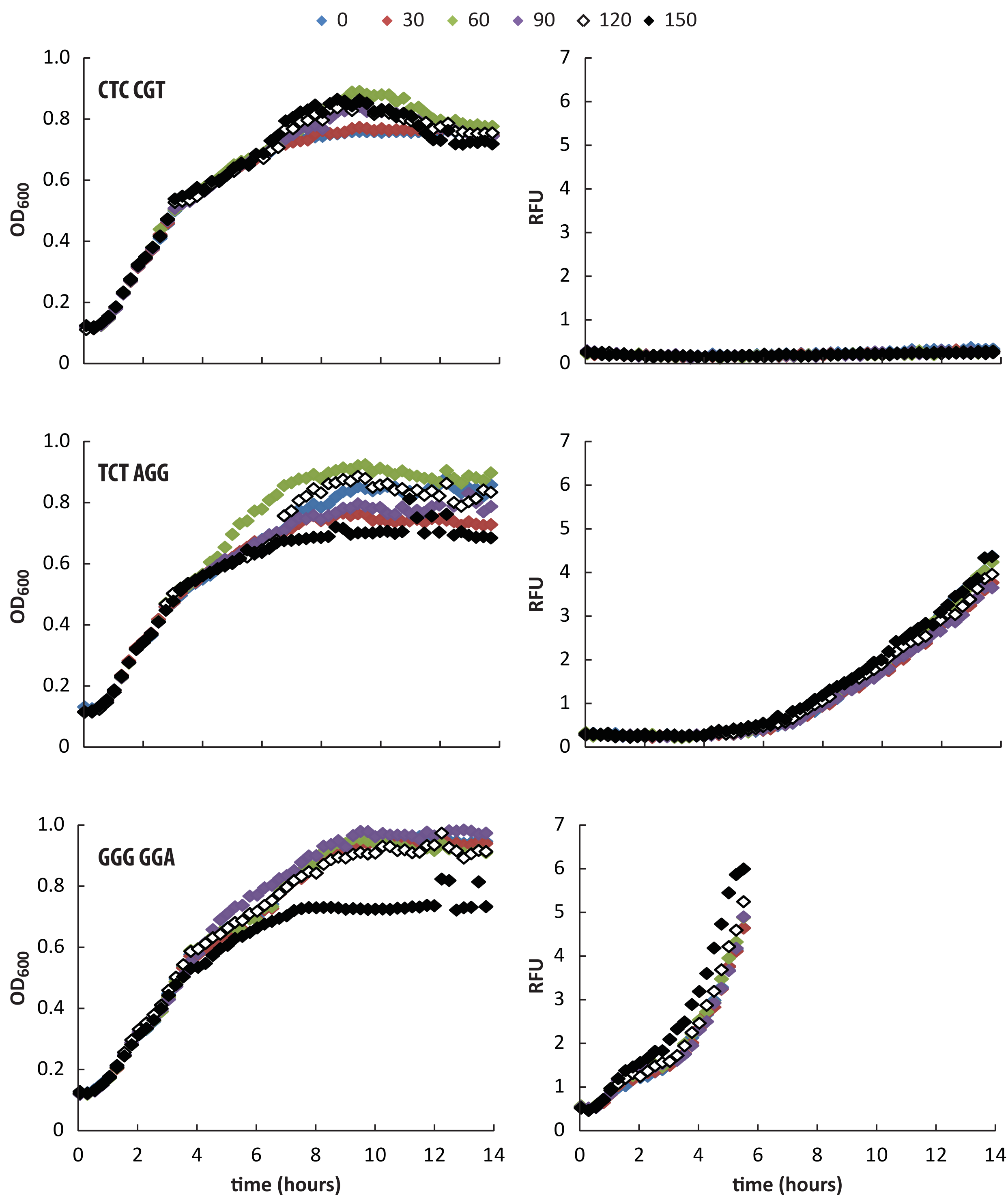

Figure 4.9. Growth curves for strains harboring the RFP translational coupling constructs in the presence of 0-150 ug/mL kanamycin and the corresponding RFP fluoresence shown to the right. Constructs for three repesentative members of the library tested are depicted with the RBS indicated in the upper left corner of the respective plot. While strains grew in the presence of all kanamycin concentrations regardless of RBS, stronger RBS sequences resulted in larger measured fluoresence values.

growth curves were indicative of a lack of translation of region A based on the inability of the strain to grow in the presence of more than 25 µg mL^{-1} kanamycin. Regions B and C exhibited improved growth capabilities in kanamycin concentrations of up to 200 µg mL^{-1}, however, there was a severe lag in growth of greater than 12 hours at the higher antibiotic concentrations.

To further probe the translational coupling system to identify the extent of translation required for varying degrees of kanamycin resistance, a library of red fluorescent protein (RFP) variants was cloned upstream of *aphII* in pLIC-KM. Library construction was achieved by PCR amplification of RFP with primers designed with a degenerate 6 bp RBS sequence. A random selection of positive clones was selected for analysis of both growth and fluorescence in the presence of 0-150 µg mL^{-1} kanamycin (Fig. 4.9). Surprising, all clones were capable of uninhibited growth in all antibiotic concentrations tested. However, a range of fluorescence phenotypes were observed including clones that exhibited no detectable fluorescence. Sequence analysis of a several representative library members revealed a direct correlation between RBS strength and relative fluorescence (Table 4.1). For example, the near consensus RBS ‘GGG GGA’ led to a sharp increase in fluorescence that tracked cell growth with little to no lag. By contrast, no fluorescence was detectable for the non-consensus ‘CTC CGT’ RBS. One explanation for this phenomenon may be the primary sequence of RFP, which contains a number of potential in-frame start codons downstream of the actual translational start site (Fig. 4.7b). The predicted RBS strength for the sequence with the greatest observable fluorescence was greater than 7000 arbitrary units. (GGG GGA). By comparison, an in-frame start codon (ATG) at position +55 relative to the translational start (+1) was predicted to have a

Table 4.1. RBS sequences for selected RFP-Kan library constiuents. Qualitative fluorescense is indicated in addition to predicited RBS strength. See Fig 3.6 for start codon numbering. RBS strengths were calculated using the Salis Lab RBS calculator (salis.psu.edu/software).

		Start Codon RBS Strength[1] (a.u.)					
RBS	**Fluorescence**	**1**	**2**	**3**	**4**	**5**	**6**
CTC CGT	None	0	32	5160	9	7373	1230
GAC GCA	None	0	22	2005	9	7373	1230
GCG TAC	None	102	67	1833	9	7373	1230
GAG AGT	High	758	64	2005	9	7373	1230
TTC AGG	High	2504	588	1917	9	7373	1230
GGG GGA	Very High	7783	1659	4933	9	7373	1230

comparable RBS strength. Translation starting at this downstream codon would result in successful translational coupling and tolerance to kanamycin but may not result in functional RFP and therefore, no observable fluorescence.

We anticipated that probing RBS strength would indicate whether minimal translation of the target gene (*e.g.,* DKS, RFP) is required to achieve a high degree of kanamycin resistance and therefore, whether the moderate antibiotic tolerance observed for niddamycin DKS regions B and C implied that translation of these fragments is very inefficient. Due to the nature of the RFP sequence, further experimentation would be required to confirm the alternate translational start hypothesis. Alternatively, a different reporter gene with no ambiguous translation signals could be substituted. Given the results of the RFP library, it is difficult to draw a conclusion about translation of the DKS regions tested, but in comparison with previous work, it is likely that the DKS constructs are not effectively translated in *E. coli* (Mendez-Perez et al., 2012).

4.2.7. Analysis of DKS expression in S. lividans

The original construction and expression of the DEBS DKS was carried out in *S. erythraea* (Ostergaard et al., 2002). The DEBS DKS construct was a derivative of the DEBS triketide synthase (Bohm et al., 1998) and a number of variations on this triketide construct have been successfully expressed in *E. coli* (Menzella et al., 2005). Yet, there is no literature precedent for the expression of the DEBS diketide construct in *E. coli*. Conditions for the functional expression of PKS in *E. coli* can be complex and some PKS may not function at all without modification. For example, both the megalomicin PKS and DEBS PKS produce 6-dEB but when expressed under the same conditions, only DEBS produced a detectable product (Murli et al., 2004).

In order to determine if the choice of *E. coli* BAP1 as a host was the cause of poor DKS expression, both niddamycin and DEBS DKS constructs were moved into pGM190 for expression in *Streptomyces lividans*. The medium-copy, broad host range shuttle vector, pGM190, is compatible with *S. lividans* and allows for thiostrepton inducible expression from the P_{tipA} promoter (Wohlleben et al., 2009). After transformation into *S. lividans* with either the DEBS or niddamycin DKS and growth under production conditions, no diketide products were detected.

4.2.8. Potential for DKS optimization

A number of challenges exist regarding the modularity of PKS in the context of engineering custom biocatalysts (Khosla et al., 2009). Regardless, if functional expression proves to be the barrier to PHA monomer biosynthesis, several strategies exist for improving the catalytic activity of the DKS. Actinomycetes such as *S. caelstis* and *S. erythraea* are characteristically guanine-cytosine (G-C) rich organisms. The G-C content for the niddamycin and DEBS DKS coding sequences is 72% and 74% respectively while the average *E. coli* gene is on the order of 50% (Muto and Osawa, 1987). The G-C content may not be the inherent problem in heterolgous expression of a given gene in *E. coli*, however, codon bias can play a role. A number of algorithms exist for determining the optimal codon usage and optimization of the DKS sequences could lead to improved heterologous expression of the niddamycin and DEBS constructs (Angov et al., 2008; Puigbo et al., 2007; Villalobos et al., 2006). Alternatively, The G-C content of *S. lividans* is 72.2% with a codon bias more closely aligned with *S. caelestis* and *S. erythraea*. If the efficacy of the translational coupling cassette could be proved in *S. lividans*, then the translation efficiency of the DKS constructs could be determined in a more closely related organism. If *S. lividans* is capable of translation of full-length DKS protein, then the functionality of these constructs might be probed without the need for codon optimization of the ~7 kb DKS coding sequences.

Recent insights into the determinants of ACP recognition dictates more rigorous design rules for creating ACP fusion proteins that retain the ability to interact with the surrounding domains (Kapur et al., 2010). Residues within helix I of the ACP mediate chain transfer while residues in loop I of the ACP interact with the KS-AT linker within the same PKS subunit to facilitate chain elongation (Fig. 4.10a,b). In the latter case, the active site of the ACP from one subunit is positioned for interaction with the KS of the opposite subunit of the PKS homodimer, which supports the existence of the conserved dimeric structure of modules of Type I PKS. Design of the ACP used in the DEBS and niddamycin DKS constructs was informed based on retaining the conserved residues and creating a seamless fusion via restriction cloning. Analysis of the ACP constructs reveals that the important recognition sites are likely intact (Fig. 4.10c-h).

Studies of ACP-TE interaction indicate that TE domains are flexible in their ability to catalyze both lactonization (cyclization) and hydrolysis of a broad range of substrates (Aggarwal et al., 1995; Weissman

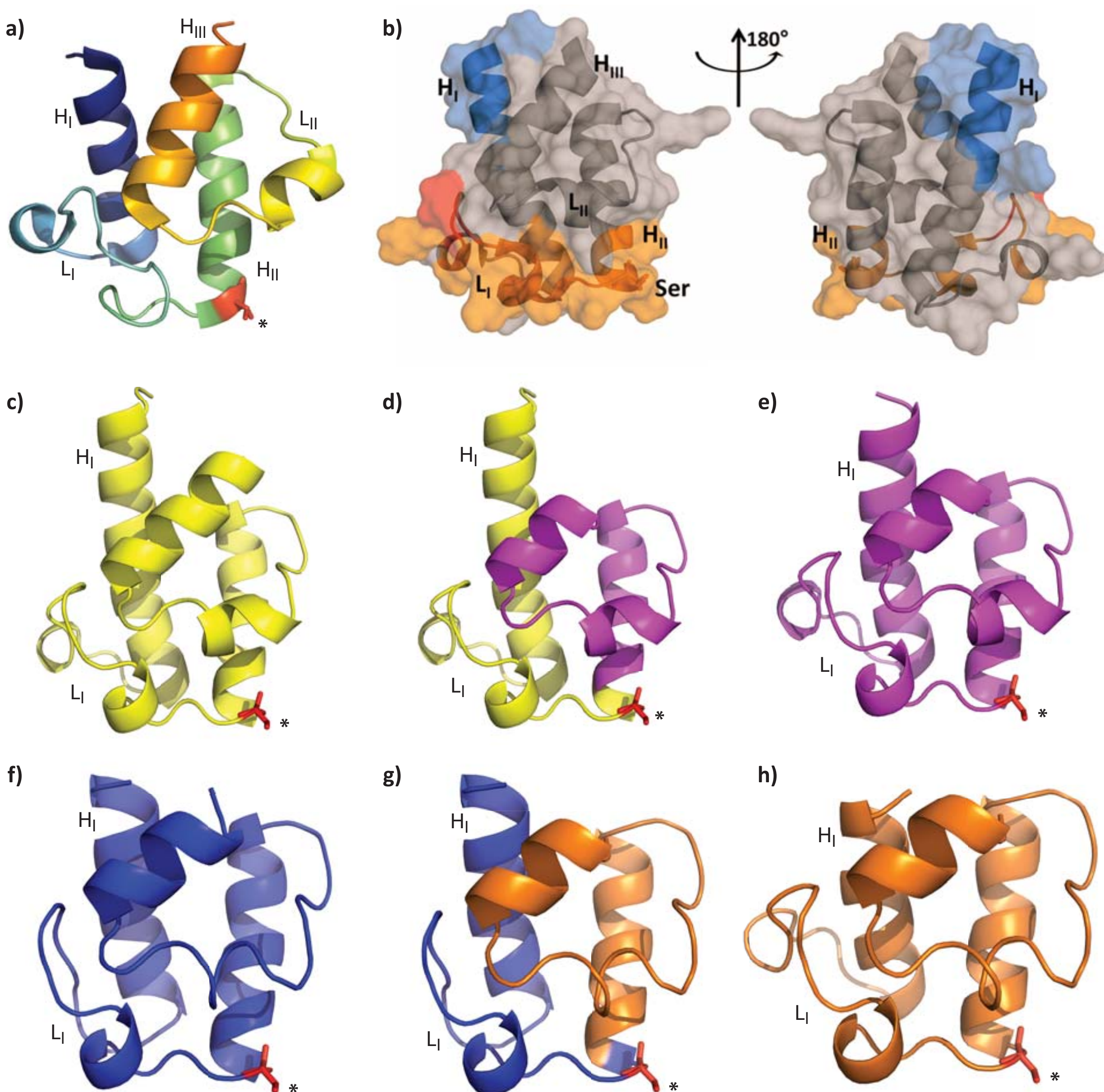

Figure 4.10. Actual and predicted ACP crystal structures. (a) Experimentally determined x-ray crystal structure of *E. coli holo*-ACP (PDB 1T8K) with helix (H) and loop (L) regions indicated. (b) The regions of the ACP that mediate chain elongation (orange) and chain transfer (blue) are shown [reproduced with permission from PNAS, (Kapur et al., 2010)]. (c-e) Predicted structures for niddamycin ACP 1, ACP 1-5 fusion and ACP 5 as well as for (f-h) DEBS ACP1, ACP 1-6 fusion and ACP 6 are shown. Coloring for (c-h) correspondes with figure 3.4. The conserved serine is depicted in red and indicated with an asterix (*).

et al., 1998). In addition, while the covalent linkage between ACP and TE improves the catalytic efficiency, protein-protein interactions between the two domains play little to no role in enhancing catalysis (Tran et al., 2010; Tran et al., 2008). The implication of these observations is that a given TE should be compatible

with both the proximal ACP and ACP-bound substrate with minimal engineering. A detailed mechanism of TE-mediated release can be found here (Du and Lou, 2010).

A further strategy for resolving issues with functional expression probes the activity of specific domains. This approach involves feeding chemically prepared N-acetylcysteamine thioester (S-NAC) intermediates and identifying whether they are loaded, elongated or reduced by the PKS (Staunton and Sutkowski, 1991; Wu et al., 2001). Additionally, this approach can be carried out either *in vivo* or *in vitro*. However, certain criteria must be met such as stability of the molecule under the experimental conditions and the capability to be processed by the PKS (usually the KS domain) (Cane et al., 2002). Use of S-NAC also benefits from preparation via both chemical and enzymatic routes (Arora et al., 2005).

In summary, PKS research is driven by the potential of this class of megasynthases as a source of clinically relevant compounds which are too complex to be synthesized using traditional organic chemistry. Apart from the production of pharmaceuticals, PKS have been called out as a potential platform for the production of a variety of fuels and chemicals (Peralta-Yahya et al., 2012; Yuzawa et al., 2012). While the ability to use engineered PKS as a source of PHA monomers has yet to be proved, existing techniques and design rules can guide the development of functional constructs for the transformation of basic cellular building blocks into diverse PHA with a defined composition.

4.2.9. A selection method for the identification of CoA-ligases

In order to successfully implement a strategy involving PKS generated monomers for PHA biosynthesis, it will be necessary to identify CoA-ligases to activate the PKS products for polymerization. CoA-ligases/transferases have activity for many organic acids found in nature but the monomer precursors derived via the proposed strategy may be unnatural or unrelated to the known substrates of characterized CoA activating enzymes. Therefore, a selection method for the identifying CoA-ligases was developed to quickly isolate an effective catalyst for monomer activation. The efficacy of the selection method is predicated upon *E. coli* short-chain fatty acid metabolism and the ability of the organism to scavenge free organic acids for growth.

In *E. coli*, the *ato* operon is associated with short chain fatty acid metabolism (Fig. 4.11). This operon encodes an $\alpha_2\beta_2$ tetrarmeric acetoacetyl-CoA transferase (*atoDA*), an inner membrane short-chain fatty acid transporter (*atoE*) and an acetoacetyl-CoA thioloase (*atoB*), which is equivalent to *phaA*. The *atoDAEB* operon is positively regulated by the response regulator half of a two component system for sensing short-chain fatty acids (*atoSC; atoS* = sensor kinase, *atoC* = response regulator).The *ato* enzymes allow for wild type growth on acetoacetate as a sole carbon source, but not on butyrate or valerate as these short-chain fatty acids do not induce expression of the *atoDAEB* operon or the *fad* genes (fatty acid degradation/β-oxidation) (Jenkins and Nunn, 1987). In fact, fatty acids with a chain-length of less than twelve carbons do not support wild type growth. To overcome this phenotype, two selectable mutations in the *fad* and *ato* regulatory genes can be made. The *E. coli* strain LS5218 contains both mutations with the first in *fadR* and the second in *atoC* which results in constitutive expression of the *fad* and *ato* genes, respectively (Spratt et al., 1981). As an aside, LS5218 is an effective strain for PHA production (Agnew et al., 2012; Tappel et al., 2012). Some of the reasons for utility of this strain are that both scl- and mcl-fatty acids are often fed as a substrate for PHA biosynthesis and the *ato* genes plays a role in affecting the molecular weight and accumulation of the resulting product (Theodorou et al., 2012).

E. coli LS5218 was used as a starting point for construction of the CoA-ligase selection strain as the *fadR* and *atoC* mutations allow for growth on short-chain organic acids as a sole carbon source. Next, a non-polar deletion of the *atoDA* genes was made (Fig. 4.12). *E. coli* LS5218 Δ*atoDA* was incapable of growth

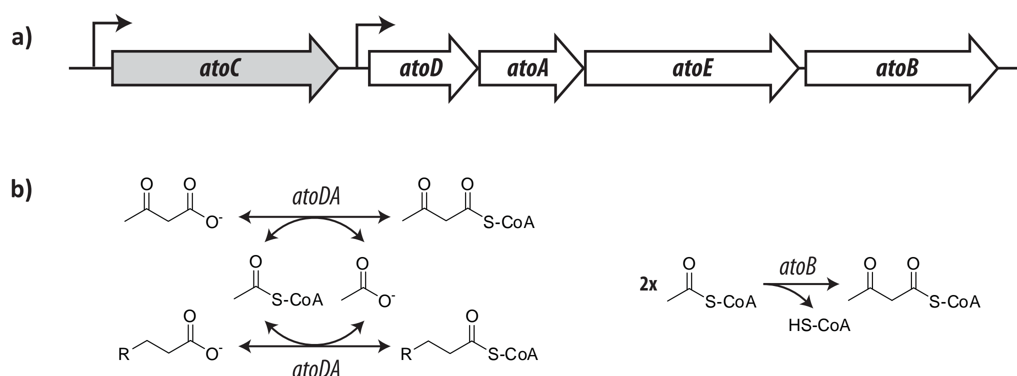

Figure 4.11. The *ato* operon is involved in short-chain fatty acid metabolism (a) Organization of the *ato* operon with the response regulator (*atoC*) and short-chain fatty acid metabolism genes (*atoDAEB*) under distinct promoter. AtoC activates translation of *atoDAEB*. (b) Catalytic role of *atoDA* and *atoB*. The product of *atoE* is a short-chain fatty acid transporter.

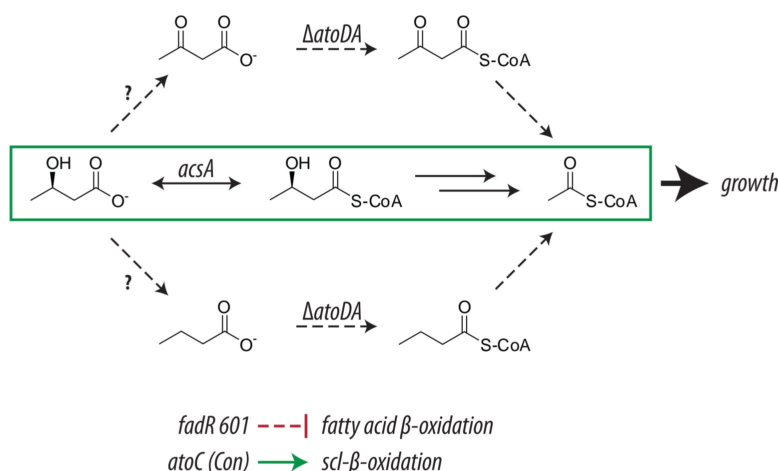

Figure 4.12. Construction of a CoA-ligase selection strain. *E. coli* LS5218 (*fadR, atoC[Con]*) Δ*atoDA* can only support growth on short-chain fatty acid substrates (*i.e.,* 3HB) as a sole carbon source when transformed with a functional CoA-ligase (*i.e., acsA*).

on both butyric acid and 3HB. However, complementation of the strain with a suitable CoA-ligase should restore growth. Therefore, given a PKS derived monomer precursor as a sole carbon source, *E. coli* LS5218 Δ*atoDA* could be transformed with a library of CoA-ligases with growth as a selection for enzymes capable of activating the monomers for polymerization. The caveat to this selection is that *E. coli* must be capable of growth on the activated monomer, or more specifically, the metabolic pathways must be present to metabolize the monomer-CoA to central metabolites capable of supporting growth. As a demonstration, we cloned the *acsA* gene from *P.putida* GPo1 which has demonstrated, albeit minimal activity towards 3HB (Ruth et al., 2008). As *E. coli* is capable of growth on 3HB as a sole carbon source, transformation of *E. coli* LS5218 Δ*atoDA* with plasmid-borne *acsA* restored the ability of the strain to grown on 3HB (Fig. 4.13).

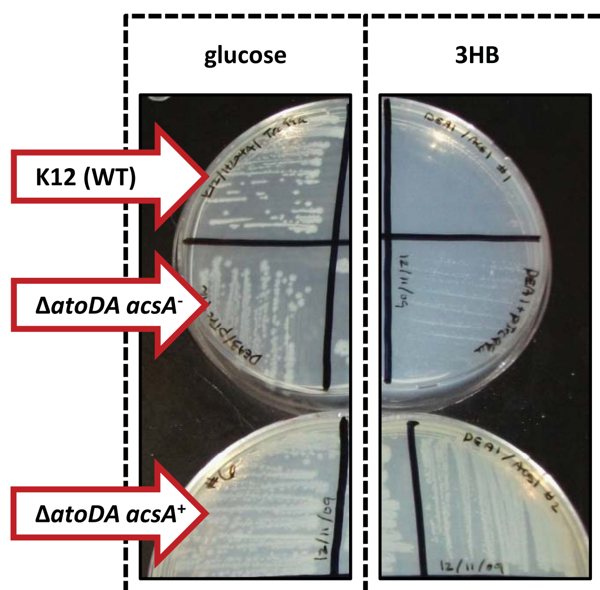

Figure 4.13. Wild-type and *atoDA* strains grown on solid media supplemented with glucose or 3HB as a sole carbon source. All strains are capable of growth on glucose. However, only LS5218 *atoDA* expressing *acsA* is capable of growth on 3HB.

The described selection for identification of CoA-ligases is an effective tool in support of pathway engineering for the biosynthesis of PHA via PKS derived monomers. CoA-ligases are a well-characterized and ubiquitous class of enzymes (Starai and Escalante-Semerena, 2004)and a large number of sequences can be identified using genome databases or alignment search tools. Directed approaches for identifying CoA-ligases include monitoring gene expression (Wang et al., 2012), homology searching (Yu et al., 2011) and deletion/overexpression analyses (Koetsier et al., 2010). In addition, many CoA-ligases consume ATP and are AMP/PP_i-forming allowing for detection of the AMP, PP_i or CoA itself using a variety of (commercially) available assays. Broader, non-rational approaches to identify CoA-ligases could also be taken. For example, *E.coli* LS5218 Δ*atoDA* could be transformed with a genomic library as the selection dictates that only colonies expressing a complementary gene or pathway will be capable of growth. While rational engineering has been used to expand the substrate range of a CoA-ligase (Wu et al., 2007), an possible alternative is random mutagenesis to quickly select for functionality from a library of degenerate enzymes.

4.2.10. Identification of PHA polymerases for unusual monomer substrates

The model systems described for the production of PKS derived PHA monomers requires a polymerase capable of activity towards 3HB and 3-hydroxy-2-methylvaleric acid, which are both demonstrated substrates for PhaC. However, PKS are capable of synthesizing an extensive library of products which have not been characterized with respect to polymerization. Methods for the identification of PHA polymerizing enzymes with activity towards unusual monomers have already been discussed (*see* 2.7.). Worth noting is the broad range of over 150 monomers already identified as substrates for PHA polymerase (Agnew and Pfleger, 2012; Steinbüchel and Valentin, 1995). In the case of unique monomers such as those with halogen or phenyl groups, the extent of incorporation in the polymer product is often minimal (Kim et al., 1996), so if it is desirable to achieve a higher degree of incorporation, polymerase engineering may prove necessary.

4.3. Conclusions

A strategy has been presented for engineering a PKS platform for the transformation of basic cellular building blocks into diverse PHA with a defined composition. The approach was broken down into

three steps in which a PKS was engineered to produce monomer precursors, the monomers were then activated via CoA-ligation and finally, the activated monomers were polymerized. This *in vivo* synthetic, metabolic pathway was envisioned as a means to both improve the types of PHA that can be made and reduce the cost of production by synthesizing the monomer from inexpensive, unrelated carbon sources. The key obstacle to the successful construction of the model system described above was the inability to express functional DKS constructs. However, the foundation is in place for accomplishing the remaining steps (activation, polymerization) given a functional monomer synthase.

Future work will be guided by ongoing developments in the PKS field as well as the existing body of knowledge discussed in detail in the previous sections. Key strategies for improving DKS functionality will likely incorporate codon optimization of the DKS sequences for *E. coli* and identification of ways to probe the activity of specific domains within the construct. A CoA-ligase selection platform will provide enzymes capable of activating the monomer precursors generated by the engineered PKS and polymerization will be carried by a broad activity PHA polymerase. In conclusion, a PKS based route to PHA has potential to advance the commercial impact of this renewable, biodegradable material if the barriers to functional PKS expression in a heterologous host can be overcome.

4.4. Materials and methods

4.4.1. Bacterial strains, reagents, media, and growth conditions

All strains used in this study are listed in Appendix 3. *E. coli* DH5α and DH10B were used to construct and propagate plasmids. *E. coli* BAP1 was used as the base strain for studying PKS expression and PHA production and was generously provided by Dr. Blaine Pfeifer (University at Buffalo – Buffalo, New York). *P. putida* GPo1 and *S. erythraea* were obtained from ATCC (Manassas, VA). *S. calestis* was obtained from the USDA ARS (Peoria, IL). *S. lividans* and pGM190 were generously provided by Dr. Michael Thomas (University of Wisconsin-Madison – Madison, WI). Chemicals and reagents were purchased from Fisher Scientific (Pittsburgh, PA) unless otherwise specified. Enzymes used for cloning were purchased from New England Biolabs (Ipswich, MA). Oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA) and sequences are listed in Appendix 4. For all growth experiments, single colonies were used to inoculate 5 mL starter cultures that were grown overnight prior to inoculation of experimental

cultures. All growth experiments were performed in a rotary shaker (250 rpm). Where necessary, cultures were supplemented with 100 µg mL^{-1} ampicillin and/or 50 µg mL^{-1} kanamycin. *E. coli* strains were routinely grown in LB medium at 37°C unless otherwise stated. *Saccharopolyspora* and *Streptomyces* strains were grown at 30°C in ISP medium 1 (Difco) or when solid media was required, ISP medium 2 (Difco). Spore preparations, growth and transformation methods for *Streptomyces* strains were carried out according to standard protocols (Kieser et al., 2000). *Pseudomonas* strains were grown at 30°C in LB or TGY medium (5g L^{-1} tryptone, 5g L^{-1} yeast extract, 1g L^{-1} K2HPO4; autoclave and add 1g L^{-1} glucose).

For plate-reader experiments, 5 mL overnight cultures were grown in culture tubes at 37°C in a rotary shaker (250 rpm) and used to inoculate fresh media to an OD_{600} of 0.05. Cultures were dispensed into a sterile, 96-well, clear polystyrene plate with lid in 200 µL aliquots. 96-well plates were incubated in a Tecan Infinite M1000 plate reader at 37°C with linear shaking. OD_{600} and fluorescence values were monitored every 15 minutes over the course of a 24 hour kinetic cycle. Cells were induced with 1 mM IPTG and 2% arabinose after approximately 2 doublings or about 1 hour after initiation of the experiment. For detection of PHA with nile red, a 1 mg mL^{-1} stock solution of nile red was prepared in DMSO. Nile red was added to wells at a 1:1000 dilution (*i.e.,* 0.2 µL nile red stock per 200 µL culture) at induction. Fluorescence excitation was carried out at 550 nm and emission data was collected at 595 nm.

For monomer synthesis and PHA production experiments, each strain was cultured in 50 mL of LB + 2% glucose with antibiotics added when necessary. Strains were inoculated at an OD_{600} of between 0.01 and 0.05 and were induced at an OD_{600} of 0.2 with 1 mM IPTG and 0.2-2.0% arabinose from 1 M and 20% stock solutions respectively. Cultures were incubated at 22°C, 30° and 37°C with shaking and 10.0 mL culture samples were taken at 24 and 48 h for PHA analysis. PHA production studies using BAP1 expressing pISA1 were carried out as described previously (Aldor and Keasling, 2001). Briefly, 50mL LB + 274mM glycerol + Amp100 was inoculated from overnight culture and grown at 37°C and 250rpm. Cultures were induced at an OD of 0.15 with 0.1% arabinose with 10 mL of sample collected for analysis at 24 and 48 hours.

4.4.2. Plasmid construction

All plasmids used in this study are listed in Appendix 3. Genomic DNA was isolated from *P. putida* GPo1, *S. erythraea S. caelstis* and *C. necator* with a Wizard® Genomic DNA Purification Kit (Promega). DEBS PKS genes *eryAI-III* and acetoacetyl-CoA ligase *acsA* were amplified from an *S.erythraea* genomic DNA template. Niddamycin PKS genes *nidAI-III* were amplified from an *S. caelestis* genomic DNA template. PHA polymeras *phaC* was amplified by PCR from a *C. necator* genomic DNA template with the respective phaC primers (Appendix 4). CoA-ligase gene *acs1* was amplified by PCR from a *P. putida* GPo1 genomic DNA template with primers acs-F/R (Appendix 4). All constructs were confirmed by DNA sequence analysis. Plasmid pDMP-MCS was constructed by PCR amplification of a fragment of pLIC-Km consisting of the translational coupling cassette using aphII as a response gene. The PCR product and plasmid pMSB6 were restriction digested and the two products were ligated together yielding plasmid pDMP-MCS. pDMP-A, -B and -C were constructed by PCR amplification of portions of pNidDKSv2 followed by restricition digest and subsequent ligation. For G-C rich templates, Phusion polymerase (New England Biolabs) was routinely used with GC Buffer and a 2M betaine solution was used in the place of water.

4.4.3. Chromosome engineering

Deletion of *atoDA* was generated as described previously using pKD13 as template (Datsenko and Wanner, 2000). For each mutant strain, resistance markers were removed by inducing FLP recombinase encoded on plasmid pCP20 which was subsequently cured by growth at a non-permissive temperature (Datsenko and Wanner, 2000). All chromosomal mutations were verified by colony PCR.

4.4.4. Monomer extraction and characterization

For monomer or monomer-CoA analysis, cultures were centrifuged at 2,500 *x g* for 10 min and 3 mL of supernatant was transferred to a 10 mL disposable glass centrifuge tube followed by addition of 20 µL concentrated HCl and 50 µL of an internal standard solution of 10 mg mL^{-1} benzoic acid in butanol. Approximately 1 g of NaCl was added to produce an oversaturated solution followed by three, 3 mL ethyl acetate extractions. Ethyl acetate fractions were combined and evaporated. Derivitization and analysis by GC/MS or GC-FID was carried out as described previously (Werker et al., 2008). Briefly, samples were

combined with 0.5 mL HCl and incubated at 100°C for 1 hour followed by addition of 1.5 mL n-butanol and further incubation at 100°C for 1 hour. Samples were cooled to room temperature and combined with 2.5 mL hexane and 4 mL deionized water. Samples were vortexed and allowed to phase separate at which point 3.5 mL of the underlying aqueous phase was removed. An additional 4 mL of deionized water was added to sample followed by vortexing and centrifugation at 2500 x g for 10 min. Finally, 1 mL of the upper hexane layer was removed to a 2 mL GC vial for analysis. Alternatively, standard FAME analysis was applied to monomer analysis (Lennen et al., 2010). In this case, 2.5 mL total culture or supernatant was analyzed as described elsewhere (*see* 3.5.4.).

4.4.5. PHA extraction and characterization

For PHA analysis, cells were harvested by centrifugation (3000 x *g* for 25 min), washed with 25 mL 1X phosphate buffered saline (PBS), and lyophilized overnight. PHA content was analyzed by GC/MS based on either of two methods described previously (Kato et al., 1996; Werker et al., 2008). A method for conversion to the butyl ester can be found above (*see* 4.4.4.) while the method for conversion to the methyl ester along with the methods used for GC/MS analysis can be found in elsewhere in this thesis (*see* 3.5.4.).

For purification of acsA, strains BL21 (DE3) or BAP1 harboring pTrc-acsA-Htag were grown in 50mL LB + antibiotics at 15°C or 37°C and 250 rpm in a rotary shaker. Cultures were inoculated at an OD of 0.05 and induced with 1 mM IPTG at an OD of 0.4. Cells were harvested at an OD of 1.0 and 5 mL of culture was centrifuged as 2500 x g for 10 min at 4°C. Supernatants were discarded and pellets were resuspended in 500 uL Bug Buster (Novagen) + 1 µL lysonase (Novagen) and transferred to a 2 mL centrifuge tube followed by 20 min of rocking at room temperature. Samples were centrifuged at maximum speed for 20 min at 4°C and supernatant was transferred to a clean 2 mL tube. Soluble, His-tagged proteins were purified using a Qiagen Ni-NTA Spin Kit (Qiagen). Proteins were analyzed using standard SDS-PAGE protocols described previously (Sambrook et al., 2001).

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Chapter 5: Alternative carbon sources for PHA production and potential for further research

5.1. Introduction

In the previous chapters of this thesis two routes for PHA biosynthesis were investigated. Chapter 2 laid the foundation for the importance of metabolic routes for PHA production that begin with unrelated carbon feedstocks, while Chapters 3 and 4 explored the potential of fatty acid and PKS based approaches respectively. Regardless of the utility of these strategies, the key feature of both approaches is that the carbon precursors required for monomer production are derived from any carbon source that the host cell, engineered or otherwise, can catabolize. Chapter 2 demonstrated that no one organism possesses the native machinery required to produce any desired intermediate. For example, propionate for P(3HV) production, or building blocks, such as methylmalonyl-CoA for PKS-based monomer synthesis. Therefore, the synthetic biologist expects that a certain degree of strain engineering is required to confer a host cell with the metabolic pathways for i) carbon source catabolism, ii) monomer biosynthesis and/or iii) PHA biosynthesis.

Beginning at the end, PHA biosynthesis can be generally defined as engineering of PHA polymerase to accept a monomer of interest. Generally, various PHA polymerases are inherently capable of incorporating a wide variety of monomers. Although, as mentioned previously (*see* 2.6), there are examples of polymerase engineering to broaden the substrate specificity (Nomura and Taguchi, 2007). In this case, the desired result was a copolymer of scl- and mcl-PHA. Still, numerous examples exist of monomers that are poor substrates for PHA polymerase (Jung et al., 2000; Kim et al., 2001; Kim et al., 2000; Kim et al., 1996). The synthetic biologist may choose to address PHA biosynthesis in cases where polymerization is the rate-limiting step in the pathway or when a poorly incorporated monomer is identified as having commercial relevance. In the case of the former, simply balancing the polymerase expression level may result in the desired outcome, thus obviating protein level engineering. Should engineering prove necessary, mutagenesis may prove valuable (Shen et al., 2011). However, while the genetics of PHA polymerase are well understood, no hi-resolution crystal structures exist. To date, only an unpublished solution NMR structure is available through the RCSB protein databank (PDB ID: 2L6P).

Monomer biosynthesis has been discussed at length in the previous chapters. The concept of the three piece puzzle introduced in Chapter 2 describes monomer synthesis and activation as distinct steps where the latter may encompass additional modifications (*e.g.,* racemization to the correct stereochemistry). In practice, most successful strategies for monomer production should rely upon oxidation/reduction reactions to generate a monomer where the only further catalytic step required is activation to the CoA form. Both the fatty acid pathway implemented in Chapter 3 and the PKS strategy explored in Chapter 4 make use of built-in β-oxidation machinery to generate (potentially non-activated) monomers. A number of other strategies for monomer biosynthesis can be envisioned. Generally, these might be classified as pathway engineering to link a particular feedstock with a desired polymer as in Chapter 3, or to engineer a new pathway for production of a non-native monomer precursor. Production of non-native, or even non-natural monomers is best exemplified by Genomatica's approach to produce 1,4-butanediol (Yim et al., 2011). While BDO is not a PHA monomer, the potential to first predict and design commercially relevant polyesters and then build the metabolic pathway shares similarities with traditional polymer design and catalysis. On the other hand, a number of opportunities exist for engineering metabolic links between inexpensive, renewable feedstocks and PHA monomers/polymers and this is the focus of Chapter 5.

Chapter 5 encompasses a discussion of several current and potential lines of research for bolstering the potential of PHA by linking production with inexpensive, renewable feedstocks. While glucose is considered an inexpensive substrate for industrial fermentations, research into utilization of lignocellulosic biomass and wastewater offers opportunities for even more cost savings. Along the lines of mcl-PHA biosynthesis explored in Chapter 3, the potential of alternative carbon sources such as acetate is examined. For PHA biosynthesis and fermentation in general, a novel carbon source derived from lignocellulosic biomass, levulinic acid, was investigated. Levulinic acid cannot serve as a sole carbon source for *E. coli*. However, it can support growth of the versatile bacterial strain, *Pseudomonas putida* KT2440. Finally, additional possibilities for improving titers, economics and PHA production in general are presented.

5.2. Results and Discussion

5.2.1. Effect of carbon source on mcl-PHA biosynthesis

In Chapter 3, the goal of defined PHA production from an unrelated carbon source was achieved with glucose as it is an inexpensive carbon source available for industrial applications (*see* Table 2.2.). Glucose is also a favorable carbon source as it lends to efficient growth of *E. coli*. While investigating both the effect of *fadR* deletion vs. *fadD* overexpression and the role of CoA-ligases in the mcl-PHA biosynthesis strategy, it was noted that certain elements of the *fad* regulon are regulated by cyclic adenosine monophosphate (cAMP) and cAMP receptor protein (CRP or CAP). Specifically, transcription of *fadD* is likely repressed in the presence of glucose (Zheng et al., 2004). Glucose blocks the activity of adenylate cyclase, which catalyzes the formation of cAMP. CRP then binds cAMP to activate transcription of *fadD*. Of note is the lack of predicted CRP-cAMP activation sites in front of the other *fad* genes (Karp et al., 2002). Tables 5.1 and 5.2 show data from previous studies that measured the activity of the *fad* regulon using a *lacZ* fusion strategy. These studies identified the effects of several carbon sources on *fad* expression levels (Clark, 1981; Pauli et al., 1974). For example, growth on glucose, acetate or succinate greatly reduced expression of *fadBA* and *fadE* compared with growth on acetate + oleate ($C_{18:1}$ fatty acid). By contrast, in a *fadR* transposon mutant strain, growth on acetate led to equivalent or slightly increased expression levels compared with growth on acetate + oleate.

As SA01 is a Δ*fadR* strain, it was hypothesized that growth on acetate might improve PHA production. Production strain SA01 transformed with either pDA-JC or pDA-JAC was grown in minimal media with either sodium acetate or glucose as a sole carbon source (Fig. 5.1). Cultures were assayed for both PHA and FFAs as described previously (*see* 3.5.4). While the residual free fatty acid concentrations were similar

Table 5.1. Expression data from Clark, 1981 for *E. coli fadA* and *fadE* when cultured in the presence of various carbon sources. Values are relative to expression in acetate + oleate medium. Expression levels were determined via β-galacosidase activity assays for *lacZ* fusion constructs with *fad* genes.

Genotype	Medium	*fadA*	*fadE*
WT	acetate+oleate	100	100
WT	acetate	12	7
WT	acetate + oleate + glucose	4	3.5
WT	glucose	4	2.5
WT	succinate	5	3
fadR	acetate+oleate	100	100
fadR	acetate	103.5	100.5
fadR	glucose	8.5	12
fadR	succinate	61	93

Table 5.2. Expression data from Pauli et al., 1974 for *E. coli fadA*, *fadB* and *fadD* when cultured in the presence of various carbon sources. Values are relative to expression in tryptone + oleate medium. Expression levels were determined via β-galacosidase activity assays for *lacZ* fusion constructs.

Genotype	Medium	*fadA*	*fadB*	*fadD*
WT	tryptone + oleate	100	100	100
WT	tryptone	12	14	37
WT	glucose	1	1	8
WT	glucose + cAMP	0.9	0.9	10
WT	glucose + oleate	1	1	11
WT	glucose + oleate + cAMP	10	15	33
crp	tryptone + oleate	13	10	13
crp	tryptone	3	2	7
crp	glucose	0.4	1	5
crp	glucose + oleate	0.5	0.8	5
crp	glucose + oleate + cAMP	0.7	1	6
fadR	tryptone	152	146	112
fadR	glucose	3	4	7
fadR	glucose + cAMP	11	10	22
fadR	glucose + oleate	3	4	7
fadR	glucose + oleate + cAMP	30	40	35
fadR crp	tryptone + oleate	20	18	18
fadR crp	tryptone	21	21	16
fadR crp	glucose	3	2	6
fadR crp	glucose + oleate	3	3	4
fadR crp	glucose + oleate + cAMP	3	3	5

between strains expressing *acs*, PHA titers in strains grown on acetate were significantly improved over strains grown on glucose. As seen previously, *acs*$^{+}$ strains accumulated more PHA when grown on glucose and the trend remained the same for growth on acetate as a sole carbon source. SA01 pDA-JC grown on acetate also exhibited the greatest accumulation of FFAs over the course of the experiment. Improvements in overall production were observed in the bioreactor experiments in the case of glucose; however, analogous experiments with acetate were not conducted.

Several questions remain to be addressed along the lines of the choice of acetate versus glucose. While the experimental results seemed to corroborate the interplay of glucose and cAMP and the resulting effect on transcription regulation, additional experiments would be required to confirm whether or not the effect is due to regulation of *fadD*. In this case, it would be straightforward to monitor transcription levels of *fadD* and other *fad* genes with a technique such as quantitative reverse transcription PCR. Another outcome of varying carbon sources was that strains grown on acetate grew more slowly and resulted in lower total CDWs compared to growth on glucose. If higher ODs are desired for industrial production, it may be worth exploring a two-phase production strategy

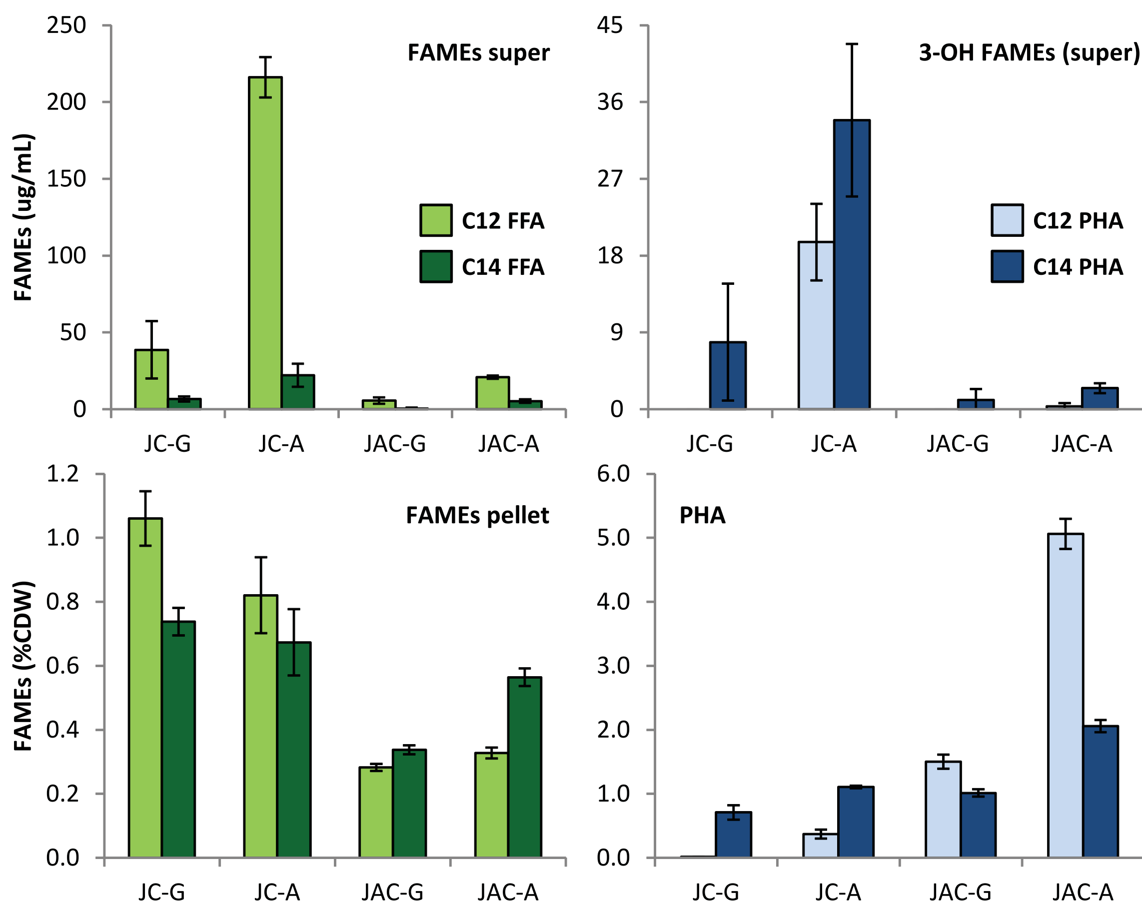

Figure 5.1. Production of mcl-PHA in *E. coli* SA01 in the presence of either acetate or glucose. Titer of PHA as a percentage of cell dry weight determined by quantifying 3-hydroxy fatty acid methyl esters from a PHA extraction. Titer of fatty acids determined by quantifying fatty acid methyl esters (FAME) from a total lipid extraction. JC, pDA-JC; JAC, pDA-JAC; A, acetate; G, glucose.

involving a growth phase on glucose to achieve high ODs followed by a production phase on acetate. In addition, acetate prices are currently on par with glucose which could be an economic benefit when considering industrial production strategies (Boswell et al., 2012), although potential requirements for neutralization of the acid may prove a disincentive. Finally, while acetate was selected to provide a non-glucose feedstock, there are likely a number of other potential carbon sources that allow for the avoidance of CRP-cAMP regulation. It would be valuable to explore other options such as glycerol in order to begin to determine how the choice of carbon source factors into final the PHA yield.

5.2.2. Pathway balancing for optimal PHA biosynthesis.

It is not uncommon in the design of production strains to require the expression of a number of genes in concert (Khosla and Keasling, 2003; Martin et al., 2003). To facilitate this approach, especially in prokaryotic hosts, genes can be grouped into a synthetic operon (Baga et al., 1988). Unfortunately, a basic construct consisting of an inducible promoter and genes cloned in series does not generally lead to optimal gene expression and metabolite production (Nishizaki et al., 2007; Salis et al., 2009). Worse, expression of poorly designed operons can lead to accumulation of undesired intermediate products, reduced growth rates, and in some cases cell death from metabolite toxicity (Kizer et al., 2008). As understanding of transcription and translation increases, the selection of appropriate regulatory sequences is becoming a more complex process. Methods of altering transcription initiation, mRNA stability, transcript secondary structure, translation initiation, and translation elongation are known (Carrier and Keasling, 1999; Guzman et al., 1995; Salis et al., 2009; Smolke et al., 2000). However, the optimal level of expression for a particular protein is frequently unknown *a priori*. This combination makes rational operon design challenging.

In building the strains and plasmids described in Chapter 3 for the biosynthses of mcl-PHA from glucose, a number of design rules were taken into consideration. The goal of the final strain design was to balance fatty acid biosynthesis with downstream monomer production and polymerization. Beginning with fatty acid biosynthesis, it was known that the accumulation of mcl-FFAs is toxic to *E. coli* and low levels of expression of the codon optimized BTE gene were suitable for maximal production (Lennen et al., 2010; Lennen et al., 2011). It was anticipated that minimal expression of BTE would be sufficient and as such, BTE was incorporated at one copy on the chromosome under the strong, IPTG inducible *trc* promoter. Previous heterologous expression studies indicated that high level expression of PHA genes is suitable for polyester production. Therefore, the medium-copy plasmid, pMSB6 was selected. Each of the three genes, *phaJ*, *acs* and *phaC*, were cloned as an operon under the same *trc* promoter. In general, consensus RBS were chosen (AGG AGG) and the relative RBS strengths were confirmed to be similar based on the RBS calculator (Salis et al., 2009). The outcome of this design was a moderate accumulation on the order of 10-20% of the CDW. With previous studies demonstrating the accumulation of nearly 90% of the CDW, it is important to consider where bottlenecks in the pathways may exist.

One directed approach relies on balancing expression levels for each of the genes involved in the pathway and a great number of tools are available to the synthetic biologist. To control transcription, one can choose from an array of promoters that have been characterized in both native and heterologous hosts (Agnew et al., 2011; Jana and Deb, 2005). Methods for producing libraries of synthetic promoters using basic PCR techniques have also been used to tune transcription levels (Hammer et al., 2006). Rates of translation initiation can be controlled by altering the sequence of the ribosome binding site (RBS) located upstream of each gene (Kozak, 2005). *In silico* models have been developed for predicting RBS strength with predictions were confirmed *in vivo* for engineered RBS (Salis et al., 2009). Translation elongation rates can be altered by the codon usage and mRNA secondary structure of sequences located immediately 3’ of the start codon (e.g., ATG) (Seo et al., 2009). Altering the secondary structure of intergenic region mRNA to include hairpins or RNase sites has also been shown to be effective for tuning expression of synthetic operons by altering the rates of transcription termination, mRNA decay, translation initiation. (Pfleger et al., 2006).

Previous work in this lab explored the outcome of integrating multiple copies of BTE into the chromosome and comparing the results with plasmid-based expression (Youngquist et al., 2012). This approach may prove valuable to identifying whether fatty acid biosynthesis represents a rate-limiting step. Although, given the accumulation of fatty acids in the bioreactor experiments, this step is likely not this issue. Addition of the *acs* gene was observed to increase PHA production substantially with a concomitant decrease in the accumulation of FFAs. Therefore, it may be worthwhile to explore increasing expression of the *acs* gene or complementing CoA-ligase activity in version of SA01 with *fadD* under control of the IPTG inducible *trc* promoter. A direct comparison of chromosomal *fadD* overexpression and plasmid-based *acs* expression has not been made but may be help to elucidate the contribution of *acs* and determine if *fadD* overexpression is equally effective. The remaining enzymes associated with the mcl-PHA biosynthesis pathway include FadE (acyl-CoA dehydrogenase), PhaJ (enoyl-CoA hydratase) and PhaC (polymerase). No manipulation of *fadE* expression levels was made in the final strain beyond deletion of *fadR* in order to derepress transcription of all β-oxidation genes. Balancing expression levels *fadE* along with *phaJ* and *phaC*, may therefore prove beneficial.

The accumulation of potentially toxic products and intermediates can prove detrimental to cell physiology but is not a concern with PHA as the polyester itself forms a relatively inert, insoluble inclusion. Therefore, overexpression of PHA polymerase is a viable approach to improving PHA biosynthesis. However, expression levels of *phaC* can have an effect on the molecular weight of the polymer produced (Sim et al., 2001). Free fatty acids and lauric acid in particular are known to be toxic to the cell, but the toxicity of enoyl-CoA and hydroxyacyl-CoA molecules is unknown. Additionally, thioesterases native to *E. coli* may have activity towards β-oxidation intermediates under conditions of intermediate accumulation. Recent work in this and other labs has demonstrated evidence of the active export of free fatty acids by native transporters (Lennen, 2012; Lennen et al., 2013; Rosenberg et al., 2003). Deletion of *acrAB* exacerbated the negative effect of endogenous free fatty acid production in a BTE expressing strain. If *acrAB* is responsible for the active export of free fatty acids, deletion of *acrAB* in a mcl-PHA accumulating strain may provide the benefit of maintaining pathway intermediates inside the cell for incorporation into the polyester.

Overall, balancing of the described pathways for mcl-PHA biosynthesis is non-trivial. The process often requires trial and error and relies upon the ability to detect intermediates as well as the final product – the engineering of yeast and *E. coli* for artemisinin biosynthesis being a prime example (Martin et al., 2003; Ro et al., 2006). One obstacle to achieving a balanced pathway is that the current method for PHA production in *E. coli* involves a 96 hour culture time. Moreover, sample processing requires an additional 2-3 days. It may be possible to reduce the culture time for the purposes of pathway balancing, but data indicate that polyester accumulates over the entirety of the time-course (Fig. 5.2). A more rapid, but qualitative detection method involves the lipophilic stain, nile red. When introduced into cellular systems, a fluorescence shift is observed

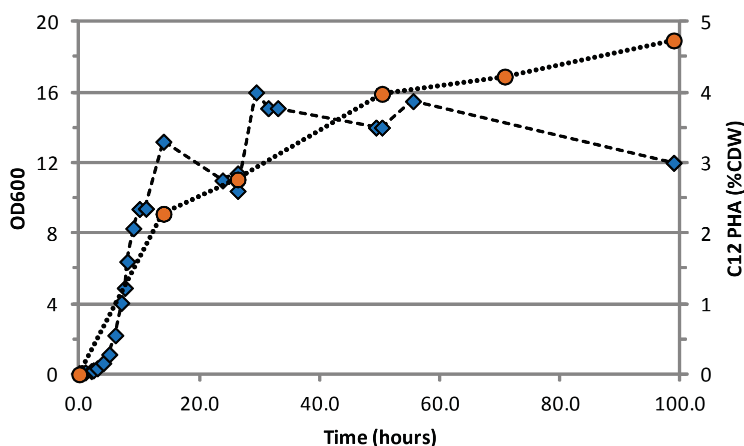

Figure 5.2. Growth curve and PHA production for SA01 pDA-JAC cultured in a bioreactor in the presence of glucose.

due to the presence of features such as lipid membranes, triacylglycerols, fatty acids, and PHA (Diaz et al., 2008). Nile red also permits real time detection of PHA accumulation *in vivo* through fluorescence measurements (Gorenflo et al., 1999). Taken altogether, for the described approach to be commercially viable, it will be necessary to increase the degree of PHA accumulation beyond the current capabilities of the strain.

5.2.3. Potential for phasins to improve PHA accumulation

In native PHA producers, PHA granules consist of not only the polymer and polymerase, but also at least one other class of proteins known as phasins. Proteins associated with the PHA granule form an approximately 4 nm layer around the outer surface of the polyester and make up 2-3% of the total weight of the granule (Griebel et al., 1968). Phasins are not required for PHA biosynthesis and are often omitted in studies involving heterologous expression for PHA production. Nonetheless, phasins play an important role in the accumulation of polyester. First, production of the phasin protein, PhaP, is mitigated by regulator protein, PhaR. PhaR is cable of binding both to DNA as well as to the PHA granule and regulates expression of both itself and *phaP* (Yamada et al., 2007). This mechanism allows for the coordination of the amount of PhaP required to effectively coat existing PHA granules. This proteinacious coating is responsible for preventing the hydrophobic PHA granule for coalescing into one large granule and may play a role in how the polyester is partitioned during cell division (Jendrossek, 2009).

Deletion and overexpression studies have elucidated some of the contributions of phasins to PHA biosynthesis. For example, the *Ralstonia eutropha* H16 genome codes for at least four phasin proteins. A *phaP1* deletion increased the rate of PHA degradation but did not affect the molecular weight of the polymer (Kuchta et al., 2007). Previous studies in which deletions of *phaP2- 4* were made had no effect on PHB properties or growth. Although, deletion strains of *phaP1* alone or in combination with *phaP2-4* exhibited similar growth but accumulated less PHB (Potter et al., 2005). Heterologous expression of *phaP* in *E. coli* with *phaBAC* from *Azotobacter sp.* F8 resulted in an increase in both cell mass and PHB when cultured on either glucose or glycerol (Almeida et al., 2007). In *Aeromonas hydrophila* 4AK4, *phaP* expression slightly increased the percent incorporation of 3-hydroxyhexanoate (C_6) monomers and resulted in a greater final poly(3HB-co-C_6) copolymer concentration (Han et al., 2004). Finally, *E. coli* expressing *phbCAB* and *phaP*

from *R. eutropha* H16 resulted in increase PHB accumulation from 16% to 57% CDW (Seo et al., 2003). As opposed to having a direct affect on polymerization (*i.e.,* on PhaC) the mechanism of action of PhaP is likely due to stabilization of the PHA granules.

The role of phasins and the apparent redundancy of these proteins in native producers are still not entirely understood. Still, the relationship between phasins and PHA granules has been exploited for a number of applications unrelated to PHA biosynthesis. One study reported the use of a green fluorescent protein (GFP)-phasin fusion for PHB affinity-based protein purification (Barnard et al., 2005). In 2006, work began on the solution of the crystal structure of PhaP from *A. hydrophila* 4AK4 which will allow for greater insight into the properties of phasins and may prove useful for further engineering endeavors (Zhao et al., 2006). In summary, the current knowledge of phasins, their ability to be successfully expressed in *E. coli* and the reported effect to increase PHA accumulation make these proteins an attractive target for improving PHA biosynthesis.

Co-expression of a phasin in the mcl-PHA production strain discussed in Chapter 3 has the potential to increase mcl-PHA accumulation (*i.e.,* increase % CDW). In the aforementioned strategy, PHA genes were cloned from *P. aeruginosa* PAO1 (*phaJ*, *phaC*) and *P. putida* KT2440 (*acs*). *P. putida* contains two adjacent ORFs predicted to code for phasins , PP_5007 and PP_5008. While *P. putida* is a native PHA producer, neither of the two predicted phasins exhibits significant identity (< 10%) to PhaP1 from *R. eutropha* H16 or PhaP from *Azotobacter sp.* F8. A similar set of ORFs can be found on the *P. aeruginosa* genome (PA_5061 and *phaF*). It is possible that a host strain's native phasins are effective in combination with the corresponding PHA biosynthesis enzymes and that *R. eutropha* PhaP1 may not be effective in a strain expression *P. aeruginosa* PhaC. Currently, an experiment is underway to answer the questions of whether a phasin can improve PHA accumulation and if phasins from one organism can work with PHA polymerase from an unrelated organism. It is important to point out that improvements in PHA production have so far only been observed for C_4 and C_6 PHA and not for C_{12} PHA, which is the major component of the mcl-PHA produced with BTE. Furthermore, it may be important to coexpress the corresponding regulatory protein, PhaR, to allow for balanced expression of PhaP.

5.2.4. Levulinic acid and lignocellulosic biomass

No discussion of inexpensive, renewable feedstocks would be complete without mention of Lignocellulosic biomass. Lignocellulose, the major component of the plant cell wall, is the most abundant organic material on earth (Fig. 5.3). It is composed of lignin, cellulose and hemicellulose, and has long been touted as a renewable source of sugars for industrial fermentation processes. Accessing the sugars within lignocellulose requires physical and/or chemical pretreatment followed by some form of hydrolysis. Lignin is a random macromolecule formed by a free-radical polymerization process and the goal of the pretreatment step is to disrupt the lignin matrix that contains the cellulose and hemicellulose. Next, cellulose, a linear polymer of glucose, and hemicellulose, a more complex polymer consisting of a variety of C_5 and C_6 sugars are hydrolyzed, often via enzymatic digestion. The hydrolysis process releases the individual sugar monomers such that they are a usable substrate for fermentation. *E. coli* and engineered strains of yeast are capable of growth on the various sugars released from lignocellulose and a number of industrial processes exist for the conversion of lignocellulosic biomass to fuels and chemicals with ethanol being a prime example (Huffer et al., 2012).

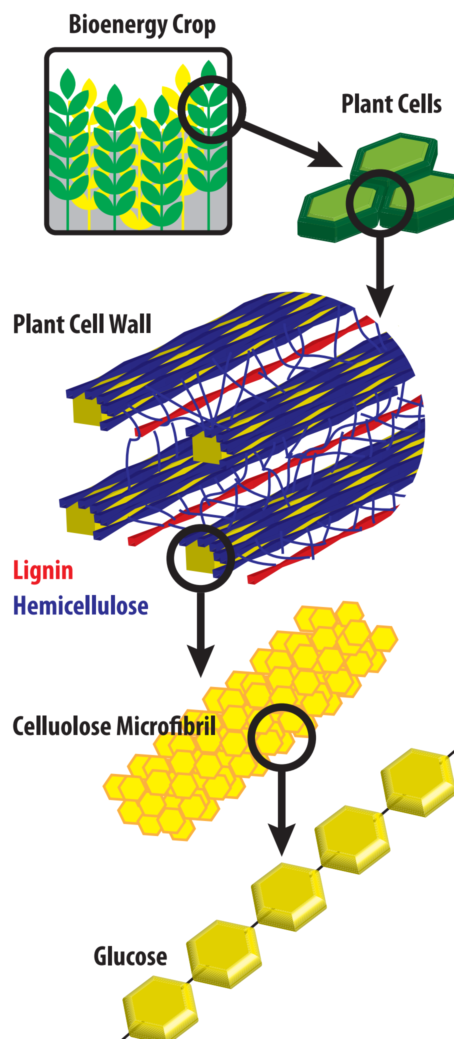

Figure 5.3. Cartoon schematic of the structure of lignocellulose. Figure adapted from a previous report (Genome Management Information System/Oak Ridge National Lab).

While lignocellulosic biomass is an advantageous feedstock for industrial fermentation – it is renewable, abundant and a non-food resource – pretreatment is involved and energy intensive, and is therefore an expensive part of the process. For production of ethanol from lignocellulose, pretreatment can comprise up to $0.30 per gallon of ethanol produced (Mosier et al., 2005). Furthermore, whether

various production processes for ethanol from biomass require as much energy input as is derived from the fuel produced is a topic of debate (Hill et al., 2006). Therefore, alternative methods for converting lignocellulose to valuable fuels and chemicals have a great deal of potential.

A number of catalytic processes exist for the conversion of lignocellulose to various products such as jet fuel, diesel and gasoline (Serrano-Ruiz and Dumesic, 2011). One of these chemicals, levulinic acid, is particularly attractive as both a platform chemical and as a potential substrate for industrial fermentation processes. Levulinic acid is traditionally derived from maleic anhydride or furfuryl alcohol. Currently, these non-biomass feedstocks for commercial production of levulinic acid are expensive, resulting in a market price of about US $10 per kg making production from inexpensive and renewable biomass feedstocks an attractive alternative (Alonso et al., 2010). In fact, recent research has proved levulinic acid as one of the most readily accessible biomass derivatives. In fact, the US DOE has identified it as one of the top 12 "value added chemicals from biomass." As lignocellulose can be less than 5% of the cost of maleic acid, it has been projected that levulinic acid production via lignocellulose could reduce the price per kg to $0.20.

Levulinic acid is obtained by catalytic (non-enzymatic) hydrolysis of the lignocellulosic biomass feedstock by way of hydroxymethylfurfural (HMF) in the presence of sulfuric acid at relatively low temperature and pressure (Fig. 5.4). This process has been optimized to the point where 70-80% of the theoretical maximum conversion of C_6 sugars to levulinic acid is achieved. The remaining material is recovered as formic acid and a mixture of organic solids known as humins. Formic acid is a low-value commodity chemical that is used in the production of such materials as formalydehyde, rubber and plasticizers and may have future utility as a feed source for hydrogen storage and production. Levulinic acid itself can be converted by traditional catalytic routes to liquid biofuels such as γ-valerolactone or methyltetrahydrofuran which can be blended with gasoline for use in standard combustion engines (Alonso et al., 2010). Alternatively, it is possible to convert levulinic acid via fermentation to chemicals such as 2-methylcitrate and 4-hydroxyvalerate for use as chiral, synthetic building blocks in the production fine chemicals and pharmaceuticals (Ewering et al., 2006; Martin and Prather, 2009).

While there are still many concerns to address regarding the production of levulinic acid from biomass, such as catalyst selectivity, recovery and yield, the interest in developing levulinic acid as a

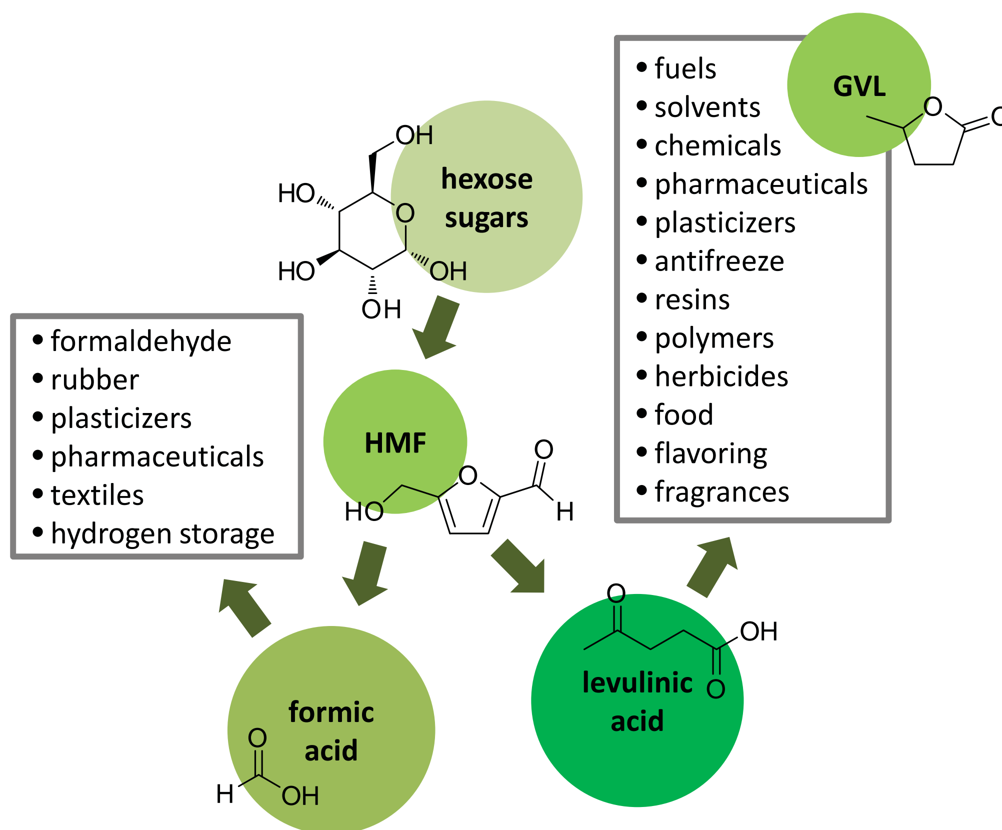

Figure 5.4. Overview of production of levulinic acid and its derivitives from hexose sugars – specifically, lignocellulosic feedstocks.

platform for production of fuels, polymers, pharmaceuticals will drive innovation. In fact, levulinic acid may have utility as an inexpensive alternative feedstock for industrial fermentation processes. This ideas stems from the fact that lignocellulosic biomass is more readily converted to levulinic acid with traditional catalysis than to pentose and hexose sugars via enzymatic hydrolysis. While levulinic acid cannot support the growth of *E. coli* as a sole carbon source, it can support growth of other strains of bacteria such as *P. putida* (Martin and Prather, 2009) and *C. necator* (Jaremko and Yu, 2011). How levulinic acid is metabolized is unknown, although some evidence exists to support pathways that result in the formation of acetyl-CoA and propionyl-CoA (Fig. 5.5). If it were possible to identify the mechanism of levulinic acid metabolism, the ability to use levulinic acid as a fermentation feedstock in industrial strains such as *E. coli* and *Saccharomyces* could be realized.

5.2.5. Levulinic acid metabolism in P. putida KT2440

P. putida is capable of growth on levulinic acid and has been used for the production of chiral 3- and 4-hydroxyacids as well as PHA consisting of these molecules (Martin and Prather, 2009; Steinbüchel et al., 1998). The identification of a route to both 4 and 5 carbon PHA monomers from levulinic acid suggests the existence of a metabolic pathway involving central metabolites. Expanding this capability of *P. putida* to metabolize levulinic acid to make a wider range of molecules, not just PHA, has a great deal of potential for industrial mircobiology. Observations of the accumulation of certain intermediates indicate that levulinic acid is metabolized by way of 4-hydroxyvalerate, 3HV and finally acetate and propionate. How levulinic might be metabolized to 3HV is unclear. Once 3HV (likely the CoA form) is produced, fatty acid degradation enzymes are likely responsible for the final catabolic steps. In *E. coli*, 3HV is a substrate for short-chain β-oxidation (*ato* and *fad* genes). The metabolism of 3HV results in one molecule of acetyl-CoA and proionyl-CoA, of which the former can support growth. While *E. coli* possesses genes that encode for propionate use, propionate is toxic at concentrations greater than 5 mM and cannot support growth as a sole carbon source. Therefore, the challenge to conferring *E. coli* with the ability to use levulinic acid as a sole carbon source is 2-fold. First, a strain must be transformed with the ability to catabolize levulinic acid, likely to acetate and propionate, and then the strain must be engineered to tolerate and ideally use the resulting propionate.

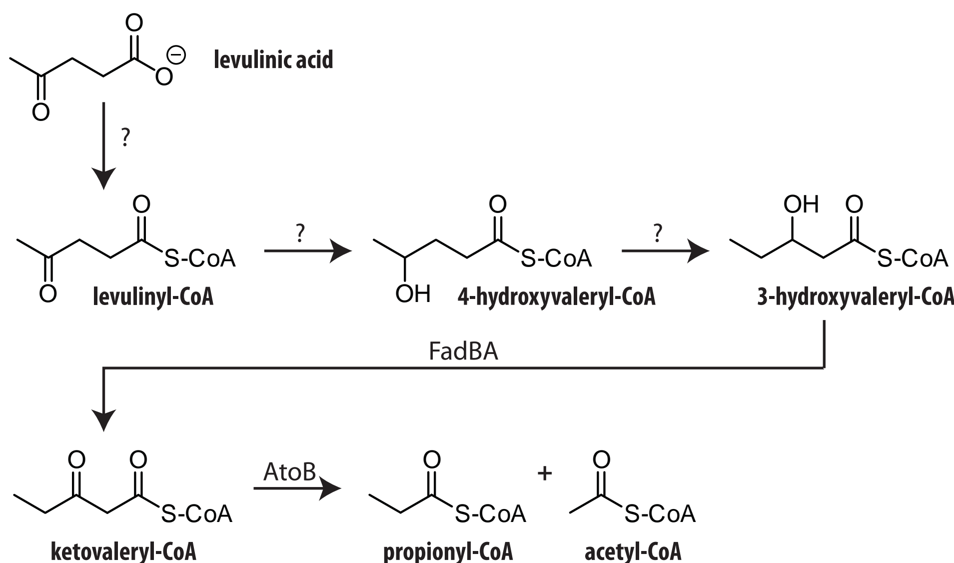

Figure 5.5. Proposed pathway for the metabolism of levulinic acid by *P. putida* KT2440.

To pinpoint genes that may be involved in levulinic acid metabolism in *P. putida*, an experiment was designed to identify loss of function mutations which resulted in growth on glucose or rich media, but not on levulinic acid as a sole carbon source. Transposon mutagenesis is a common approach for generating libraries of random mutations that result in loss of function. The strain of interest is transformed with a small piece of genetic material known as a transposon which is semi-randomly integrated into the host genome by a transposase which is co-transformed. The transposon is designed to contain a selectable marker for recovery of strains that have acquired the transposon. A library of transposon mutants of *P. putida* KT2440 was created and over 7,500 members were screened for growth on glucose, levulinic acid and rich media. *P. putida* KT2440 consists of a predicted 5,516 genes so the number of colonies screened represents approximately 1.5X coverage of the genome.

Colonies capable of growth on glucose but not levulinic acid were sequenced to identify the location of the transposon insertion in the host genome. The process resulted in the identification of roughly a dozen genes of both predicted and unknown function. In particular, transposon insertions were identified in genes associated with propionate which corroborates the observation that levulinic acid is metabolized to acetate and propionate. Additional insertions were identified in a variety of locations with cryptic relationships to levulinic acid metabolism. However, more than one insertion was identified in a putative operon consisting of several open reading frames. This operon codes for putative proteins predicted to have dehydrogenase and oxidoreductase activity. This finding supports a mechanism in which levulinic acid is converted to 4- and 3-hydroxyvalerate.

Characterization of the putative operon is currently underway. As transposon mutangensis often results in polar effects on up and downstream genes, clean deletions need to be constructed to confirm which gene knock-outs result in a loss of the ability to growth on levulinic acid. In addition, complementation of the knock-outs is important to determine if the original phenotype can be restored. Finally, members of the putative operon may be necessary but not sufficient for levulinic acid metabolism. Further screening of the transposon library may reveal additional genes that play a role, but it is also possible that certain steps in the metabolic pathway can be accomplished by enzymes with complementary activities. For example, the promiscuous activity of CoA-ligases has already been discussed in the context of activation of PHA monomers. It is likely that levulinic acid must first be activated to levulinyl-CoA before further enzymatic

reaction can take place. As levulinic acid is a non-natural carbon source, it is also likely that *P. putida* does not possess a dedicated levulinic acid CoA ligase and instead may have a number of complementary enzymes that can perform this activation step.

5.2.6. Propionate toxicity in E. coli and Salmonella

Propionate metabolism in bacteria is known to occur via several routes with the most notable being the conversion to pyruvate via the 2-methylcitric acid cycle (Fig. 5.6). This metabolic pathway is perhaps best characterized in *Salmonella* and involves the analogous *prp* operon present in *E. coli* (Horswill and Escalante-Semerena, 1999). The pathway proceeds first via the ATP dependent activation of propionate to propionyl-CoA catalyzed by PrpE. PrpC then catalyzes the condensation of propionyl-CoA with an intermediate from the TCA cycle – oxaloacetate – to yield 2-methylcitrate. PrpD and AcnB then catalyze the isomerization of 2-methylcitrate to 2-methylisocitrate via the unusual *syn* elimination of a molecule of water. Finally, 2-methylisocitrate is hydrolyzed to pyruvate and succinate by PrpB. Pyruvate can then be converted to acetyl-CoA which, along with succinate, enters the TCA cycle.

Propionate is known to affect the growth of *E. coli* at µM concentrations. The mechanism of toxicity in bacteria has yet to be completely characterized, although in *Salmonella*, both propionate and 2-methylcitrate likely play a role by blocking enzymes involved in linking glycolysis and the TCA cycle (pyruvate

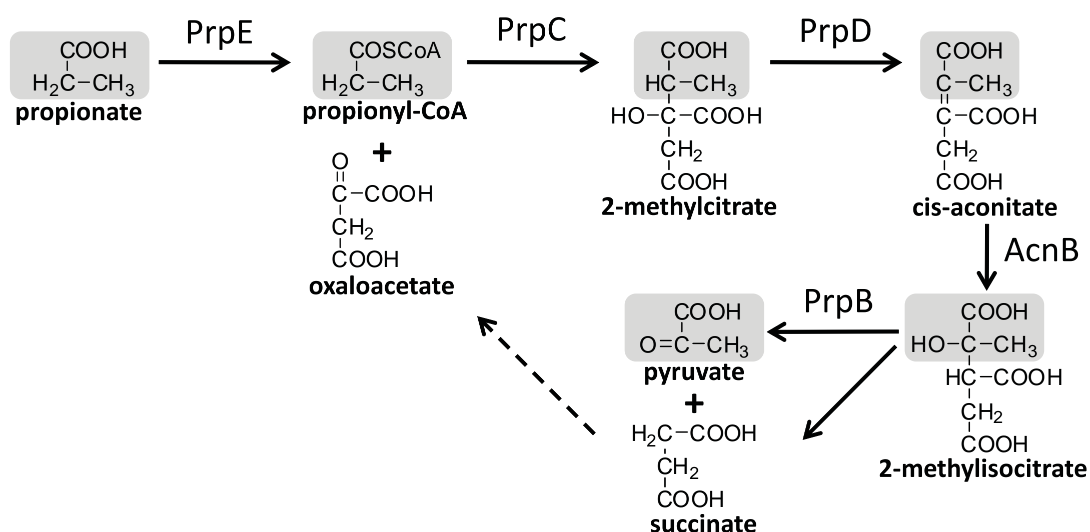

Figure 5.6. Propionate metabolism via the 2-methylcitrate cycle.

dehydrogenase, AceEF & LpdA), the TCA cycle itself (citrate synthase, GltA) and gluconeogenesis (fructose-1,6-bisphosphatase, Fbp) (Rocco and Escalante-Semerena, 2010). However, *E. coli* is able to tolerate propionate as a metabolite involved in the overproduction of polyketides such as 6-deoxyerythronolide B (6-dEB) and polyesters such as poly(3-hydroxyvalerate). It is worth noting that the metabolism of propionate in both of these cases occurs separate from the 2-methylcitric acid cycle.

As propionate is likely a key intermediate in the metabolism of levulinic acid, it will be necessary to engineer propionate tolerance in *E. coli*. This might be accomplished through the overexpression of key enzymes involved in the 2-methylcitric acid cycle to prevent the accumulation of potential toxic intermediates such as 2-methylcitrate. In *Salmonella*, propionate has been identified as substrate of citrate synthase which is a key enzyme in the TCA cycle. The action of citrate synthase on propionate results in the production of the (2S,3R) and the (2R,3S) stereoisomers of 2-methylcitric acid which are distinct from the (2S,3S) stereoisomer produced by 2-methylcitrate synthase. Ultimately, this is an issue due to stereospecificity associated with downstream enzymes in the 2-methylcitric acid cycle. Accumulation of these alternative enzymes may play a role in the inhibition of fructose-1,6-bisphosphatase.

Once the mechanism of toxicity is determined, steps can be taken to engineer tolerance. If the mechanism turns out to be the same as for *Salmonella*, one approach would be to alter the native fructose-1,6-bisphosphatase with an S123F mutation which was shown to restore growth on propionate as a sole carbon source in *Salmonella* (Rocco and Escalante-Semerena, 2010). Whether or not the mechanism differs from *Salmonella*, it may prove worthwhile to circumvent native propionate metabolism with an alternative pathway. One approach would involve heterologous expression of a known pathway involving propionyl-CoA carboxylase from *Streptomyces* wherein propionate would be converted to methylmalonyl-CoA which can in turn be metabolized to succinyl-CoA – another TCA cycle intermediate. As a final point, it is possible that little to no engineering may be required. Once the genes from *P. putida* KT2440 responsible for levulinic acid metabolism to 3-hydroxyvaleryl-CoA are determined, these genes can be overexpressed in *E. coli*. If little to no accumulation occurs for intermediates of levulinic acid metabolism, namely propionate, then the toxic effects may be avoided altogether.

5.3. Conclusions

There is no lack of opportunities to advance the commercial prospects of PHA. This thesis focused on a number of targeted approaches to improve the link between inexpensive unrelated feedstocks and commercially relevant polyesters that are often derived from structurally related carbon sources. The importance of this works stems from the premium price necessitated by the existing technology implemented in industrial PHA production. At costs 2-3 times greater than traditional, petroleum derived plastics, a number of considerable obstacles must be overcome for PHA to be competitive in the marketplace. To begin to address these issues, Chapter 1 made an argument for the need for energy security and sustainable production of commodity fuels and chemicals. These needs have resulted in an industrial biotechnology revolution and the current state of the industry in the context of plastics manufacturing was described. An argument was made for PHA as a viable alternative to traditional, petroleum derived plastics, the need for ways to reduce production costs and the potential of engineering pathways for novel PHA that begin with these feedstocks.

The remainder of the thesis focused on what options currently exist for PHA production from structurally unrelated carbon sources and the potential for the use of PKS and fatty acid-based routes. Chapter 2 updated the list of known monomers incorporated into PHA over the last two decades. This list sets the stage for the potential of PHA and the problems, namely cost, associated with producing these monomers. Engineering strategies to produce novel PHA from starting materials such as glucose and glycerol were reviewed and new strategies and ideas were described. Chapters 3 and 4 implemented the new ideas described in Chapter 2. Chapter 3 synthesized strategies for fatty acid overproduction and PHA biosynthesis culminating in a strain of *E. coli* capable of production of defined mcl-PHA from glucose. The success of this strategy paves the way to generate defined PHA based on the product profile of a given thioesterase. The results of this research chapter also provide examples of the need for an understanding of basic science to answer engineering problems as was the case for construction of the library of β-oxidation deletion strains. In Chapter 4, the potential of modified type I PKS to function as endlessly reconfigurable monomer synthases for the production of PHA with a defined composition was explored. The main outcome of this work was the need for a better understanding of PKS biochemistry. In

time, an advanced knowledge of the modularity of PKS will pave the way for the production of a plethora of novel chemicals, drugs and perhaps PHA as well.

Finally, Chapter 5 outlines future directions for continued research. This chapter broadens the scope of pathway engineering to include, for example, ways to use non-natural carbon sources that may not be readily metabolized by industrially relevant microorganisms. In the context of the greater production process, the ability to use inexpensive feedstocks in conjunction with strain engineering for improvements in production is only one part of the story. The limited discussion found in this thesis may not adequately convey the importance of the potential for improving the downstream recovery and purification processes following fermentation. A number of ideas, including polymer excretion, autolysis and chlorinated solvent-free recovery methods may yet play a role in the commercial viability of PHA. Nevertheless, it is hoped that the work presented in this thesis has provided a significant contribution towards the development of a sustainable, biodegradable alternative to traditional plastic products.

5.4. Materials and Methods

5.4.1. Effect of carbon source on mcl-PHA biosynthesis

Experiments comparing acetate and glucose as carbon sources for mcl-PHA biosynthesis involved strain SA01 and plasmids pDA-JC and pDA-JAC listed in Appendix 3. Acetate was purchased from Fisher Scientific (Pittsburgh, PA). Growth experiments were performed as described previously (*see* 4.5.1). For PHA production experiments, MOPS minimal media was prepared with 1% casamino acids and either 1% (D)-glucose or 1.366% Na acetate. Casamino acids, glucose and Na acetate were prepared as 20% solution in water and adjusted to pH 7.0 with 5 M HCl or 5 M NaOH. In 250 mL baffled flasks, 50mL media supplemented with 100 ug mL^{-1} ampicillin was inoculated to an OD_{600} of 0.05 and induced with 1mM IPTG at an OD_{600} of 0.2. After induction, cultures were incubated for 96 h at 37 °C with shaking at which point, cultures were harvested for PHA and FAME analysis. Fatty acid and PHA extraction and characterization were performed as described previously (*see* 4.5.4).

5.4.2. Levulinic acid metabolism in P. putida KT2440

Pseudomonas putida KT2440 was obtained from ATCC (Manassas, VA) (ATCC® Number 47054). Chemicals and reagents were purchased from Fisher Scientific (Pittsburgh, PA) unless otherwise specified. Enzymes used for cloning were purchased from New England Biolabs (Ipswich, MA). Oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA) and sequences are listed in Appendix 4. For all experiments, single colonies were used to inoculate 5 mL starter cultures that were grown overnight prior to inoculation of experimental cultures. *P. putida* was maintained at 30°C and E. coli at 37°C. Liquid culturing was performed in a rotary shaker (250 rpm). Where necessary, cultures were supplemented with 50 μg mL^{-1} kanamycin. Levulinic acid solutions were prepared as a 20% stock in water and adjusted to pH 7.0 with 5 M NaOH. Stock solutions prepared from Na levulinate formed a precipitate when combined with minimal media so only levulinic acid was used. Furthermore, levulinic acid was stored at 30°C to maintain the reagent in the liquid phase. M9 minimal media was prepared according to standard recipes (Sambrook et al., 2001).

For generation of the transposon library, plasmid pBAM1 was obtained from Esteban Martínez-García (Centro Nacional de Biotecnología – Madrid, Spain) along with *E. coli* strains CC118 λ-pir, HB101 (pRK600) and S17-1 λ-pir. Plasmid pBAM1 was transformed into *P. putida* by either electroporation or mating as described previously (Martinez-Garcia et al., 2011). Colonies containing the Tn5 transposon were selected for on LB + 50 μg mL^{-1} kanamycin. Colonies deficient in the ability to use levulinic acid as a sole carbon source were screened for on 1.5% agar solid media composed of M9 + 1% glucose, M9 + 1% levulinic acid or LB. All solid media contained 50 μg mL^{-1} kanamycin. Colonies of interest were sequenced to determine the location of the transposon insertion site as described previously (Martinez-Garcia et al., 2011). Sequence data was aligned to the *P. putida* KT2440 genome with BLAST (blast.ncbi.nlm.nih.gov).

Plasmid pK18mobsacB was acquired from Jeffrey Gardner (Great Lake Bioenergy Research Center – Madison, WI) for the construction of scarless deletions using a sucrose based counter-selection method described previously (Schäfer et al., 1994). Eventually, a 5-fluorouracil based counter-selection method proved to be more tractable compared with the use of pK18mobsacB (Graf and Altenbuchner, 2011). Gene

deletions were designed to leave intact the start codon seamlessly fused to the terminal 7 codons of the ORF.

5.4.3. Propionate toxicity in E. coli and Salmonella

Chromosomal gene deletions were created in *E. coli* K12 MG1655 as by P1 transduction (Thomason et al., 2007) using phage lysates generated from members of the KEIO collection (Baba et al., 2006). Resistance markers were removed by inducing FLP recombinase encoded on plasmid pCP20 which was subsequently cured by growth at a non-permissive temperature (Datsenko and Wanner, 2000). Chromosomal deletions were verified by colony PCR. Strains were grown in MOPS minimal media supplemented with 30 mM succinate and 0-30 mM propionate from 5% and 20% stock solutions, respectively. All solutions were adjusted to pH 7.0 with 5 M HCl or 5 M NaOH where necessary.

For plate-reader experiments, 5 mL overnight cultures were grown in culture tubes at 37°C in a rotary shaker (250 rpm) and used to inoculate fresh media to an OD_{600} of 0.05. Cultures were dispensed into a sterile, 96-well, clear polystyrene plate with lid in 200 µL aliquots. 96-well plates were incubated in a Tecan Infinite M1000 plate reader at 37°C with linear shaking. OD_{600} was monitored every 15 minutes over the course of a 24 hour kinetic cycle.

5.5. References

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Appendix 1: Abbreviations used in this work

Abbreviation	Term
3HB	3-hydroxybutyrate
3HP	3-hydroxypropionate
3HV	3-hydroxyvalerate
4HB	4-hydroxybutyrate
6-dEB	6-deoxyerythronolide B
ACP	acyl-carrier protein
ADM	Archer Daniels Midland
AT	acyltransferase
BDO	1,4-butanediol
BTE	California Bay laurel thioesterase
CDW	cell dry weight
CoA	coenzyme A
DEBS	6-deoxyerythronolide B synthase
DH	dehydratase
DKS	diketide synthase
DMT	dimethylterephthalate
DO2	dissolved oxygen
ER	enoylreductase
FAME	fatty acid methyl ester
FAS	fatty acid synthase
FFA	free fatty acids
GBL	γ-butyrolactone
G-C	guanine-cytosine
HDPE	high density polyethylene
HMF	hydroxymethylfurfural
KR	ketoreductase
KS	ketosynthase
LB	lysogeny broth
mcl	medium-chain-length
NMR	nuclear magnetic-resonance
ORF	open reading frame
PBS	polybutylene succinate
PDO	1,3-Propanediol
PE	polyethylene
PET	polyethylene terephthalate
PHA	polyhydroxyalkanoates
PHB	polyhydroxybutyrate
PKS	polyketide synthases
PLA	Polylactic acid
PP	polypropylene
PPT	phosphopantetheine
PPT	polypropylene terephthalate
PS	polystyrene
PTA	purified terephthalic acid
PVC	polyvinylchloride
RBS	Ribosome binding site
RFP	red fluorescent protein

Appendix 1: (continued)

Abbreviation	Term
scl	short-chain-length
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
S-NAC	N-acetylcysteamine thioester
TCA	tricarboxylic acid
TE	thioesterase
T_g	glass transition temperature
T_m	melting temperature

Appendix 2: List of new PHA monomers described since 1995.

1. Delta 8,9-epoxy-3-hydroxy-5c-tetradecenoic acid; (Eggink et al., 1995)
2. Delta 6.7-epoxy-3-hydroxydodecanoic acid; (Eggink et al., 1995)
3. Delta 4,5-epoxy-3-hydroxydecanoic acid; (Eggink et al., 1995)
4. 3,8-dihydroxy-5c-tetradecenoic acid; (Eggink et al., 1995)
5. 3,6-dihydroxydodecanoic acid; (Eggink et al., 1995)
6. 6-hydroxy-3c-dodecenoic acid; (Eggink et al., 1995)
7. 4-hydroxydecanoic acid; (Eggink et al., 1995)
8. 3-hydroxy-6,6,6-trifluorohexanoic acid; (Kim et al., 1996)
9. 3-hydroxy-6,6,7,7,8,8,8-heptafluorooctanoic acid; (Kim et al., 1996)
10. 3-hydroxy-6,6,7,7,8,8,9,9,9-nonafluorononanoic acid; (Kim et al., 1996)
11. 3-hydroxy-6,6,7,7,8,8,9,9, 10,10,11,11,11-tridecafluoroundecanoic acid; (Kim et al., 1996)
12. Terminal Epoxy-3-hydroxyalkanoic acids; (Bear et al., 1997)
13. 3-hydroxy-2,2-dimethylpropanoic acid (2-hydroxypivalic acid); (Fuechtenbusch et al., 1998)
14. 3,6-dihydroxy-7-*cis*-nonenoic acid; (He et al., 1998)
15. 3,6-dihydroxy-7-*trans*-nonenoic acid); (He et al., 1998)
16. 3-hydroxy-6-phenylhexanoic acid; (Garcia et al., 1999)
17. 3-hydroxy-8-phenyloctanoic acid; (Garcia et al., 1999)
18. 3-hydroxy-10-phenyldecanoic acid; (Garcia et al., 1999)
19. 3-hydroxy-5-thiophenoxypentanoic acid; (Takagi et al., 1999)
20. 3-hydroxy-7-thiophenoxyheptanoic acid; (Takagi et al., 1999)
21. 6-para-methylphenoxyhexanoic acid (Kim et al., 2000)
22. 8-para-methylphenoxyoctanoic acid (Kim et al., 2000)

Appendix 2: (continued)

23. 8-meta-methylphenoxyoctanoic acid (Kim et al., 2000)
24. 8-ortho-methylphenoxyhexanoic acid (Kim et al., 2000)
25. 10-undecynoic acid (Kim et al., 2000)
26. 3-hydroxy-7-oxooctanoic acid; (Jung et al., 2000)
27. 3-hydroxy-5-oxohexanoic acid; (Jung et al., 2000)
28. 6-acetoxy-3-hydroxyhexanoic acid; (Jung et al., 2000)
29. 4-acetoxy-3-hydroxybutyric acid; (Jung et al., 2000)
30. 3-hydroxy-7-phenylheptanoic acid; (Abraham et al., 2001)
31. 3-hydroxy-4-phenylbutyric acid; (Song et al., 2001)
32. 3-hydroxy-4-cyclohexylbutyric acid; (Kim et al., 2001)
33. 4-hydroxyhexanoic acid; (Lee and Lee, 2003)
34. 3-hydroxy-5-(2,4-difluorophenoxy)pentanoic acid; (Takagi et al., 2004)
35. 3-hydroxy-7-(3-fluorophenoxy)heptanoic acid; (Takagi et al., 2004)
36. 3-hydroxy-5-(3-fluoropheoxy)pentanoic acid; (Takagi et al., 2004)
37. 3-hydroxy-7-(4-fluorophenoxy)heptanoic acid; (Takagi et al., 2004)
38. 3-hydroxy-5-(4-fluoropheoxy)pentanoic acid; (Takagi et al., 2004)
39. 3-hydroxyoctadecanoic acid; (Singh and Mallick, 2008)
40. 3-hydroxy-9-phenoxynonanoic acid; (Choi et al., 2009)
41. 3-hydroxy-5-methylheptanoic acid; (Ashby et al., 2009)
42. 3-hydroxy-5-methylnonanoic acid; (Ashby et al., 2009)
43. 3-hydroxy-5-methylundecanoic acid; (Ashby et al., 2009)
44. 3-hydroxy-6-methylundecanoic acid; (Ashby et al., 2009)

Appendix 2: (continued)

45. 3-hydroxy-7-methylundecanoic acid; (Ashby et al., 2009)
46. 3-hydroxy-8-methylundecanoic acid; (Ashby et al., 2009)
47. 3-hydroxy-9-methylundecanoic acid; (Ashby et al., 2009)
48. 3-hydroxy-10-methylundecanoic acid; (Ashby et al., 2009)
49. 3-hydroxy-5-methyltridecanoic acid; (Ashby et al., 2009)
50. 3-hydroxy-6-methyltridecanoic acid; (Ashby et al., 2009)
51. 3-hydroxy-7-methyltridecanoic acid; (Ashby et al., 2009)
52. 3-hydroxy-8-methyltridecanoic acid; (Ashby et al., 2009)
53. 3-hydroxy-9-methyltridecanoic acid; (Ashby et al., 2009)
54. 3-hydroxy-10-methyltridecanoic acid; (Ashby et al., 2009)
55. 3-hydroxy-11-methyltridecanoic acid; (Ashby et al., 2009)
56. 3-hydroxy-11-phenoxyundecanoic acid; (Choi et al., 2010)
57. 2-hydroxybutyric acid *in vitro;* (Han et al., 2011) *in vivo*; (Park et al., 2011)
58. 3-hydroxy-5-octenoic acid; (Impallomeni et al., 2011)
59. 3-hydroxy-7-decenoic acid; (Impallomeni et al., 2011)
60. 3-hydroxy-5,8,11-tetradecenoic acid; (Impallomeni et al., 2011)
61. 3-hydroxy-6-acetylthiohexanoic acid; (Escapa et al., 2011)
62. 3-hydroxy-4-acetylthiobutanoic acid; (Escapa et al., 2011)
63. 2-hydroxyethanoic acid (glycolic acid); (Matsumoto et al., 2011)
64. 3-hydroxy-methylpropionic acid *in vitro*; (Song et al., 2012)

Appendix 3: Strains and plasmids used in this study.

Strain/Plasmid	Relevant Genotype/Property	Source or Reference
Strains		
Cupriavidus necator	*a.k.a. Ralstonia eutropha*; source for *phaCAB*	ATCC 17699™
E. coli BAP1	F-*ompT hsdSB* (*rB-mB-*) *gal dcm* (DE3) *prpRBCD*:: T7prom-*sfp*,T7prom-*prpE*	(Pfeifer et al., 2001)
E. coli BL21 (DE3)	*fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS* *λ DE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5*	(Studier and Moffatt, 1986)
E. coli CC118 λ-*pir*	Δ(*ara-leu*), *araD, ΔlacX174, galE, galK, phoA, thi1, rpsE, rpoB, argE* (*Am*), *recA1,* lysogenic, λ*pir*	(Herrero et al., 1990)
E. coli DH10B	F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*lacZ*ΔM15 Δ*lacX*74 *recA*1 *endA*1 *araD*139 Δ(*ara, leu*)7697 *galU galK* λ^- *rpsL nupG*	Invitrogen
E. coli DH5α	F^- Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *recA1 endA1 hsdR17* (r_k-, m_k+) *pho*A *sup*E44 λ^- *thi*$^-$1 *gyr*A96 *rel*A1	Invitrogen
E. coli DY330	F^- λ^- *rph*-1 INV(*rrnD*, *rrnE*) Δ*lacU169 gal490 pgl*Δ*8* λcI857 Δ(*cro-bioA*)	(Yu et al., 2000)
E. coli ECF530	WM2949: attB_::PBAD-*pir*P106L^F107S *rrnB*PI(CTC-AGA)-*lacY*A177C	(Bowers et al., 2004)
E. coli HB101	Sm^R, *hsdR*$^-$$M^+$, *pro*, *leu*, *thi*, *recA*	(Boyer and Roulland.D, 1969)
E. coli K-12 MG1655	F^- λ^- *ilvG*$^-$ *rfb*-50 *rph*-1	ECGSC
E. coli LS5218	F^+ *fadR601 atoC512*(Const)	ECGSC
E. coli S17-1 λ-pir	(F^-) RP4-2-Tc::Mu *aphA*::Tn*7recA*λ*pir*lysogen, Sm^R, Tp^R	(Simon et al., 1983)
NRD204	MG1655 *ΔaraBAD::cat*	(De Lay and Cronan, 2007)
Pseudomonas aeruginosa	(*mucosus*); Source for *exaDE*	ATCC 17933™
Pseudomonas aeruginosa PAO1	Source for *phaC1-2, phaJ1-4*	ATCC BAA-47™
Pseudomonas putida GPo1	(a.k.a. *Pseudomonas oleovorans* NRRL B-14683), source for *acs1*	ATCC 29347™
Pseudomonas putida KT2440	Source for PP_0763	ATCC 47054™
Saccharopolyspora erythraea	Source of *eryAI-III*, *acsA*	ATCC 11635™
Streptomyces caelestis	NRRL 2418; Source of *nidAI-III*	USDA ARS Culture Collection
Streptomyces lividans 1326	Wild Type	M. Thomas
E. coli K12 Δ*atoDA*	MG1655 Δ*atoDA*	This work
araBAD	MG1655 Δ*araBAD*	This work
A	MG1655 Δ*araBAD* Δ*fadA*	This work
B	MG1655 Δ*araBAD* Δ*fadB*	This work
E	MG1655 Δ*araBAD* Δ*fadE*	This work
I	MG1655 Δ*araBAD* Δ*fadI*	This work
J	MG1655 Δ*araBAD* Δ*fadJ*	This work

Appendix 3: (continued)

Strain/Plasmid	Relevant Genotype/Property	Source or Reference
Strains		
R	MG1655 Δ*araBAD* Δ*fadR*	This work
RA	MG1655 Δ*araBAD* Δ*fadR* Δ*fadA*	This work
RB	MG1655 Δ*araBAD* Δ*fadR* Δ*fadB*	This work
RE	MG1655 Δ*araBAD* Δ*fadR* Δ*fadE*	This work
RI	MG1655 Δ*araBAD* Δ*fadR* Δ*fadI*	This work
RJ	MG1655 Δ*araBAD* Δ*fadR* Δ*fadJ*	This work
AI	MG1655 Δ*araBAD* Δ*fadA* Δ*fadI*	This work
BJ	MG1655 Δ*araBAD* Δ*fadB* Δ*fadJ*	This work
AB	MG1655 Δ*araBAD* Δ*fadAB*	This work
IJ	MG1655 Δ*araBAD* Δ*fadIJ*	This work
RAI	MG1655 Δ*araBAD* Δ*fadR* Δ*fadA* Δ*fadI*	This work
RBJ	MG1655 Δ*araBAD* Δ*fadR* Δ*fadB* Δ*fadJ*	This work
RAB	MG1655 Δ*araBAD* Δ*fadR* Δ*fadA* Δ*fadB*	This work
RIJ	MG1655 Δ*araBAD* Δ*fadR* Δ*fadIJ*	This work
ABIJ	MG1655 Δ*araBAD* Δ*fadAB* Δ*fadIJ*	This work
RABIJ	MG1655 Δ*araBAD* Δ*fadR* Δ*fadAB* Δ*fadIJ*	This work
Φ(P_{trc}-fadD)	MG1655 Δ*araBAD* Φ(P_{trc}-*fadD*)	This work
SA01	MG1655 Δ*araBAD* Δ*fadR* Δ*fadIJ* *fadBA::*Φ(P_{trc}-BTE)	This work
Plasmids		
pBAD33	P_{BAD} promoter, pACYC origin, Cm^R	(Guzman et al., 1995)
pBAD33*-C1	pBAD33-C280* containing *phaC1* gene (*P. aeruginosa*)	This work
pBAD33*-C2	pBAD33-C280* containing *phaC2* gene (*P. aeruginosa*)	This work
pBAD33-C280*	pBAD33 *araE* C280* Δ281-292	(Lee et al., 2007)
pBAD35 (pBT2bad18)	pBT-2, P_{BAD} promoter, MCS, terminator	
pBAM1	Kan^RA, Amp^R, *ori*R6K, *tnpA, oriT*	(Martinez-Garcia et al., 2011)
pBT-2	pBBR1 origin, Kan^R	
pBTE-int	pTrc99A containing BTE with *cat*-FRT cassette from pKD3 (Datsenko and Wanner, 2000) inserted 5' of $lacI^Q$	(Youngquist et al., 2012)
pCP20	FLP^+, λ $cI857^+$, λ p_R Rep^{ts}, Ap^R, Cm^R	(Cherepanov and Wackernagel, 1995)
pDA-JAC	pDA-JC with PP_0763 cloned between *phaJ3* and *phaC2*	This work
pDA-JC	pMSB6 containing *phaJ3* and *phaC2* genes (*P. aeruginosa*)	This work
pDMP-A	pDMP-MCS with Niddamycin DKS region A (see text)	This work
pDMP-B	pDMP-MCS with Niddamycin DKS region B (see text)	This work
pDMP-C	pDMP-MCS with Niddamycin DKS region C (see text)	This work
pDMP-MCS	pMSB6 with translational coupling cassette from pLIC-Km cloned after MCS	This work
pEryDKS	pTrc99a, *eryAI-eryTE* (*ery* DKS)	This work
pFL506a	γ *ori*, *cat*, *rrnB*T_1T_2, wt *lacPO* with mutagenized SD sequence, tL3	(Bowers et al., 2004)

Appendix 3: (continued)

Strain/Plasmid	Relevant Genotype/Property	Source or Reference
Plasmids		
pGM190	*Streptomyces-E.coli* shuttle vector, *tsr*, *aphII*, pSG5 derivative (Muth et al., 1988), *tipA* promoter	(Wohlleben et al., 2009)
pGM202	pGM190 derivative encoding C-terminal 6X His-tag	(Wohlleben et al., 2009)
pISA1	Amp^R, *Acinetobacter sp.* RA3849 *phaBCA* in pBAD24	(Aldor and Keasling, 2001)
pK18*mobsacB*	Suicide plasmid for gene deletion, kan^R	(Schäfer et al., 1994)
pKD13	Template plasmid for gene disruption. Kan^R cassette flanked by FRT sites. Amp^R	(Datsenko and Wanner, 2000)
pLIC-Km	Translational coupling cassette using aphII as response gene	(Mendez-Perez et al., 2012)
pMSB6	pTrc99A with altered MCS	This work
pMSB6-J1	pMSB6 containing *phaJ1* gene (*P. aeruginosa*)	This work
pMSB6-J2	pMSB6 containing *phaJ2* gene (*P. aeruginosa*)	This work
pMSB6-J3	pMSB6 containing *phaJ3* gene (*P. aeruginosa*)	This work
pMSB6-J4	pMSB6 containing *phaJ4* gene (*P. aeruginosa*)	This work
pNidDKSv2	pTrc99a, *nidAI-nidTE* (*nid* DKS)	This work
pRK600	Cm^R; *ori*ColE1, RK2, mob^+, tra^+	(Delorenzo and Timmis, 1994)
pSIM5	pSC101ts *gam exo bet* chloramphenicol resistance (CmR)	(Datta et al., 2006)
pSIM6	pSC101ts *gam exo bet* ampicillin resistance (AmpR)	(Datta et al., 2006)
pSP01	pBAD35, $phaC_{Cne}$, $acsA_{Ser}$	This work
pTrc99A	P_{trc} promoter, pBR322 origin, Amp^R	(Amann et al., 1988)
pTrc99A-BTE	pTrc99A carrying BTE under P*trc* control, Amp^R	(Hoover et al., 2011)
pTrc99A-*fadD*	*fadD* cloned as a *Kpn I –Xba I* fragment into pTrc99a	This work
pTrc-*acs1*	pTrc99a with *acs1* from *P. putida GPo1*	This work
pTrc-acsA-Htag	6X C term his tag acsA?	This work
pYW7011	T7prom-*metK*-T7term	(Wang et al., 2007)

Appendix 4: Primers used in this study.

Primer Name	Sequence
acs-F	GGGGGTACCAGGAGGTATAATTAATGTTGCAGACACGCATCATC
acs-R	GGGTCTAGATTACAACGTGGAAAGGAACGC
atoDA_pKD4_fwd	CTGACTGTACCCACAACGGTGTATGCAAGAGGGATAAAAAGTGTAGGCTGGAGCTGCTTC
atoDA_pKD4_rev	GGCTGACAAAACGCGTCATAAAACGCGATATGCGACCAATCATATGAATATCCTCCTTAG
C280*-F	GGGCTCGAGTTAACCGGCACGGAACTCGCTCG
C280*-R	GGGCTCGAGTTGGTAACGAATCAGACAATTGACGGC
Cne_phaC_fwd’	GATGCAGCTAGCAGGAGGTATAATTAATGGCGACCGGCAAAGGCGCG
Cne_phaC_rev’	GTATCACATATGTCATGCCTTGGCTTTGACGTATCGCCC
fadIJ::Cm-F	CAGGTCAGACCACTTTATTTATTTTTTTACAGGGGAGTGTGAAGCGGCATGCGTTCCTATTCC
fadIJ::Cm-R	TTGCAGGTCAGTTGCAGTTGTTTTCCAAAAACTTTCCCCAGTGTAGGCTGGAGCTGCTTC
fadR::Cm-F	TCTGGTACGACCAGATCACCTTGCGGATTCAGGAGACTGAGAAGCGGCATGCGTTCCTATTCC
fadR::Cm-R	AACCCGCTCAAACACCGTCGCAATACCCTGACCCAGACCGGTGTAGGCTGGAGCTGCTTC
GPo1_acs1_fwd	GATGTGCCCGGGAGGAGGTATAATTAATGATCGAAAATTTTTGGAAGG
GPo1_acs1_rev	GATGCTTCTAGATCAGGCGATCTTCTTCAAA
IJ::BTE-F	GGTCAGACCACTTTATTTATTTTTTTACAGGGGAGTGTTAGCGGCATGCGTTCCTATTCC
IJ::BTE-R	CTCCGCCATTCAGCGCGGATTCATATAGCTTTGACCTTCTTAAACACGAGGTTCCGCCGG
NidA1_GM_fwd	GACGCACATATGGCAGGGCATGGTGACGCC
NidA1_GM_rev	GACGTTAAGCTTCCACATGGCAATTGGTGCCGCC
NidDKS_3'	CATCGGTGTACATCAGCGTCC
NidDKS_3'fwd	GACGAGGCGGTGTCCCTGATG
NidDKS_3'rev	GTTGTCACGGACGGTGGACGC
NidDKS_3'RT	CAACCGGAGGGCACGCTGCTC
NidDKS_5'	CTTGACCGAGCCCACCAGGAG
NidDKS_5'fwd	GGGTCCGATGCCATCGCTGTC
NidDKS_5'rev	CTTGACCGAGCCCACCAGGAG
NidDKS_5'RT	GCCACCGCGCAAAAGGCGCAG
NidDKS_mid	GCCCTGTTGGTGTAGTTGCTG
NidDKS_mid-fwd	CAAGTCATCCGCGACGCCCTG
NidDKS_mid-rev	CCGGACAGGACGCAGTGGGTG
NidDKS_mid-RT	GCCAGCAACGGCCTCACCGCC
N-RFP_fwd	GATGATGAATTCNNNNNNTATATTTAATGGTGTCTAAGGGCGAAGAG
N-RFP_rev	GATGAAACTAGTCTTGTACAGCTCGTCCATGCC
pDMP_F	GTGTCACTGCAGACTAGTCATCATCACCACCATC
pDMP_R	GTCGTCAAGCTTAGAAAAACTCATCGAGCA
PfadD-kan-F	TGAATAATTGCTTGTTTTTAAAGAAAAAGAAACAGCGGCTGGTCCGCTGTGTGTAGGCTGGAGCTGCTTC
PfadD-kan-R	TCGATGGTGTCAACGTAAATGATTCCGGGGATCCGTCGACC
PfadD-sew-R	TCAGGCTTTATTGTCCACTTTG
PfadD-Trc-F	CATTTACGTTGACACCATCGA
phaC1-F	GGGGAGCTCAGGAGGTATAATTAATGAGTCAGAAGAACAATAACGAG
phaC1-R	GGGGGTACCTCATCGTTCATGCACGTAGGT

Appendix 4: (continued)

Primer Name	**Sequence**
phaC2-F	GGGGAGCTCAGGAGGTATAATTAATGCGAGAAAAGCAGGAATCGGG
phaC2-F2	GGGTCTAGAAGGAGGTATAATTAATGCGAGAAAAGCAGGAATCGGG
phaC2-R	GGGGGTACCTCAGCGTATATGCACGTAGGTGC
phaC2-R2	GGGAAGCTTTCAGCGTATATGCACGTAGGTGC
phaJ1-F	GACGATGAATTCAGGAGGTATTAATAATGAGCCAGGTCCAGAACATTC
phaJ1-R	GACGATGGATCCGGCCCGACGGTAGGGAAA
phaJ2-F	GACGATGAATTCAGGAGGTATTAATAATGGCGCTCGATCCTGAGGTGC
phaJ2-R	GACGATGGATCCCTTCGCTTCAGTCCGGCCGCT
phaJ3-F	GACGATGAATTCAGGAGGTATTAATAATGCCCACCGCCTGGCTCGAC
phaJ3-R	GACGAAGGATCCTCAGCCCTGTAGCCGGCTCCA
phaJ4-F	GACGATGAATTCAGGAGGTATTAATAATGCCATTCGTACCCGTAGCAG
phaJ4-R	GACGATGGATCCTCAGACGAAGCAGAGGCTGAG
pMSB6_F	GTGTGGAATTGTGAGCGGATAAC
pMSB6_R	CATCCGCCAAAACAGCCAAG
pNidDKS_fwd1	GACGCATCTAGAAGGAGGTATAATTAATGGCAGGGCATGGTGACGCCACC
pNidDKS_fwd2	GACGATCTGCAGTGGACGCGCGGGCCTCCTTC
pNidDKS_rev1	GACGATCTGCAGTGGCCGAGCCGTGCCGAAGCAC
pNidDKS_rev2	GATCATAAGCTTTCATCGGTGTACATCAGCGTCCTCCG
R::BTE-F	GAGTCCAACTTTGTTTTGCTGTGTTATGGAAATCTCACTAGCGGCATGCGTTCCTATTCC
R::BTE-R	ACCCCTCGTTTGAGGGGTTTGCTCTTTAAACGGAAGGGATTAAACACGAGGTTCCGCCGG
regionA_rev	GGGGATACTAGTGTTCACCTCCTGGTCGGCATC
regionB_fwd	GAACACATCGATAGGAGGTATAATTAATGGCCGACCAGGAGGTGAACGGC
regionB_rev	GACGTTACTAGTTCCTGCAGTGGCCGAGCCGTGCCGAAGCAC
regionB2_fwd	GGGAGATCTAGGAGGTATAATTAATGGATCCAATTGCCGTCATCGGTATCGGATGC
regionC_fwd	GTTCAATCTAGAGGAGGTATAATTAATGGCTGCAGTGGACGCGCGGGCCTCCTTC
regionC_rev	GATCATACTAGTTCGGTGTACATCAGCGTCCTCCGG
Ser_acsA_fwd’	GATCCAGAATTCCATATGAGGAGGTATAATTAATGACTTCAAGCACCACTCCTGAGCTGC
Ser_acsA_Htag’	GTCGTTTCTAGATCAGTGGTGGTGGTGGTGGTGGGCTCCGGAATGCCTGCTGATCTG
Ser_acsA_rev’	GTCGTTTCTAGATCAGGCTCCGGAATGCCTGCTGATC

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