Enhancing polyhydroxyalkanoate biosynthesis in Escherichia coli: A genetic engineering and process optimization approach towards functionalized polymeric nanomedicine

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Abstract

R. A. Scheel. Enhancing Polyhydroxyalkanoate Biosynthesis in *Escherichia coli*: A Genetic Engineering and Process Optimization Approach Towards Functionalized Polymeric Nanomedicine, 264 pages, 10 tables (1 in Appendix F), 34 figures (53 in Appendices), 2020. Harvard (author-date) citation style guide was used, with minor formatting changes for publication in Frontiers in Bioengineering and Biotechnology.

Recombinant *Escherichia coli* is a desirable bacterial chassis for the biosynthesis of many biological compounds including poly(3-hydroxyalkanoates) (PHAs), a class of naturally occurring biodegradable polyesters with promising biomedical and material applications. PHAs are very structurally diverse, and control over their composition allows the production of unique materials with tunable physical and chemical properties. The major limitation to widespread use of PHAs is their high cost and low volumetric productivity, particularly for a desirable class of PHAs with medium chain-length (MCL) side chains. In this work, transcriptional regulators of fatty acid degradation were removed from *E. coli* in an effort to enhance MCL PHA biosynthesis. Deletion of *arcA* was successful in improving yields due to increased expression of *fadD* and *fadL* in the mutant strains. Although modest improvements in yield were observed in this mutant, a fed-batch high-density fermentation process was developed to further enhance MCL PHA yields using an industrially relevant bioprocess. Through process optimization, some of the highest yields and productivities were achieved to date, including for PHAs with chemically modifiable functional groups. One of these novel functional polymers, a copolymer of 3-hydroxyoctanoate and 3-hydroxy-10-azidodecanoate, was investigated as a targeted nanoparticle drug delivery system. Preliminary results show high encapsulation efficiency of paclitaxel and a surface chemistry amenable to rapid and efficient Click chemistry. In addition to work with MCL PHAs, short chain-length PHAs from a lignocellulosic waste stream, hydrolyzed paper mill waste fines. This work in its entirety demonstrates several successful strategies for improving the availability of PHAs with controlled compositions.

Keywords: poly[(R)-3-hydroxyalkanoates], high-density fermentation, metabolic engineering, biopolymer, targeted drug delivery, nanoparticles

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Chapter 1: Introduction and literature review for polyhydroxyalkanoates

1.1 Introduction to polyhydroxyalkanoates

Petroleum-based plastics have been rapidly accumulating in the environment for the last 50 years, a process which has only been accelerating. An estimated 250,000 tons of plastic contaminates the world’s oceans, posing a significant threat to wildlife that we are unable to remediate, and that may ultimately end up in our own food supply (Barboza et al., 2018; Eriksen et al., 2014). This has led to growing concerns for the health of the environment, and there is increasing demand for sustainable plastic alternatives (Lazarevic et al., 2010).

Poly-[(R)-3-hydroxyalkanoates] (PHAs) comprise a large class of natural polyesters originally found in bacteria. In native PHA-producing microorganisms, these materials are biosynthesized from intracellular fatty acid derivatives or central metabolites and accumulated as a response to increased levels of carbon rich nutrients while experiencing a shortage of other essential elements in the environment (e.g. N, P, O, etc.) (Lu et al., 2009). These biopolymers have enjoyed increasing interest worldwide and have influenced and driven studies in a multitude of disciplines such as microbiology, metabolic engineering, biochemistry, and materials chemistry. Much of the research to date has focused on the organisms, pathways, and enzymes used to produce PHA materials.

The predominant class of PHA materials are characterized by a polyester backbone product of the biosynthetic polycondensation of (R)-3-hydroxyacyl-CoA thioesters. These polymers and their bulk physical properties are differentiated and classified by the length of their hydrocarbon sidechains. Whereas PHAs with sidechains containing 0-2 carbon atoms are referred to as short-chain-length (SCL) PHAs, those with sidechains containing 3-11 carbon atoms are commonly
identified as medium-chain-length (MCL) PHAs. The former display physical properties typically associated with crystalline thermoplastics while the latter generally possess low crystallinity and elastomeric character (Barham et al., 1992). Poly[(R)-3-hydroxybutyrate] (PHB) is the most well characterized, being the first PHA discovered and the most commonly produced in nature (Lemoigne, 1926). However, the physical properties of PHB are relatively unattractive, producing a stiff and brittle material (Barham et al., 1992). Copolymerization of PHB and with other monomers such as MCL 3-hydroxyacyl species can significantly increase the flexibility and toughness, and current research is increasingly dominated by these endeavors (Hassan et al., 2006; Noda et al., 2010).

1.2 PHA composition and physical properties

There is great diversity in the structure of PHAs, which is predominantly localized to the side chains. As previously mentioned, these are most often categorized as SCL (0-2 carbons per side chain) and MCL (3-11 carbons per side chain), although long chain-length (LCL) PHAs have been observed with side chains up to 13 carbons (Lee et al., 1995). Most PHAs form an ester linkage via a β-hydroxyl group, but notable exceptions such as poly(4-hydroxybutyrate) do exist (Hein et al., 1997; Söhling and Gottschalk, 1996; Valentin and Dennis, 1997). A selection of some representative PHAs is shown in Fig. 1.1, which includes the commercially available group of SCL-co-MCL PHAs known as Nodax™, in addition to some common SCL and MCL PHAs.
Figure 1.1: Chemical structures of common representative polyhydroxyalkanoates (PHAs). Abbreviations: P3HB, poly[(R)-3-hydroxybutyrate]; P4HB, poly(4-hydroxybutyrate); PHHx, poly[(R)-3-hydroxyhexanoate]; PHO, poly[(R)-3-hydroxyoctanoate]; PHD, poly[(R)-3-hydroxydecanoate]; PHBV, poly[(R)-3-hydroxybutyrate-co-3-hydroxyvalerate]; PHB-co-4HB, poly[(R)-3-hydroxybutyrate-co-4-hydroxyvalerate]; PHBHHx, poly[(R)-3-hydroxybutyrate-co-3-hydroxyhexanoate].
In addition to the common PHAs shown in Fig. 1.1, numerous examples are documented in the literature of uncommon PHAs containing functional groups. These are addressed in greater detail at the end of this Chapter. The composition of PHAs depends largely on the type of microorganism that produces them; *Pseudomonas putida* and *P. oleovorans* are two well-studied examples of MCL PHA producers that can incorporate 3-hydroxyacyl monomers of varying lengths and degrees of saturation, while other commonly described organisms such as *Cupriavidus necator* and *Aeromonas caviae* biosynthesize PHB and PHBHHx, respectively (Lu et al., 2009). Through metabolic engineering and the selection of unique carbon substrates, an even greater variety than what is observed in nature can be achieved (Dong et al., 2019; Matsumoto and Kageyama, 2019; Mizuno et al., 2018; Sparks and Scholz, 2008; Thuronyi et al., 2017). Some examples of these can be found in Fig. 1.2.

**Figure 1.2:** Examples of uncommon and functional PHAs. Abbreviations: P2M3HB, poly(2-methyl-3-hydroxybutyrate); P2MHV, poly(2-methyl-3-hydroxyvalerate); P2H4MV, poly(2-hydroxy-4-methylvalerate); P2F3HB, poly(2-fluoro-3-hydroxybutyrate); PHUe, poly(3-hydroxy-10-undecenoate); PHUy, poly(3-hydroxy-10-undecynoate); PHDN3, poly(3-hydroxy-10-azidodecanoate); PHDBr, poly(3-hydroxy-10-bromodecanoate).
The physical properties of PHAs are largely determined by their chemical composition. Homopolymeric PHB is highly crystalline, generally ranging from 60-80\% crystallinity depending on the method of extraction and/or post-isolation processing (Chanprateep, 2010; Doi and Abe, 1990; Tappel et al., 2012b). In addition, PHB has a high Young’s modulus similar to the petrochemically-derived plastic polypropylene (Werber and Baptist, 1964). However, PHB has considerably lower toughness and elongation to break than polypropylene, rendering it quite brittle (Chanprateep, 2010; Sudesh et al., 2000). In comparison, several other PHA homopolymers exhibit elastomeric properties and a greater degree of toughness. Poly(4-hydroxybutyrate), a PHA first isolated from the anaerobic bacterium *Clostridium kluyverii*, has an elongation to break of 1000\%, and the copolymerization of 3HB with 4HB monomers confers an increasing degree of toughness to the resulting material as the monomer \% of 4HB increases (Doi and Abe, 1990; Saito and Doi, 1994). Copolymers of 3HB and MCL monomer units also have attractive physical properties, with increasing MCL monomer content conferring increasingly elastomeric qualities, although these are relatively uncommon in nature (Noda et al., 2005). MCL copolymers, which are most often ter- and quadripolymers of various MCL monomers, are amorphous and characterized by low or indiscernible melting points (Gagnon et al., 1992). A summary of the physical properties of some common PHAs and conventional plastics is listed in Table 1.1.
Table 1.1: Physical properties of some representative PHAs and conventional plastics.

<table>
<thead>
<tr>
<th></th>
<th>Crystallinity (%)</th>
<th>Young’s Modulus (MPa)</th>
<th>Tensile Strength (MPa)</th>
<th>Elongation to Break (%)</th>
<th>Tₙ (°C)</th>
<th>Tₒ (°C)</th>
<th>Tₘ (°C)</th>
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<td><strong>PHB</strong></td>
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<td>3500</td>
<td>40</td>
<td>0.4-45</td>
<td>-1.2 to 10</td>
<td>45 to 95</td>
<td>177</td>
<td>(Chanprateep, 2010; Doi and Abe, 1990; Tappel et al., 2012b)</td>
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<td><strong>PHBV (%3HV)</strong></td>
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<td>-125</td>
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<td>~14</td>
<td>~10</td>
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<td><strong>MCL PHAs (dominant monomers)</strong></td>
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<td>-37</td>
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<td>(Preusting et al., 1990)</td>
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<td>12 to 15</td>
<td>7 to 10</td>
<td>300 to 450</td>
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<td><strong>Conventional Plastics</strong></td>
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<td>PP</td>
<td>50 to 70</td>
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<td>176</td>
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<td>PETF</td>
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<td>PS</td>
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Blank cells indicate the value was not determined from the referenced study. Tₙ (glass-transition temperature), Tₒ (crystallization temperature by differential scanning calorimetry, DSC), Tₘ (melting temperature). Polymer abbreviations; PHB, poly[(R)-3-hydroxybutyrate]; PHBV, poly[(R)-3-hydroxybutyrate-co-3-hydroxyvalerate]; P4HB, poly(4-hydroxybutyrate); PHBHHx, poly[(R)-3-hydroxybutyrate-co-3-hydroxyhexanoate]; 3HO, 3-hydroxyoctanoate; 3HHx, 3-hydroxyhexanoate; 3HOU, 3-hydroxy-7-octenoate; 3HHU, 3-hydroxy-5-hexenoate; 3HD, 3-hydroxydecanoate; 3HB, 3-hydroxybutyrate; LDPE, low-density poly(ethylene); PP, poly(propylene); PETF, poly(ethylene terephthalate); PS, poly(styrene).
1.3 PHA biosynthesis in native producers

PHA biosynthesis pathways in native organisms generally fall into one of two categories, SCL biosynthesis and MCL biosynthesis. PHB, the most common SCL PHA, is produced by a wide variety of organisms using the same set of three enzymes; (1) a β-ketothiolase (PhaA) that catalyzes the condensation of two molecules of acetyl-CoA to form acetoacetyl-CoA, (2) an acetoacetyl-CoA reductase (PhaB) that catalyzes the reduction of acetoacetyl-CoA to form (R)-3-hydroxybutyryl-CoA, and (3) a PHA synthase (PhaC) which catalyzes the formation of an ester bond between two (R)-3-hydroxybutyryl-CoA molecules and begins the elongation of a growing polymer chain (Lu et al., 2009).

PhaC can often accept other hydroxyacyl-CoA substrates, resulting in the formation of PHV, P4HB, or the copolymers PHBV and P3HB4HB. PHV is formed from the metabolism of propionate, which can be supplied exogenously or formed from oxaloacetate in a reversible reaction of gluconeogenesis (Robbins, 1988). Propionate is then converted into propionyl-CoA and condensed with a molecule of acetyl-CoA by an alternative β-ketothiolase (BktB) to form β-ketovaleryl-CoA (Slater et al., 1998). This intermediate is then reduced by PhaB and subsequently polymerized by PhaC, resulting in PHV or PHBV.

P4HB is produced by Clostridium kluyverii from succinyl-CoA, which is converted into succinate semialdehyde by a succinate semialdehyde dehydrogenase (SucD) (Steinbüchel and Valentin, 1995). This intermediate is reduced to 4HB by 4-hydroxybutyrate dehydrogenase (4hbD), converted to 4-hydroxybutyryl-CoA by a succinyl-CoA:CoA transferase (OrfZ, aka Cat1), and is polymerized by PhaC. These pathways are shown in Fig. 1.3.
Figure 1.3: Examples of SCL PHA biosynthesis pathways in native producers. (A) Production of PHB from sugars (acetyl-CoA). (B) Production of PHV from oxaloacetate. (C) Biosynthesis of P4HB from succinyl-CoA.
1.4 PHA biosynthesis in *Escherichia coli* LSBJ

In order to address the difficulties observed with controlling PHA composition, Tappel *et al.* engineered a recombinant *E. coli* strain that was able to exhibit precise control in both SCL and MCL monomer incorporation from fatty acid substrates (2012b). *E. coli* was chosen as a production chassis for several reasons: its genome is very well understood and there are simple methods described to manipulate it, it can grow rapidly to high density and tolerate a wide variety of growth conditions and media, and it cannot naturally produce PHA which allows for greater control over the PHA biosynthesis pathway. The construction of this strain is briefly described below.

### 1.4.1 Engineering of *E. coli* LS5218

Since control of repeating unit composition is critical to produce polymers with desirable, reproducible properties, the Nomura lab engineered *E. coli* to produce PHA polymers with defined repeating unit compositions from fatty acids (Tappel *et al.*, 2012a). The parental strain used was *E. coli* LS5218 (Spratt *et al.*, 1981), which carries two significant mutations that make it ideal for producing PHA polymers from fatty acids: (1) a *fadR* mutation for enhanced expression of genes encoding enzymes involved in β-oxidation, described in more detail below; and (2) an *atoC(Con)* mutation which results in the constitutive expression of the Ato short-chain-fatty acid uptake enzymes in *E. coli*. This strain was further engineered to control the production of enoyl-CoA by deleting the *fadB* (Yang *et al.*, 1995) and *fadJ* (Campbell *et al.*, 2003) genes (*E. coli* LSBJ), both of which can catalyze the (*S*)-specific hydration of enoyl-CoA, resulting in a roadblock in β-oxidation ([Figure 1.5](#)). This resulted in a strain where any exogenous fatty acid supplied was pooled as its enoyl-CoA intermediate with no loss of carbon from the substrate. For example, supplementation with a 4-carbon fatty acid (butyrate) results in a butenoyl-CoA intermediate, and
supplementation with an 8-carbon fatty acid (octanoate) results in pooling of an octenoyl-CoA intermediate. Thus, specific fatty acid feeding regimens, coupled with expression of the (R)-specific enoyl-CoA hydratase (PhaJ4) and PhaC1(STQK) engineered synthase, results in production of PHA polymers with defined repeating unit compositions (Takase et al., 2003; Tappel et al., 2012b).

1.4.2 SCL and MCL biosynthesis pathways

SCL PHA biosynthesis can be accomplished in *E. coli* LSBJ from glycolytically-derived acetyl-CoA via the heterologous expression of three enzymes: a β-ketothiolase (PhaA), an acetoacetyl-CoA reductase (PhaB), and a PHA synthase (PhaC). This process is functionally the same as that described above for native producers (Fig. 1.4).
The biosynthesis of PHA in *E. coli* LSBJ utilizing short-chain-length (SCL) and medium-chain-length (MCL) fatty acids is shown in Fig. 1.5. The absence of *fadB* and *fadJ* in *E. coli* LSBJ in combination with the plasmid-borne recombinant enzymes PhaC1(STQK) and PhaJ4 establishes a linear pathway for the production of PHA polymers from free fatty acids. Extracellular fatty acids are transported across the outer membrane dependent on size; SCL and shorter MCL fatty acids can diffuse across the outer membrane, while longer MCL fatty acids (12 carbons and higher) can be transported by the long-chain fatty acid transporter FadL (Lepore et
al., 2011). Inner membrane transport and activation is accomplished by the SCL-specific Ato system (AtoEAD) or the MCL-specific acyl-CoA synthetase FadD (Kameda and Nunn, 1981; Theodorou et al., 2006). Acyl-CoA substrates are converted into enoyl-CoA by the acyl-CoA dehydrogenase enzyme FadE (Campbell and Cronan, 2002), and are unable to proceed further through β-oxidation due to the absence of FadB and FadJ. The enoyl-CoA pool is then converted to \((R)\)-3-hydroxyacyl-CoA by the \(R\)-specific enoyl-CoA hydratase PhaJ4 (Tsuge et al., 2003), and finally polymerized by the PHA synthase PhaC1(STQK) (Takase et al., 2004, 2003). This system allows for the biosynthesis of PHA polymers with tightly controlled repeating unit composition, as the number of carbons present in the fatty acid substrate is retained as the total number of carbons in each repeating unit. This was previously demonstrated by Tappel et al. with both single fatty acid substrates, resulting in PHA homopolymers with the same number of carbon atoms as the fatty acid precursors, as well as dual fatty acid co-substrates to produce PHA copolymers with controlled composition (2012a, 2012b).
Figure 1.5: Biosynthesis of PHA in E. coli LSBJ utilizing short-chain-length (SCL) and medium-chain-length (MCL) fatty acids.

1.4.3 Transcriptional regulation of MCL PHA biosynthesis pathway

Due to the inability of *E. coli* to natively biosynthesize PHA, regulation of MCL PHA biosynthesis primarily occurs at the level of fatty acid uptake and catabolism. These processes are tightly regulated by a number of transcription factors, which will be described here.
1.4.3.1 Regulation by FadR

One of the primary regulators of fatty acid metabolism is the transcriptional dual regulator FadR, or the fatty acid degradation regulon (Overath et al., 1969). FadR is responsible for repressing genes necessary for fatty acid degradation (β-oxidation) and activating genes necessary for fatty acid biosynthesis to control carbon metabolism in response to exogenous fatty acids. FadR interacts with the upstream region of genes within its regulon by binding to the palindromic consensus binding sequence -TGGNNNNNCCA- (Xu et al., 2001). When this consensus sequence overlaps the -35 or -10 region (and the region immediately upstream of the -10), as is the case for fad genes, FadR binding prevents the recruitment of RNA polymerase (RNAP) and inhibits transcription (Black and DiRusso, 1994; Cronan and Subrahmanyam, 1998; Henry and Cronan, 1992). If the FadR binding site lie upstream of the -35 region, as it does for the fab genes (fatty acid biosynthesis), then the binding of FadR activates transcription by enhancing RNAP recruitment.

Crystal structures have been determined for FadR, the FadR-acyl-CoA complex, and FadR bound to DNA, which have helped to develop a mechanistic understanding of this enzyme (van Aalten et al., 2000; Xu et al., 2001). The FadR regulator functions as a homodimer, and binds to DNA through the interaction of the two DNA-Binding domains of the apoenzyme (Fig. 1.6) (Raman et al., 1997). The DNA-binding domain, which has been determined to be within the first 66 amino acid residues of the N-terminus (Raman et al., 1997), forms a winged helix motif common in prokaryotic transcriptional regulators and is highly conserved in fadR homologs of other prokaryotes (Brennan, 1993; Iram and Cronan, 2005). A large region of the C-terminus forms the dimerization and acyl-CoA binding domains; although the exact residues involved in dimerization are currently unknown, three residues necessary for acyl-CoA binding (G216, S219,
W223) have been identified in *E. coli* MG1655 (DiRusso et al., 1998). This region is much less conserved than the DNA binding domain (~25% homology), and is responsible for significant differences in the preferred fatty acid substrates among bacteria (Iram and Cronan, 2005). In *E. coli* LS5218, the parental strain of LSBJ, a single nucleotide polymorphism (SNP, L55Q) in the DNA binding domain prevents DNA binding and relieves *fad* repression by FadR (Rand et al., 2017). This mutation, also known as *fadR601*, is believed to disrupt a hydrophobic interaction among three neighboring residues based on evidence from other *E. coli* *fadR* mutants (Zhang et al., 2014).

**Figure 1.6** FadR domain map. N-Terminal DNA binding domain contains a SNP in *E. coli* LS5218 and its derivatives that results in a nonfunctioning FadR. This domain is separated from the C-terminal dimerization / acyl-CoA binding domain by a short linker. Acyl-CoA binding requires three residues in *E. coli* MG1655 and LS5218, G216, S219, and W223.

The FadR sensing mechanism hinges on the binding of fatty acyl-CoA molecules to the bound apo-enzyme complex (Xu et al., 2001). The binding of a fatty acyl-CoA triggers conformational changes in the protein which releases DNA from the N-terminal DNA-binding domain (**Fig. 1.7**). This means that the presence of fatty acyl-CoA molecules relieves the repression of β-oxidation genes while simultaneously abolishing FadR-mediated activation of fatty
acid biosynthesis. In *E. coli* MG1655 and many related strains, FadR will only bind long chain-length (LCL) fatty acyl-CoAs, and has the highest affinity towards oleoyl-CoA (Feng and Cronan, 2012; Iram and Cronan, 2006). For this reason, these strains cannot grow on MCL fatty acids such as decanoate due to the inhibition of the β-oxidation pathway. The FadR601 mutation is therefore necessary for the production of MCL PHAs in *E. coli* LSBJ from fatty acids of 6 to 14 carbons.

**Figure 1.7:** Mechanism of transcriptional regulation by FadR and FadR601(L55Q). (A) In the absence of acyl-CoA, FadR binds to the promoter region of fad genes and represses transcription. DNA binding is blocked in the nonfunctioning FadR601. (B) In the presence of acyl-CoA, FadR binds acyl-CoA and releases DNA through due to conformational changes. Acyl-CoA binding is not blocked in FadR601, however it has no effect on transcription. This figure was adapted from Feng and Cronan (2012).

1.4.3.2 *Regulation by ArcA/ArcB*

Another important regulator of fatty acid catabolism is the transcriptional dual regulator ArcA, which forms the central hypothesis in Chapter 2 and will therefore be reviewed in detail. In *E. coli*, ArcA serves as the response regulator of the ArcAB two-component signal transduction
system, and plays a pivotal role in controlling the switch from aerobic to anaerobic metabolic processes in response to oxygen availability (Iuchi and Lin, 1988; Lynch and Lin, 1996).

Due to the large number of genes affected by ArcA, researchers initially had difficulty characterizing the protein, and subsequently there are several synonyms present in the literature. In 1973 and 1976, researchers from Australia published articles in which they identified and named the *seg* gene (*arcA*), so named because a strain harboring a mutant gene prevented segregation and replication of the F-factors (Hathaway and Bergquist, 1973; Jamieson and Bergquist, 1976). In 1978, a separate group found that strains with this particular mutation were resistant to male-specific bacteriophages, and later studies in the early 1980’s revealed that these mutants showed inhibition of F-pilus expression, leading to the classification *msp* and *fex*, respectively (Buxton et al., 1978; Lerner and Zinder, 1982). Within the same time period, several studies explored the effects of this gene using chemical mutagenesis, producing a set of mutants with varying impacts on sex factor regulation, which led to the new synonym *sfrA* (Beutin et al., 1981; Beutin and Achtman, 1979).

The realization that this putative sex factor gene had a larger role in transcriptional regulation did not occur until 1983, when an article was published which made connections between the sex factor regulation studies and a seemingly unrelated paper on the *dye* gene. Mutants lacking this *dye* gene were previously shown in 1979 to have increased sensitivity to the dye toluidine blue and several antibiotics (Roeder and Somerville, 1979). Researchers from the UK (Buxton *et al.*) found that the putative *dye* gene occupied the same chromosomal position as the putative *fex* gene, and through a series of complementation studies determined that *dye*, *fex*, and *sfrA* were all the same gene (Buxton et al., 1983; Buxton and Drury, 1983). They also revealed that the envelope protein profile in Δ*dye* mutants had several differences compared to strains with
functional dye protein product, which was believed to be the cause for increased sensitivity to dyes and antibiotics, and the researchers concluded that the dye gene may be involved in the regulation of envelope proteins in addition to sex factor expression (Buxton et al., 1983). This team of researchers continued their exploration of the now-named dye gene, publishing several more articles in which they characterized the Dye protein, analyzed the DNA and amino acid sequences, and found some similarity to the transcriptional regulator OmpR (Buxton and Drury, 1984; Drury and Buxton, 1985).

The final piece of evidence necessary for discovering the true function of the dye(arcA) gene came from an unrelated study published in 1988. Researchers from Harvard Medical School under Dr. Edmund Lin, while studying the transcriptional regulation of aerobic and anaerobic metabolic pathways, created a mutant E. coli strain that retained very high anaerobic expression of many enzymes normally expressed predominantly during aerobic growth (Iuchi and Lin, 1988). They isolated the gene responsible for this phenotype, which they labelled as arcA (aerobic respiration control), and by mapping the gene to the genome they were able to determine that it occupied the same locus as the previously described dye gene. The group confirmed that the two genes were identical by observing that the arcA mutant was both sensitive to the dye toluidine blue and resistant to the male-specific phage M13 (Iuchi and Lin, 1988). This study became the foundation for the modern understanding of arcA, and its role as a transcriptional dual-regulator.

ArcA belongs to the OmpR family of proteins, which is a typical family of two-component response regulators (Cho et al., 2006; Itou and Tanaka, 2001; West and Stock, 2001). The arcA gene has a nucleotide sequence of 717 bases, encoding a protein of 238 amino acids in length and a molecular weight (Mw) of 27,346 Da, as determined computationally and analytically (Buxton
and Drury, 1984; Drury and Buxton, 1985). The ArcA protein purifies along with other soluble cytosolic proteins (Buxton and Drury, 1984).

The domain structure for ArcA is relatively simple; as in other OmpR-family proteins, the active enzyme forms a homodimer, and the two domains present include a receiver domain and a DNA-binding domain (Fig. 1.8) (Cho et al., 2006; Drury and Buxton, 1985). The crystal structure of ArcA has been solved by X-ray crystallography, enhancing our understanding of these domain interactions (Toro-Roman et al., 2005). The receiver domain, also known as the dimerization domain (D1’), is located from amino acids 6-113 (N-terminal) and serves both as the site of dimerization and as the site where the ArcB histidine kinase interacts with and transphosphorylates ArcA (Drury and Buxton, 1985; Finn et al., 2014; West and Stock, 2001). It is within this domain that the catalytic aspartic acid residue (D54) resides, which activates the enzyme upon its phosphorylation (Drury and Buxton, 1985; Finn et al., 2014). The DNA-binding domain (DB1) is responsible for interacting with the promoter regions of regulons under the control of ArcA, and is located from amino acids 156-232 (C-terminal) (Drury and Buxton, 1985; Finn et al., 2014). The bound ArcA-P acts as a dual regulator, capable of inhibiting or activating various genes (Cho et al., 2006).

**Figure 1.8:** ArcA domain map. N-Terminal dimerization domain contains a catalytic aspartic acid residue (D54), and is separated from the C-terminal DNA-Binding domain by a short linker.
For a complete understanding of the function of ArcA, it is also necessary to discuss the structure of ArcB due to its important role in the activation of ArcA. The sensor protein ArcB is classified as a tripartite hybrid histidine kinase, and contains several important domains; two transmembrane domains (TM1 & 2) separated by a short periplasmic domain (P1), a dimerization domain (D1) which also acts as a linker, and primary and secondary transmitter domains (T1 & 2) separated by a receiver domain (R1), shown below in Fig. 1.9 (Ishige et al., 1994; Iuchi and Lin, 1992; Kwon et al., 2000; Nuñez Oreza et al., 2012). The two transmembrane domains serve to anchor the complete enzyme in the inner membrane, but neither they nor the periplasmic domain play an active role in sensing; instead, the cytosolic portion of the enzyme interacts directly with quinones involved in the electron transport chain (Ishige et al., 1994; Iuchi and Lin, 1992; Kwon et al., 2000; Nuñez Oreza et al., 2012). The dimerization domain has the characteristics of a leucine zipper, and forms a homodimer by a coiled-coil motif (Nuñez Oreza et al., 2012). The two transmitter domains along with the receiver all play a role in the phosphorelay system (Nuñez Oreza et al., 2012; Peña-Sandoval and Georgellis, 2010).

Figure 1.9: ArcB domain map. The N-terminal transmembrane domain consists of two membrane spanning regions (TM1&2) separated by a short periplasmic linker. The dimerization domain (D1) separates the transmembrane domain from the cytosolic phosphorelay domains, which contains the primary and secondary transmitter domains (T1&2) separated by a receiver domain (R1), each with catalytic residues of H292, D576, and H717, respectively.
ArcA is activated by phosphorylation under reducing conditions, or conditions of low oxygen, by a phosphorelay system involving the electron transport chain, ArcB, and an aspartic acid residue of ArcA (Iuchi and Lin, 1992; Peña-Sandoval and Georgellis, 2010). Under reducing conditions, the His292 residue of ArcB (T1, Fig. 1.10A) autophosphorylates through interaction with cytosolic ATP (Iuchi and Lin, 1992; Peña-Sandoval and Georgellis, 2010). Through intramolecular reactions, the phosphoryl group is passed to the Asp576 residue (R1, Fig. 1.10A) and finally to the His717 residue (T2, Fig. 1.10A) located near the C-Terminus (Iuchi and Lin, 1992; Peña-Sandoval and Georgellis, 2010). The phosphoryl group is then transferred to the Asp54 residue in the dimerization domain of ArcA (D1’, Fig. 1.10A), resulting in the activated form of ArcA, ArcA-P (Iuchi and Lin, 1992; Peña-Sandoval and Georgellis, 2010). This activated ArcA-P then proceeds to bind DNA in order to alter gene expression.

Under aerobic conditions, ArcA-P undergoes a reverse phosphorelay and is de-activated. First, quinones involved in electron transport become oxidized, and silence the kinase function of ArcB by forming disulfide bonds in the dimerization domain, preventing the phosphorelay in the activating direction (Georgellis et al., 2001, 1998). The phosphorylated ArcA-P proceeds to transfer its phosphoryl group back to the His717 residue of ArcB (T2, Fig. 1.10B), which then transfers it to the receiver domain (R1, Fig. 1.10B) at Asp576 (Georgellis et al., 2001, 1998). Together, the forward and reverse phosphorelay systems serve to control gene regulation by ArcA in response to the oxygen available in the environment.
Figure 1.10: Simplified, proposed model of the activation of ArcA by ArcB. QH$_2$ and Q refer to the reduced and oxidized forms of quinones, respectively. (A) Autophosphorylation of ArcB (The membrane-bound protein) cleaves a phosphoryl group (yellow “P”) from ATP, then transfers this phosphoryl group along the relay to ArcA (orange), which allows DNA binding through allosteric modification. (B) De-phosphorylation of ArcA occurs in aerobic conditions, where quinone-induced disulfide bonds form between ArcB molecules, preventing the cleavage of ATP and forcing the relay in the opposing direction, preventing DNA binding (Georgellis et al., 2001, 1998; Malpica et al., 2004; Nuñez Oreza et al., 2012; Peña-Sandoval and Georgellis, 2010).

As a transcriptional dual regulator, ArcA has the capacity to both repress and activate certain genetic elements. In order to determine which genes ArcA was most likely to regulate, it was necessary for researchers to identify a consensus binding sequence; an extensive 1996 article written by Lynch and Lin used a series of electrophoretic mobility shift assays and restriction enzyme digests to hone in on where ArcA was binding in relation to several model genes, and to
determine the consensus binding sequence (Lynch and Lin, 1996). They characterized a putative consensus binding site with the sequence [5’ (A/T)GTTAATTA(A/T) 3’] and used this information to verify that genes known to be regulated by ArcA contained sequences with high similarity to this consensus (Lynch and Lin, 1996).

Once a putative sequence was determined, computational analysis revealed that several genes contained two or three potential ArcA binding sites rather than just one (Lynch and Lin, 1996). These sequences generally appear upstream of the transcription start site in the promoter region, though they can also appear downstream or overlapping the transcription start site as well (Lynch and Lin, 1996). Since this initial experiment, more in-depth computational analyses have determined that there are as many as 150 potential operons under the direct influence of ArcA, and a revised consensus sequence of [5’ GTTAATTAAATGTT 3’] was proposed and is used as the current consensus for genetic analysis (Keseler et al., 2013; Liu and Wulf, 2004).

Due to the high number of genes and operons affected by ArcA, the overall transcriptional effects are generally reported as large families involved in certain metabolic functions. In anaerobic conditions, when ArcA is phosphorylated and active, several metabolic pathways required for efficient energy utilization in aerobic conditions are repressed (Jiang et al., 2015; Salmon et al., 2005). These pathways include; the tricarboxylic acid cycle, glycolysis, amino acid transport and catabolism, fatty acid transport and degradation, and quinone oxidation, among many others (Salmon et al., 2005). There are also several metabolic functions that are activated by ArcA under anaerobic conditions, which mainly involved small molecule transport and metabolism, nitrate reduction, and DNA repair (Salmon et al., 2005).

The implications of these changes in cellular metabolism is that cells are able to utilize various energy sources more efficiently in response to oxygen availability. Pathways involving
oxygen utilization become down-regulated as the availability of oxygen decreases, and concurrently the expression of pathways needed for alternative electron transport (such as nitrate) and fermentation become up-regulated (Salmon et al., 2005).

Among the many genes regulated by ArcA, there are several transcriptional regulators; this greatly increases the complexity of ArcA regulation through the addition of indirect effects (Keseler et al., 2013; Salmon et al., 2005). Perhaps the most interesting of these indirect effects is the suppression of the sigma factor RpoS through the down-regulation of the small regulatory RNA, arcZ (Mandin and Gottesman, 2010; Soper et al., 2010). The small regulatory RNA arcZ acts as a translational activator of rpoS, binding to the rpoS mRNA and recruiting the Hfq chaperone protein, which stabilizes the molecular construct in an “active” conformation where translation can proceed (Soper et al., 2010).

Under anaerobic conditions, when ArcA is active, the expression of arcZ is highly down-regulated, which in turn inhibits the translation of rpoS (Mandin and Gottesman, 2010; Soper et al., 2010). In addition, activated ArcA-P binds to the promoter region of rpoS and inhibits transcription, which further down-regulates rpoS expression (Liu and Wulf, 2004; Mandin and Gottesman, 2010). Conversely, under aerobic conditions rpoS expression is up-regulated both by the removal of ArcA-P inhibition and by the translational activation of arcZ (Liu and Wulf, 2004; Mandin and Gottesman, 2010; Soper et al., 2010). However, determining the whole transcriptome effects of this indirect regulation is difficult to discern.

RpoS, or sigma-factor S (σS), is involved in the transcription of a diverse group of genes making up approximately 10% of the E. coli genome (Franchini et al., 2015; Patten et al., 2004; Rahman et al., 2006). Many of these genes are involved in stress response, and there is a large amount of regulatory overlap between σS and σ70, which have similar consensus sequences, and
other stress-response sigma factors \((\sigma^{54} \text{ and } \sigma^{28})\) (Dong et al., 2011; Franchini et al., 2015; Patten et al., 2004; Rahman et al., 2006). This complex regulatory network makes it difficult to definitively attribute transcriptional effects to \(\sigma^5\). However, studies have found that RpoS (\(\sigma^5\)) is involved in functions such as stress response, secondary metabolism, nucleic acid synthesis, as well as sugar and polyamine metabolism in response to stress (Keseler et al., 2013; Maciag et al., 2011). The control of RpoS by ArcA increases the widespread transcriptional effects of the ArcAB two-component signal transduction pathway, and likely assists in the metabolic shift that occurs between anaerobic and aerobic conditions. **Fig. 1.11** below displays a summary of the ArcA regulon, which includes aerobic respiration, the tricarboxylic acid cycle, glycolysis, amino acid transport and catabolism, fatty acid transport and degradation, RpoS expression, and quinone oxidation.
Figure 1.11: Summary of ArcA transcriptional regulation. Transphosphorylation of ArcA by ArcB leads to the up-regulation of small molecule metabolism and nitrate reduction, the down regulation of aerobic respiration, fatty acid degradation, amino acid catabolism, and RpoS expression, among other pathways (Jiang et al., 2015; Liu and Wulf, 2004; Mandin and Gottesman, 2010; Salmon et al., 2005; Soper et al., 2010). Green arrows refer to activation, and red arrows refer to inhibition.

1.4.3.3 Regulation by CRP-cAMP

The cyclic AMP receptor protein (CRP) – cyclic AMP complex (CRP-cAMP) is another important regulator of fatty acid metabolism in E. coli, responsible for activating transcription of the fad regulon in the absence of a preferred carbon source, glucose (Fic et al., 2009). This is part of larger process known as carbon catabolite repression, which is mediated by a protein phosphorylation cascade via components of the phosphoenolpyruvate (PEP):carbohydrate phosphotransferase system, or PTS (Deutscher, 2008; Franchini et al., 2015). Glucose is transported into the cell by the glucose-specific membrane permease EIIC\textsuperscript{Glc}, concomitant with its
phosphorylation by the EIIB subunit to form glucose-6-phosphate (Kundig et al., 1964). Transphosphorylation of EIIB is catalyzed by EIIA to ensure continued glucose transport, which results in an elevated pool of the dephosphorylated EIIA. This in turn reduces the activity of adenylate cyclase (CyaA), which requires phosphorylation to catalyze the synthesis of cAMP. A reduction in available cAMP in reduces the concentration of CRP-cAMP, ultimately down-regulating fad regulon expression. Conversely, when glucose in unavailable the pool of CRP-cAMP in the cytosol is high, activating the transcription of both fadD and fadE (Feng and Cronan, 2012). This has implications for the biosynthesis of MCL PHAs in E. coli LSBJ.

1.4.3.4 Regulation by OmpR/EnvZ

One member of the fad regulon, the LCL fatty acid transporter FadL, is additionally regulated by the OmpR/EnvZ two component response regulator (Aiba and Mizuno, 1990; Forst and Roberts, 1994). Although this regulatory system was implicated in the hypotheses in Chapter 2, it was found to not play a role in MCL PHA biosynthesis in E. coli LSBJ and will therefore only be reviewed briefly.

The OmpR/EnvZ regulatory system is primarily responsible for controlling the expression of outer membrane porins in response to osmolarity, with OmpR serving as the response regulator for this system (Mizuno and Mizushima, 1990). OmpR is a well-characterized transcriptional regulator in E. coli, belonging to a large family of transcription factors with a winged helix DNA binding motif (the OmpR family) (Kato et al., 1995). The outer membrane porins OmpF and OmpC, both of which form ion-permeable channels and aid in the diffusion of small hydrophilic molecules across the inner membrane, are the primary targets of OmpR regulation (Foo et al., 2015). Although both porins allow transport of similar molecules, OmpF is characterized by a faster flow rate and is its expression is activated in conditions of low osmolarity. OmpC plays an
opposing role; in conditions of high osmolarity \textit{ompC} expression is activated, and slower flow rates through OmpC help maintain ion homeostasis (Nikaido, 1992; Silhavy and Pratt, 1995).

Regulation by OmpR is controlled by the sensor kinase EnvZ, which autophosphorylates in response to extracellular and intracellular osmolarity and transphosphorylates OmpR, which proceeds to bind to the upstream promoter region of target genes (Forst and Roberts, 1994; Matsubara and Mizuno, 1999; Wang et al., 2012). Although \textit{ompF} and \textit{ompC} are the primary targets for OmpR binding, it has also been shown to modulate the expression of amino acid metabolism, flagellar biosynthesis, and LCL fatty acid transport via the outer membrane porin FadL (Higashitani et al., 1993; Oshima et al., 2002). Although FadL is sometimes implicated in MCL PHA biosynthesis, it has been shown to exhibit no activity on fatty acids of 12 carbons or less (Black, 1990).

\textbf{1.5 Recent developments in the biosynthesis of chemically modifiable PHAs}

Significant research effort has centered on expanding the capability of PHA-producing bacteria to incorporate, in a controlled fashion, MCL/SCL and unnatural monomers into copolymers by engineering biosynthetic pathways and expanding the substrate specificities of their enzymatic constituents. There have been several successful examples of this approach, including chimeric PHA synthases with broad substrate specificity (Matsumoto et al., 2009), the development of controllable production platforms such as \textit{E. coli} LSBJ (Tappel et al., 2012b), and engineered recombinant pathways capable of synthesizing unique copolymers (Li et al., 2017, 2016). These research efforts have yielded slow-yet-steady gains in the production of uncommon PHA copolymers from a variety of related and unrelated feedstocks.

An underdeveloped area of PHA research, however, has been that of finding solutions to the limited chemical tractability of PHA side chains. Thus far this challenge has been circumvented.
by applying multistep synthetic routes post-polymerization to adapt them to specific applications and designs. Besides some excellent examples from a few laboratories, meaningful efforts to augment the chemical tractability of PHAs via incorporation of unnatural or uncommon monomers have been sporadic and inadequate. Similarly, organisms such as *Pseudomonas oleovorans* have been shown to produce exotic and uncommon PHAs biosynthetically, however there has been little progress towards scaling up their production (Lenz et al., 1992).

A great variety of uncommon functional groups have been observed in PHAs, including; terminal alkenes and alkynes (Lageveen et al., 1988; Levine et al., 2015; Tappel et al., 2012b), halogens (Doi and Abe, 1990; Kim et al., 1996; Lenz et al., 1992), esters (Scholz et al., 1994), epoxides (Bear et al., 1997), aromatic rings (Aróstegui et al., 1999; Kim et al., 1991; Takagi et al., 1999), and nitrile groups (Lenz et al., 1992). Although these are fascinating examples of the diversity of PHAs, several more recent advances in functionalization have great potential for commercial significance and more advances applications.

1.5.1 Fluorination of the PHA α-carbon

Fluoropolymers are incredibly useful materials that have transformed the fields of polymer chemistry and materials science, with the classic example being that of poly(tetrafluoroethylene) (PTFE) which has extraordinary physical properties (Plunkett, 1941). These properties arise from the C-F bond, which is the strongest single bond to carbon that can be made, and the unique electronic interactions that result from the high electronegativity of fluorine (O’Hagan, 2008). Organofluorine compounds have also been used extensively in the pharmaceutical industry, where substitutions with fluorine often dramatically alter enzymatic interactions (Kirsch, 2005; Purser et al., 2008). This utility is enhanced by the dearth of organofluorine compounds found in nature; very few metabolic pathways exist that can manipulate C-F bonds, and there are a limited number
of biologically produced organofluorine structures (Dong et al., 2004; Leong et al., 2017; Twigg and Socha, 2001; Walker and Bong Chui Lien, 1981).

Recently, a biosynthetic approach for producing fluorinated PHAs was reported by Thuronyi et al. (2017). This novel recombinant pathway has been included here as an excellent example of the chemical modification of existing PHAs through non-synthetic means (Fig. 1.12), and because there is increasing interest in developing chemistries for modification of the notoriously unreactive C-F bond.
Figure 1.12: Recombinant pathway for 2-fluoro-PHA synthesis in Escherichia coli. Using biologically available fluoromalonate as a feedstock, poly[2-fluoro-(R)-3-hydroxybutyrate] can be synthesized as a copolymer with (R)-3-hydroxybutyrate comonomers (Thuronyi et al., 2017).
Exogenous fluoromalonate is imported into the cell by one of several potential transporter systems; Thuronyi et al. found that the most effective system was the two-component Na⁺:malonate symporter MadLM isolated from *Pseudomonas fluorescens* (Schaffitzel et al., 1998; Thuronyi et al., 2017). The intracellular fluoromalonate is then converted to fluoromalonyl coenzyme A (fluoromalonyl-CoA) in a two-step reaction by the malonate-CoA ligase MatB isolated from *Rhodopseudomonas palustris* (Crosby et al., 2012). The unique acetoacetyl-CoA synthase NphT7 isolated from *Streptomyces sp.* CL190 catalyzes the condensation of one molecule of acetyl-CoA with one molecule of fluoromalonyl-CoA to yield one molecule of acetofluoroacetyl-CoA concomitant with the release of CO₂ and free coenzyme A (Okamura et al., 2010; Walker et al., 2013). PhaB, an acetoacetyl-CoA reductase isolated from *Ralstonia eutropha* (Peoples and Sinskey, 1989a), catalyzes the chemoselective reduction of the beta keto group of acetofluoroacetyl-CoA to yield 2-fluoro-(R)-3-hydroxybutyryl-CoA. This substrate, along with the non-fluorinated analog (R)-3-hydroxybutyrate which occurs due to the presence of the key fatty acid biosynthesis metabolite malonyl-CoA, is finally polymerized by the PHA synthase PhaC isolated from *Ralstonia eutropha* (Peoples and Sinskey, 1989b) to generate the copolymer poly[2-fluoro-(R)-3-hydroxybutyrate-co-(R)-3-hydroxybutyrate]. This is the first described biosynthesis of a fluoro-PHA, and represents a novel approach to diversifying the portfolio of chemically modified PHAs by the manipulation of the α-carbon.

With the increasing number of synthetic organofluorine compounds, driven in large part by the development of pharmaceuticals, there has been increasing interest in the activation and scission of C-F bonds for further modification. This is a challenge however, given the strength of the C-F bond and its unsuitability as a leaving group (O’Hagan, 2008), which necessitates the use
of harsh reaction conditions such as strong nucleophiles (e.g. organolithium reagents) and high temperatures (Fuchibe et al., 2009; Perutz and Braun, 2007; Pigeon et al., 2010). More recently, mild reaction conditions have been found that exploit the fluorophilicity of silicon to cleave non-activated, aliphatic C-F bonds (Tanaka et al., 2016).

Defluorosilylation of alkyl fluorides with relatively mild conditions has been recently achieved independently by several groups (Cui et al., 2018; Liu et al., 2019; Mallick et al., 2019). Two examples of these defluorosilylation reactions with alkyl fluorides are shown in Fig. 1.13. It is possible that these reaction conditions could be applied to poly[2-fluoro-(R)-3-hydroxybutyrate] in which the fluorine is in an activated position on the α-carbon, allowing further modification of the organosilicon moiety (Komiyama et al., 2017). However, as the ester linkage in PHAs exhibits poor acid/base resistance, more research is needed to determine whether these new methods employed for defluorosilylation are capable of cleaving the C-F bond without destruction of the polymer. Chemical modification of the PHA α-carbon, if possible, would offer an interesting
alternative to the current portfolio of functionalizations, most of which utilize reactive groups on the branch chain.

**Figure 1.13:** Recent methods for base-mediated defluorosilylation. (A) Two examples of independently-devised methods for defluorosilylation of alkyl fluorides (Cui et al., 2018; Liu et al., 2019). (B) It may be possible to adapt these methods for the modification of 2-fluoro-PHA.

An important aspect to consider in the development of fluoropolymers is their environmental impact. Perfluoropolymers such as poly(tetrafluoroethylene) are persistent in the environment, owing to their high stability and chemical inertness. However, many of these materials have been found to be nontoxic and meet the criteria for “polymers of low concern,” which has been extensively reviewed by Henry et al. (2018). The primary concern of these materials, with respect to environmental toxicology, is the toxicity of perfluoroalkyl acids (PFAAs) which are used as surfactants for fluoropolymer synthesis, or in the case of side-chain fluorinated polymers can be generated by polymer degradation (Liu and Mejia Avendaño, 2013). PFAAs are regarded as persistent bioaccumulative toxins and have been shown to be carcinogenic (Takacs and Abbott, 2007), hepatotoxic (Seacat et al., 2002), and teratogenic (Wolf et al., 2007).
in a variety of mammalian studies. These compounds have also been implicated in thyroid disease in humans (Melzer David et al., 2010; Winquist and Steenland, 2014), and these combined toxicological effects make it imperative to prevent environmental pollution with PFAAs and potential fluoropolymer precursors. In the case of α-fluorinated PHAs, there are documented pathways of microbial defluorination that may be capable of maintaining the biodegradability typical of PHA polymers, however the toxic potential of 2-fluorobutyrate and its degradation products must still be investigated (Leong et al., 2017; Walker and Bong Chui Lien, 1981). For these reasons, we should exercise caution in the development of fluoro-PHAs.

1.5.2 Development and biosynthesis of azido-PHAs

Click reactions comprise a group of synthetic, chemoselective strategies that have no side products, occur under mild reaction conditions, and proceed rapidly with high yields (Kolb et al., 2001). Considered the paradigm among click reactions is the so-called copper alkyne-azide cycloaddition reaction (CuAAC), which reliably yields 1,2,3-triazole moieties under mild conditions (Fig. 1.14) (Rostovtsev et al., 2002). The reaction involves a thermodynamically-favored non-concerted cycloaddition featuring Cu(I) as the reactive coordination center by which alkynes and organoazides rearrange to regioselectively form triazole heterocycles. The extraordinary chemoselectivity of the CuAAC and its recognized value as a model reaction in pharmaceutical applications has led to an expanded availability of commercial sources of azido- or alkynyl-conjugated biomolecules. More recently, strain-promoted variants of the alkyne-azide cycloaddition (SPAAC) were developed, with substituted cyclooctynes as their centerpiece, born out of the desire to employ these popular reactions in organisms, avoid the use of deleterious copper, and preserve the reaction’s kinetic parameters in vivo (Agard et al., 2004; Baskin et al., 2007). Progress in the budding field of orthogonal and bioorthogonal chemical transformations
has been rapidly developing and has produced examples which include, but are not limited to; UV-promoted thiol-ene click reactions between thiols and alkenes/alkynes (Dondoni, 2008; Hoyle and Bowman, 2010), (4+2) cycloadditions between dienes and dienophiles such as normal and reverse-electron-demand Diels-Alder reactions (Pipkorn et al., 2009; Shi and Shoichet, 2008), and the Staudinger ligation between organoazides and organophosphorus reagents (Saxon and Bertozzi, 2000; Schilling et al., 2011).
Figure 1.14: Examples of how click reactions can be used to expand functionality of PHA polymers. In addition to the copper catalyzed azide-alkyne cycloaddition reaction, thiol-ene click reactions and copper-free cycloadditions with strained alkynes are also possible.

In view of the need to develop simple and efficient solutions to the chemical modification of PHAs, Nomura et al. led an effort to produce PHA\textsubscript{MCL} polymers containing sidechains with reactive organoazide functional groups (Pinto et al., 2016). This modification would allow researchers to bridge the latent reactivity of these polymers with a vast library of click-chemistry
based reagents, commercial and otherwise, and offers the potential to expand the reach of PHA applications into high-value areas such as biomedicine, advanced materials, and others.

The implementation of a bacterial strain like *E. coli* LSBJ, able to uptake and redirect fatty acid feedstocks towards the production of PHA as described previously in this chapter, inspired the development of a short synthetic route to produce chemically tractable azidofatty acids. For this purpose, 8 and 12-carbon α,ω-diols were converted in three steps to the corresponding ω-azidofatty acids in approximately 35% overall yields (Pinto et al., 2016). In addition to the ω-azidofatty acids, 10-azidodecanoic acid was synthesized from commercially available 10-bromodecanoic acid in a single step and with 87% yield. The metabolic incorporation of the synthetic azidofatty acids into PHA copolymers was assessed by fermentation studies employing *E. coli* LSBJ and octanoic acid as a primary monomer. *E. coli* LSBJ was able to uptake all three azidofatty acids, leading to the production of copolymers of variable % azide compositions. Of the materials obtained, poly-[(R)-3-hydroxyoctanoate-co-10-azido-(R)-3-hydroxydecanoate] was employed by the researchers in SPAAC conjugation experiments with the commercially-available cyclooctyne (1R,8S,9s)-bicyclo[6.1.0]non-4-yn-9-ylmethanol.

Nomura et al.’s exploratory study provided a clear answer about the feasibility of introducing organoazides into PHA copolymers and using strain-promoted click chemistry conditions to modify them. More recently, Scholz et al. expanded on this work by providing examples of how copper catalyzed click chemistry conditions could be applied to the modification of PHAs (Nkrumah-Agyeefi and Scholz, 2017). Their study centered on the evaluation of two distinct yet functionally complementary copolymers with nonanoate as the primary monomer: one displaying an azide, and another an alkyne functional group (Fig. 1.15). Optimized production of the alkyne-containing polyester was performed by fermentation with *P. oleovorans* ATCC 29347.
in the presence of a 1:1 molar ratio of sodium nonanoate and 10-undecynoic acid (a 40 mM total concentration), and 10 mM sodium acetate over 20h. As opposed to employing synthetic azidofatty acid feedstocks to fermentatively incorporate into PHA, Scholz et al reported an alternative approach that involved the production of a brominated PHA intermediate to be subsequently converted to AzidoPHA by nucleophilic substitution with NaN₃. Incorporation of brominated fatty acids into PHA was confirmed by ¹H NMR as represented by the methylene signal at 3.41 ppm (CH₂Br). The brominated side chains were converted by substitution to organoazides by reaction with sodium azide in DMF and their presence confirmed by their characteristic FTIR stretch at 2093 cm⁻¹.

![Figure 1.15: Examples of alkynyl-PHA and bromo-PHA biosynthesis by Pseudomonas oleovorans.](image)

The azido-PHA copolymers obtained were modified by copper-catalyzed click chemistry. The authors emphasized the need to test methodologies for this step as they found difficulties when attempting to use a single copper-catalyzed method across polymer systems. The alkynyl-PHA copolymer was conjugated with methyl-2-azidoacetate in the presence of CuBr(PPh₃)₃, a copper catalyst soluble in tetrahydrofuran (THF) (Malkoch et al., 2005). The reaction progress was monitored by the disappearance of the stretch at 3292 cm⁻¹, representative of terminal alkynes in
FTIR spectroscopy. Formation of the triazole adduct was confirmed by $^1$H NMR spectroscopy. An analogous method was employed in the click chemistry reaction of azido-PHA copolymer with propargyl benzoate. The key difference was the use of the more common click chemistry conditions of CuSO$_4$ 5H$_2$O as catalyst in an aqueous/THF solvent mixture containing sodium ascorbate. Under similar reaction conditions the conjugation of azido-PHA with propargyl acetate failed to yield product.

1.5.3 Biosynthesis and modifications of unsaturated PHAs

One type of chemically modifiable monomer unit that has been incorporated into PHAs are $\omega$-unsaturated fatty-acids of varying chain lengths (e.g. 10-undecenoic acid and 7-octenoic acid). The terminal alkenes featured in these polymers have become an attractive chemical handle for their modification post polymerization, mainly by oxidative cleavage reactions. These strategies have been the subject of much scrutiny and have been featured and well described elsewhere (Kai and Loh, 2014).

This notwithstanding, a few salient examples have surfaced in recent years such as that by Scholz et al. which showed that PHAs containing 10-undecenoate repeating units can be modified to produce ionizable and hydrophilic PHAs (Sparks and Scholz, 2008). In their study, the terminal alkene moieties in poly-[($R$)-3-hydroxyoctanoate-$co$-($R$)-3-hydroxyundecenoate] were epoxidized by treatment with 3-chloroperbenzoic acid and ring-opened by nucleophilic reaction with diethanolamine. The resulting ionizable PHA polymer was characterized by 1D and 2D NMR spectroscopy and solubility tests confirmed its miscibility in water. The polycationic character of the modified PHA polymer served as the basis for investigating its value as a plasmid DNA delivery system. The authors demonstrated the polymer’s ability to tightly bind and protect plasmid DNA from endonuclease degradation (Sparks and Scholz, 2009).
Other novel approaches for the modification and application of unsaturated PHA polymers have been performed by employing thiol-ene click chemistry. This type of click reaction involves the radical-driven covalent coupling of alkene and thiol functional groups in the presence of ultraviolet light (UV) and in the presence of a photoinitiator (Kade et al., 2010). This reaction has been successfully demonstrated by Levine et al. using poly(3-hydroxybutyrate-co-3-hydroxy-10-undecenoate) (PHBUe) copolymers biosynthesized by *E. coli* LSBJ (2015). Copolymers were cross-linked using pentaerythritol tetrakis(3-mercaptopropionate) (PETMP) and the radical photoinitiator 2,2-dimethoxy-2-phenylacetophenone (DMPA) (Fig. 1.16), resulting in a 200% increase in tensile strength. Cytotoxicity assays conducted with human mesenchymal stem cells (hMSC) showed that cell viability was unaffected by the cross-linking, with hMSC-seeded PHBUe and PHBUx scaffolds exhibiting no difference between each other or a positive control. This represents an exciting new PHA material with potential use in tissue engineering.

**Figure 1.16:** Cross-linked poly(3-hydroxybutyrate-co-3-hydroxy-10-undecenoate) (PHBUe) produced via thiol-ene click chemistry.

The potential use of PHAs in biomedical applications such as drug delivery and tissue engineering has generally been limited by the methodologies available to chemically modify and
fine-tune their properties (Hazer et al., 2012; Poletto et al., 2008). Recent advances in the biosynthesis of azide-, fluorine-, and alkyne-containing PHAs are an excellent first step towards the production of useful chemically modifiable materials.

1.6 Objectives of this dissertation

A number of PHAs have been studied in great detail as biodegradable alternatives to conventional plastics, particularly SCL PHAs such as PHB and PHBV or the SCL-co-MCL polymer PHBHHx, which can be more readily produced at an industrial scale (Chanprateep, 2010; Noda et al., 2010). MCL PHAs and diverse SCL-co-MCL PHAs offer exciting new material properties and chemical handles for functionalization; however, controlled biosynthesis of these polymers has only recently been achieved and is hindered by low yields and prohibitive production costs (Chen and Albertsson, 2019; Tappel et al., 2012b).

MCL PHA biosynthesis in recombinant E. coli LSBJ most commonly occurs via β-oxidation intermediates, and can produce a variety of MCL homopolymers and copolymers with controlled compositions. This process is limited in its production capacity due to the poor growth of E. coli on fatty acid substrates and the limiting effect of catabolite repression when supplemented with a preferred carbon source such as glucose (Deutscher, 2008; Feng and Cronan, 2012; Nunn, 1986). The first objective of this dissertation is to evaluate the role of transcriptional regulators of fatty acid catabolism in the PHA biosynthesis pathway of E. coli LSBJ, with a focus on enhancing MCL PHA yields (Chapter 2). The hypothesis presented in this chapter is that the deletion of the negative transcriptional regulators arcA and ompR from E. coli LSBJ will increase expression of the fad genes necessary for PHA biosynthesis from fatty and improve polymer yields.
In a separate effort to address the prohibitive cost of PHA biosynthesis, the second objective of this dissertation is to explore alternative lignocellulosic feedstocks as cheaper alternatives to pure sugars (Chapter 3). Using a combination of the *de novo* biosynthesis pathway (Fig. 1.4) from acetyl-CoA and PhaG-mediated MCL monomer incorporation, both PHB and PHB-*co*-MCL PHAs were successfully produced from hydrolyzed paper mill waste pulp.

Biosynthesis of many desired chemicals and materials can be achieved in high yields using fed-batch, high-density fermentation processes in a scalable vessel such as a stirred tank reactor (Lau et al., 2004; Lee, 1996; Riesenberg and Guthke, 1999). These techniques have been applied to PHA biosynthesis as well, resulting in significant improvements to productivity primarily for SCL polymers (Amini et al., 2020; Blunt et al., 2018; Koller, 2018; Lee et al., 1999). However, there have been comparatively few studies to investigate the production of MCL PHAs using high-density fermentation, and fewer still that have maintained control over monomer composition (Follonier et al., 2015; Gao et al., 2018; Gopi et al., 2018; Jiang et al., 2013). The work presented in Chapter 4 describes a process optimization for the biosynthesis of MCL PHAs with controlled composition via high-density fed-batch fermentation with *E. coli* LSBJ. The objective of this work was to significantly improve MCL PHA yields from those observed in previous studies that relied on unscalable shake flask culture techniques, including the biosynthesis of functional azido PHA polymers.

Chapter 5 expands on the novel azido PHAs produced in Chapter 4 and in previous work by Pinto *et al.* to develop functionalized polymeric nanoparticles for targeted drug delivery (2016). This chapter presents promising preliminary data for the production of endotoxin-free nanoparticles capable of encapsulating the chemotherapeutic drug paclitaxel at a high efficiency.


1.7 References


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Chapter 2: Enhancing poly(3-hydroxyalkanoate) production in *Escherichia coli* by the removal of the regulatory gene *arcA*

The work presented here was published in AMB Express (2016). Please see Appendix G for publication cover. My contributions include the gene deletion experiments, the growth and harvest of all bacterial cultures, the GC preparation and analysis, the polymer extraction and purification, the compositional verification using H-NMR, the molecular weight analysis using GPC, the gene expression analysis, and the drafting of the manuscript in its entirety.

2.1 Abstract

Recombinant *Escherichia coli* is a desirable platform for the production of many biological compounds including poly(3-hydroxyalkanoates), a class of naturally occurring biodegradable polyesters with promising biomedical and material applications. Although the controlled production of desirable polymers is possible with the utilization of fatty acid feedstocks, a central challenge to this biosynthetic route is the improvement of the relatively low polymer yield, a necessary factor of decreasing the production costs. In this study we sought to address this challenge by deleting *arcA* and *ompR*, two global regulators with the capacity to inhibit the uptake and activation of exogenous fatty acids. We found that polymer yields in a Δ*arcA* mutant increased significantly with respect to the parental strain. In the parental strain, PHV yields were very low but improved 64-fold in the Δ*arcA* mutant (1.92 mg L⁻¹ to 124 mg L⁻¹). The Δ*arcA* mutant also allowed for modest increases in some medium chain length polymer yields, while weight average molecular weights improved by approximately 1.5-fold to 12-fold depending on the fatty acid substrate utilized. These results were supported by an analysis of differential gene expression, which showed that the key genes (*fadD, fadL, and fadE*) encoding fatty acid degradation enzymes were all upregulated by 2-, 10-, and 31-fold in an Δ*arcA* mutant, respectively. Additionally, the
short chain length fatty acid uptake genes \textit{atoA}, \textit{atoE} and \textit{atoD} were upregulated by 103-, 119-, and 303-fold respectively, though these values are somewhat inflated due to low expression in the parental strain. Overall, this study demonstrates that \textit{arcA} is an important target to improve PHA production from fatty acids.

\textbf{2.2 Introduction}

Poly(3-hydroxyalkanoates), or PHAs, are a group of biodegradable polyesters produced by a variety of microorganisms as a form of carbon storage (Lu et al. 2009). These PHAs are typically classified as short chain-length (SCL) PHAs, which contain repeating units of 3 to 5 carbons, and medium chain-length (MCL) PHAs containing 6 to 14 carbons. The physical properties of PHAs are dependent on monomer composition; SCL PHAs are generally stiff and brittle while MCL PHAs are more elastomeric, and co-polymerization of the two groups allows for great variability in material properties (Laycock et al. 2013). Previous studies have shown that MCL PHAs can be effectively produced in recombinant \textit{E. coli} lacking the fatty acid degradation gene \textit{fadB} when utilizing a related carbon source such as fatty acids, although the monomer composition of the resulting polymers was heterogenous and uncontrolled (Langenbach et al. 1997; Qi et al. 1997).

Recently, it was shown that the monomer identity can be precisely controlled in both PHA homo- and co-polymers synthesized by recombinant \textit{Escherichia coli} strain LSBJ (Tappel et al. 2012a; Tappel et al. 2012b). This was accomplished by deleting both the \textit{fadB} and \textit{fadI} genes in \textit{E. coli} LS5218, recombinantly co-expressing the \textit{phaJ4} gene from \textit{Pseudomonas putida} KT2440 with the highly active and broad substrate utilizing \textit{phaC1(STQK)} genes, and feeding in specific ratios of fatty acids for conversion to PHAs (Fig. 2.1). This system allowed for strict control of repeating unit composition which enables great control over the physical properties of PHA polymers produced using this system, unlocking the potential for tailoring PHA materials for click-
chemistry modifications (Levine et al. 2015; Levine et al. 2016; Pinto et al. 2016). Although these previous studies addressed control of the monomer composition and thus physical and chemical properties of PHA polymers, overall polymer yields were still relatively low and some fatty acid substrates had poor incorporation into either PHA homo- or copolymers. The previously defined system relied heavily on the deletion of the fadR gene in E. coli LS5218 for constitutive expression of the genes encoding enzymes from the β-oxidation pathway (Spratt et al. 1981). In a previous study, researchers demonstrated that the inhibition of β-oxidation intermediates using acrylic acid was an effective strategy for improving PHA biosynthesis, particularly in combination with a fadR deletion (Qi et al. 1998). In addition to FadR, there are three other transcriptional regulators, ArcA, OmpR, and CRP-cAMP, that are known to inhibit the expression of genes involved in β-oxidation. For this study, we focused on the regulators ArcA and OmpR because CRP-cAMP is known to act as a transcriptional activator of β-oxidation in the absence of glucose, and only exhibits repression when glucose is present (Fic et al. 2009). We hypothesized that removal of transcriptional regulators that inhibit expression of β-oxidation related genes would result in improved flux through fatty acid catabolic pathways to increase PHA polymer yields in our engineered system. Therefore, in this study E. coli LSBJ was engineered to improve the biosynthesis of PHA from fatty acid substrates by removing the global regulatory genes arcA and ompR.
Figure 2.1: Biosynthesis of PHA in *E. coli* LSBJ utilizing short-chain-length (SCL) and medium-chain-length (MCL) fatty acids. The absence of *fadB* and *fadJ* in *E. coli* LSBJ in combination with the plasmid-borne recombinant enzymes PhaC1(STQK) and PhaJ4 establishes a linear pathway for the production of PHA polymers from free fatty acids. Extracellular fatty acids are transported across the outer membrane dependent on size; SCL and shorter MCL fatty acids can diffuse across the outer membrane, while longer MCL fatty acids can be transported by the long-chain fatty acid transporter FadL (Lepore et al. 2011). Inner membrane transport and activation is accomplished by the SCL-specific Ato system (AtoEAD) or the MCL-specific acyl-CoA synthetase FadD (Kameda and Nunn 1981; Theodorou et al. 2006). Acyl-CoA substrates are converted into enoyl-CoA by the acyl-CoA dehydrogenase enzyme FadE (Campbell and Cronan 2002), and are unable to proceed further through β-oxidation due to the absence of FadB and FadJ. The enoyl-CoA pool is then converted to (R)-3-hydroxyacyl-CoA by the R-specific enoyl-CoA hydratase PhaJ4 (Tsuge et al. 2003), and finally polymerized by the PHA synthase PhaC1(STQK) (Takase et al. 2003; Takase et al. 2004). This system allows for the biosynthesis of PHA polymers with tightly controlled repeating unit composition, as the number of carbons present in the fatty acid substrate is retained as the total number of carbons in each repeating unit.
The transcriptional regulator OmpR functions as a response regulator of the two component regulatory EnvZ/OmpR system, which exhibits control over the expression of outer membrane porins in response to osmolarity (Mizuno and Mizushima 1990). The sensor kinase EnvZ autophosphorylates in response to extracellular osmolarity and transphosphorylates OmpR (OmpR-P), which binds to DNA and alters expression of genes within the OmpR regulon (Forst and Roberts 1994; Matsubara and Mizuno 1999). The most well-studied members of the OmpR regulon are OmpF and OmpC, outer membrane porins that control the diffusion of small hydrophilic molecules (Aiba and Mizuno 1990; Mizuno and Mizushima 1990; Silhavy and Pratt 1995). However, OmpR controls numerous other transporter genes including fadL (Table 2.1), as well as genes involved in amino acid metabolism and flagellar biosynthesis (Higashitani et al. 1993; Oshima et al. 2002).

The global transcriptional dual regulator ArcA functions as the response regulator of the two-component regulatory ArcAB system, which regulates the expression of genes involved in aerobic and anaerobic metabolism in response to oxygen availability (Iuchi and Lin 1988; Lynch and Lin 1996). During conditions of decreasing oxygen availability, ArcA is activated through a phosphorelay mechanism and binds to the consensus sequence 5’-wGTTAATTAw-3’ (w is A or T) located in numerous genes, including several genes involved in β-oxidation shown in Table 2.1, and acts as either a repressor or activator (Iuchi and Lin 1992; Lynch and Lin 1996; Liu and Wulf 2004; Cho et al. 2006; Peña-Sandoval and Georgellis 2010). It has previously been shown that the redox biochemistry and transcriptional regulation of an ΔarcA mutant strain is significantly altered during microaerobic growth conditions, and to a lesser degree during aerobic growth conditions (Oshima et al. 2002; Alexeeva et al. 2003; Shalel-Levanon et al. 2005). The work
presented here demonstrates for the first time an *arcA* deletion mutant combined with a *fadR* mutation to improve the biosynthesis of PHA polymers derived from fatty acid substrates.

### Table 2.1: Regulation Targets of ArcA and OmpR

<table>
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<tr>
<th>Target Gene</th>
<th>Description</th>
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<tr>
<td>arcA</td>
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<tr>
<td><em>fadL</em></td>
<td>Long-chain fatty acid transporter, experimental evidence.</td>
<td>Cho et al., 2006</td>
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<tr>
<td><em>fadD</em></td>
<td>Acyl-CoA synthetase, experimental evidence.</td>
<td>Cho et al., 2006</td>
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<tr>
<td><em>fadE</em></td>
<td>Acyl-CoA dehydrogenase, experimental evidence.</td>
<td>Cho et al., 2006</td>
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<tr>
<td>ompR</td>
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<tr>
<td><em>fadL</em></td>
<td>Long-chain fatty acid transporter, predicted.</td>
<td>Higashitani et al., 1993</td>
</tr>
</tbody>
</table>

### 2.3 Methods

#### 2.3.1 Materials

A complete list of strains, plasmids, and primers used for this study is shown in Table 2.2. All strains were grown in Lennox Broth (LB; composition per liter: 10 g tryptone, 5 g yeast extract, and 5 g sodium chloride, pH 7.0) purchased from Difco, and the antibiotics kanamycin (50 mg L\(^{-1}\)) and ampicillin (100 mg L\(^{-1}\)) were added to media throughout the experiment as appropriate. The fatty acids sodium butyrate (Alfa Aesar), pentanoic acid (Alfa Aesar), hexanoic acid (Alfa Aesar), heptanoic acid (Alfa Aesar), sodium octanoate (Sigma Aldrich), decanoic acid (Alfa Aesar), and dodecanoic acid (Acros Organics) were used as feed supplements for PHA production (12 mM), along with the surfactant Brij-35 (Fisher Scientific, 4.0 g L\(^{-1}\)). Sodium phosphate dodecahydrate (Acros Organics, 8 mM) was added when noted. Sodium hydroxide (5 M) was used to adjust the pH to 7.0 when necessary. Primers were ordered from Integrated DNA Technologies (IDT). ACS HPLC-grade chloroform and methanol were used for gas chromatography (GC) sample preparation and polymer purification.
Table 2.2: Strains, plasmids, and primers.

<table>
<thead>
<tr>
<th>Strains, plasmids, and primers</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
</tr>
<tr>
<td>LSBJ ΔfadB, ΔfadJ, atoC512 (Const), fadR601</td>
<td>Tappel et al., 2012</td>
</tr>
<tr>
<td>RSC02 ΔarcA LSBJ</td>
<td>This study</td>
</tr>
<tr>
<td>RSC04 ΔompR LSBJ</td>
<td>This study</td>
</tr>
<tr>
<td>RSC06 ΔarcA, ΔompR LSBJ</td>
<td>This study</td>
</tr>
</tbody>
</table>

**Plasmids**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKD46</td>
<td>λ Red recombinase expression plasmid; expresses exo, θ, and γ genes from λ phage; P&lt;sub&gt;araB&lt;/sub&gt; promoter; araC; Amp&lt;sup&gt;R&lt;/sup&gt;; temperature sensitive replicon</td>
<td>Datsenko and Wanner, 2000</td>
</tr>
<tr>
<td>pKD13</td>
<td>Neomycin phosphotransferase flanked by FLP recombinase recognition targets, Amp&lt;sup&gt;R&lt;/sup&gt;, Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Datsenko and Wanner, 2000</td>
</tr>
<tr>
<td>pCP20</td>
<td>FLP recombinase expression plasmid, Amp&lt;sup&gt;R&lt;/sup&gt;, temperature sensitive replicon</td>
<td>Datsenko and Wanner, 2000</td>
</tr>
<tr>
<td>pBBR-C1J4SiI</td>
<td>pBBR1MCS-2 derivative ΔphaAB, phaI4, phaC1 (STQK)</td>
<td>Tappel et al. 2012</td>
</tr>
</tbody>
</table>

**Primers**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKD13.F.arcA</td>
<td>ATGCAGACCCCCGCACATTTCTTATCGTGAAACAGCGATTGTAACACGCAAGTGGCTGAAGCTGCTTC</td>
</tr>
<tr>
<td>pKD13.R.arcA</td>
<td>TTAATCTTCCGATCCGGAGAAGCGATAACCTCACCCTACGTGAATGGTGGATTCCGTGGATCCGTCGACC</td>
</tr>
<tr>
<td>pKD13.F.ompR</td>
<td>ATGCAAGAGAACTACAAGATTCTGGTGGTCGATGACGACATGCGCCTGCGGTGTAGGCTGGAGCTGCTTC</td>
</tr>
<tr>
<td>pKD13.R.ompR</td>
<td>TTAGAACATTACCTTAGAAGGTACTGCTCAAGAAATGTCCTTACCGGCTGGATCCGTCGACC</td>
</tr>
<tr>
<td>arcA.check.F/R</td>
<td>GTTAATTTGAGCATGCAATCAGG / GACGATGAGTTACGTATCTGG</td>
</tr>
<tr>
<td>ompR.check.F/R</td>
<td>AAATTGTGGCGAACCTTGGG / GCAATAAGCTACCGGCAAT</td>
</tr>
<tr>
<td>qAtoA.F/R</td>
<td>GGTGCGAGCCATTTGTGATAG / CGCGAGGTTTGGCTTTC</td>
</tr>
<tr>
<td>qAtoD.F/R</td>
<td>ACTTGGCAACCTGACCTATC / GACCAGTTTCATCTGGCTTAC</td>
</tr>
<tr>
<td>qAtoE.F/R</td>
<td>ACTCCTGTACCTTCC / GCAGACCGGCAATCATAA</td>
</tr>
<tr>
<td>qFadD.F/R</td>
<td>TCTCGGCAGCTGATCTTCC / CCATAGCCCTCAGCAGGATAC</td>
</tr>
<tr>
<td>qFadE.F/R</td>
<td>TTACCCGTCTGAGTAACG / GACGGGTTCATCTCGCTTAC</td>
</tr>
<tr>
<td>qFadL.F/R</td>
<td>GGGCCCTCTACCTACCTAA / TTTCAAGGTGGTGGTACC</td>
</tr>
<tr>
<td>qRpoD.F/R</td>
<td>GAGCAGGCTTATCTGCACCTATG / GCCCATGTCGTTGGATGTTT</td>
</tr>
</tbody>
</table>

<sup>a</sup> Underlined sequences are homologous to the gene to be deleted.

<sup>b</sup> Forward and reverse primers are denoted with an F or R, respectively, and primers used for qPCR are denoted with a q.

### 2.3.2 Gene deletions

The deletion of the arcA and ompR genes was accomplished using the λ red recombinase protocol, a commonly used method for nonpolar gene deletion, as previously described (Datsenko and Wanner 2000; Tappel et al. 2012b). Briefly, knockout cassettes were generated using PCR with gene-specific primers and the kanamycin resistance marker from pKD13 (Table 2.2). PCR was performed using PrimeSTAR HS polymerase (Takara) following the manufacturers
recommended protocol. The λ red recombinase was expressed using plasmid pKD46, and knockout cassettes introduced by electroporation (1500V, 5ms; BTX ECM 399). Successful recombination was determined by antibiotic selection and loci screening using check primers (Table 2.2). Antibiotic resistance was removed by the expression of FLP recombinase from the pCP20 plasmid, and successful deletions were confirmed by loss of antibiotic resistance and by PCR using loci check primers (Table 2.2). Deletion mutants ΔarcA, ΔompR, and the double deletion ΔarcA ΔompR were named RSC02, RSC04, and RSC06, respectively (Table 2.2).

2.3.3 PHA production

Protocols for PHA production and cell harvest were adapted from a previous study (Tappel et al. 2012b), with several modifications. For initial preliminary experiments, LSBJ, RSC02, RSC04, and RSC06 were made chemically competent and transformed with pBBR-C1J4SII following standard procedures (Sambrook and Russell 2001), to express PHA synthase and enoyl-CoA hydratase. Transformants were grown on LB-agar plates, and single colonies were used to inoculate separate 2 mL LB seed cultures, in triplicate for each strain. Seed cultures were grown for 16 h at 37°C and 200 rpm, and used to inoculate 100 mL of growth media in 500-mL baffled shake flasks (to final concentration of 0.5%). Growth media contained LB, Brij-35, decanoic acid, and kanamycin. Cultures were grown for 48 h at 30°C and 250 rpm on a rotary shaker, and were then harvested following previously published methods (Tappel et al. 2012b).

A more robust test of PHA homopolymer production was performed between LSBJ and RSC02 using the methods described above, with two key differences. A variety of fatty acids were tested in the growth media: sodium butyrate, pentanoic, hexanoic, heptanoic, sodium octanoate, decanoic, and dodecanoic. In addition, sodium phosphate dodecahydrate was added to the growth media for these experiments, which acts as a buffer for the shorter chain fatty acids. This addition
also provides a significant source of phosphate to the media, so to keep growth conditions consistent, sodium phosphate dodecahydrate was added to every other experiment in this study.

### 2.3.4 GC analysis

The yields and repeating unit compositions of PHA polymers were determined using GC, as previously described (Braunegg et al. 1978; Tappel et al. 2012b). Briefly, dried cells (15-20 mg) were dissolved in 2 mL of sulfuric acid: methanol solution (15:85) and 2 mL of chloroform and heated at 100°C for 140 min in a 10 mL pressure vial (Kimax). The samples were cooled to room temperature followed by the addition of 1 mL of Nanopure filtered water, after which all samples were mixed by vortex. Aqueous and organic layers were allowed to separate for 20 min. The organic layer was passed through a 0.45 μm polytetrafluoroethylene (PTFE) syringe filter (Restek). An aliquot of 500 μL of each filtered sample was mixed with 500 μL of methyl octanoate standard (1 g/L) in chloroform in a 2 mL GC vial. Samples were injected and separated using a GC 2010 Gas Chromatograph with an AOC-20i autoinjector with a flame ionization detector. Shimadzu’s GCSolution software was used to analyze the data, and statistical significance of triplicate samples was determined using a two-tailed Student’s t-test with a 95% confidence interval (α = 0.05).

### 2.3.5 Polymer purification and molecular weight determination

PHA homopolymers were extracted from residual dried cell samples from the LSBJ and RSC02 biosynthesis experiments by combining each set of triplicate samples into single 10 mL pressure vials (Kimax), adding 6 mL of chloroform, and incubating at 100 °C for 1 h. Each sample was filtered through a 0.45 μm PTFE syringe filter (Restek) into a 20-mL scintillation vial and rinsed twice with 2 mL aliquots of chloroform. Samples were concentrated to relative dryness using a rotary evaporator, and redissolved in 1 mL chloroform. Crude polymers were purified by non-solvent precipitation in cold methanol as described previously (Pinto et al. 2016), with several
modifications. Briefly, dissolved samples were added dropwise to 10 mL of ice-cold methanol (4°C) with rapid stirring. The solution was centrifuged (3,452 x g, 30 min, 4°C) to pellet the PHA, decanted and washed with an additional 5 mL of methanol, and re-centrifuged. The supernatant was decanted, and the pellet dissolved in approximately 2 mL of chloroform to transfer to a scintillation vial. Samples were concentrated in a rotary evaporator and evaporated to dryness under high vacuum for 4 h.

The weight average (M<sub>w</sub>) and number average (M<sub>n</sub>) molecular weights for each sample were determined by gel permeation chromatography (GPC) as described previously (Pinto et al. 2016). Briefly, PHA solutions of approximately 1.0 g L<sup>-1</sup> were prepared by dissolution in chloroform and passed through a syringe filter (0.45 μm PTFE). Samples were injected (50 μL) into a Shimadzu LC-20AD liquid chromatograph equipped with a Shimadzu SIL-20A autosampler, a Shimadzu CTO-20A column oven, and a Shimadzu RID-10A refractive index detector. Samples were passed through an 8 x 50 mm styrenedivinylbenzene (SDV) guard column (5 μm particles; Polymer Standards Service) and an 8 x 300 mm SDV analytical column (5 μm particles; mixed bed porosity; max molecular weight 1E6 Da; Polymer Standards Service product sda0830051lim). The column oven was maintained at 40°C with a 1 mL min<sup>-1</sup> mobile phase of chloroform. Molecular weight standards of polystyrene with a narrow polydispersity index were used for calibration. Shimadzu’s LCsolution software was used to analyze the data. GPC chromatograms are available as supplemental material (Appendix C).

2.3.6 Growth profiles of LSBJ and RSC02

An analysis of the growth profiles for LSBJ and RSC02 was performed under the PHA homopolymer biosynthesis conditions utilizing two separate substrates, sodium butyrate and decanoic acid. Media and growth conditions were identical to those in the PHA Production section.
A 1 mL aliquot was removed from each culture to measure the OD_{600} every hour using a spectrophotometer (Genesys 10S) until the stationary phase was observed. A 5 mL aliquot was removed from each culture at both 24 and 48 h to analyze PHA concentrations. The 5 mL samples were harvested and analyzed by GC as described above.

2.3.7 RNA extraction and qPCR analysis

RNA from \textit{E. coli} LSBJ and RSC02 was isolated and purified as described previously (Lundgren et al. 2013; Sarwar et al. 2016). To isolate RNA for real-time quantitative PCR (qPCR), each strain was grown in duplicate in 100 ml of growth media (as described above, with sodium phosphate dodecahydrate and decanoic acid) in 500-ml baffled shake flasks at 30°C and 250 rpm to an OD_{600} of ~0.6. Cultures were immediately stabilized by adding 1 ml of RNAProtect Bacteria reagent (Qiagen) to 0.5 ml of culture. Cells were then lysed with lysozyme and proteinase K as described in the manufacturer’s protocol. The total RNA was subsequently purified from the lysed cells with the RNEasy minikit (Qiagen) by using an on-column DNase digestion step. PCR and a Bioanalyzer were used to check the RNA for DNA contamination, quality, and concentration.

The iScript cDNA synthesis kit (Bio-Rad) was used to generate cDNA from 1 μg of the purified RNA samples. A 10-fold dilution series of the pooled cDNA from the two duplicate RNA samples from LSBJ or RSC02 was used for the qPCR experiments. The qPCR experiments were performed in triplicate. The expression of several important genes for β-oxidation was normalized to \textit{rpoD}, a housekeeping gene with stable expression during exponential growth (Jishage et al. 1996). Primers for qPCR were designed to produce ~100 bp amplicons of each of the following genes: \textit{atoA}, \textit{atoD}, \textit{atoE}, \textit{fadD}, \textit{fadE}, \textit{fadL}, and \textit{rpoD} (\textbf{Table 2.2}). The qPCR mixtures contained 300 mM each primer, 10 μl of the 2× iQ SYBR green Supermix (Bio-Rad), 5 μl of diluted cDNA, and nuclease-free water to a total volume of 20 μl. qPCR was performed on the MiniOpticon
system (Bio-Rad) with the following conditions: 1 cycle of 95°C for 2 min and 40 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s. Window-of-linearity $R^2$ values and amplification efficiency values ranged from 0.990 to 1.0 and 90.0% to >100%, respectively. The dilution series with the highest $R^2$ values was used to calculate relative gene expression of RSC02 compared to LSBJ using the Pfaffl method (Fleige et al. 2006). Amplification efficiencies and threshold cycle (Cq) values were calculated using the program LinRegPCR (Ruijter et al. 2009).

2.4 Results

2.4.1 PHA homopolymer production

Our goal was to develop a strain capable of producing PHA polymers with controlled repeating unit compositions and increased yields. To achieve this, the mutant strains RSC02, RSC04, and RSC06 were derived from E. coli LSBJ by the deletion of arcA, ompR, and a tandem arcA/ompR deletion, respectively. The amount of poly(3-hydroxydecanoate) (PHD) produced by these strains was then compared to E. coli LSBJ while expressing PhaJ4 and PhaC1(STQK) in a set of preliminary experiments. RSC02 produced significantly more PHD than other strains, with a yield of 0.353 g L$^{-1}$ (Fig. 2.2). Strains RSC04 and RSC06 were not found to be significantly different from LSBJ (Fig. 2.2) (Table F1, Appendix F).
**Figure 2.2:** Comparison of poly(3-hydroxydecanoate) (PHD) yield between LSBJ and mutant strains RSC02 (ΔarcA), RSC04 (ΔompR), and RSC06 (ΔarcA, ΔompR). The average yield achieved by RSC02 is significantly greater than that of LSBJ, while RSC04 and RSC06 are not significantly different from LSBJ. All values are averages of triplicate experiments plus or minus the standard deviation around those averages. An asterisk denotes a statistically significant difference compared to LSBJ (Student’s *t*-test, two-tailed, α = 0.05).

To further investigate the effect of the ΔarcA mutation on PHA polymer production, PHA homopolymer biosynthesis was characterized utilizing a variety of fatty acids. The fatty acids used for the production of PHA homopolymers were sodium butyrate, valeric acid, hexanoic acid, heptanoic acid, sodium octanoate, decanoic acid, and dodecanoic acid respectively. Analysis by GC showed significant increases in the amount of polymer produced by *E. coli* RSC02 when
compared to *E. coli* LSBJ for nearly all fatty acid substrates, particularly for those with six or fewer carbons (Table 2.3). Overall the parental strain, LSBJ, produced very little short chain-length PHAs, with yields of only 3.04 mg L\(^{-1}\) of poly(3-hydroxybutyrate) (PHB) and 1.92 mg L\(^{-1}\) of poly(3-hydroxyvalerate) (PHV), and only 44.8 mg L\(^{-1}\) of poly(3-hydroxyhexanoate) (PHHx), the shortest medium chain-length polymer (Table 2.3). We observed significant increases in the amount of PHA synthesized by RSC02 of approximately 3750\%, 6360\%, and 485\% when cells utilized sodium butyrate, pentanoic acid, or hexanoic acid as substrates, respectively (Table 2.3).

For these shorter chain-length PHA polymers the identity was confirmed by \(^1\)H-NMR due to the large differences in production between strains (Fig. A1, Appendix A). Of those PHA polymers tested with greater than 6 carbons per repeating unit, only poly(3-hydroxyheptanoate) (PHHp) and PHD yields were significantly different between strains, with an increase of approximately 61 and 115 percent observed for RSC02, respectively (Table 2.3).

### Table 2.3: PHA yield comparison between LSBJ and RSC02.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Strain</th>
<th>CDW(^{a}) (g/L)</th>
<th>PHA(^{a}) (wt.%)</th>
<th>PHA Concentration(^{a}) (mg/L)</th>
<th>Percent Increase(^{b})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium butyrate</td>
<td>LSBJ</td>
<td>0.80 ± 0.03</td>
<td>0.38 ± 0.05</td>
<td>3.04 ± 0.27</td>
<td>3750</td>
</tr>
<tr>
<td></td>
<td>RSC02</td>
<td>0.85 ± 0.03</td>
<td>13.7 ± 0.95</td>
<td>117 ± 11.1 *</td>
<td></td>
</tr>
<tr>
<td>Pentanoic acid</td>
<td>LSBJ</td>
<td>0.76 ± 0.05</td>
<td>0.23 ± 0.02</td>
<td>1.92 ± 0.42</td>
<td>6360</td>
</tr>
<tr>
<td></td>
<td>RSC02</td>
<td>1.14 ± 0.04 *</td>
<td>11.1 ± 1.01</td>
<td>124 ± 7.25 *</td>
<td></td>
</tr>
<tr>
<td>Hexanoic acid</td>
<td>LSBJ</td>
<td>0.85 ± 0.01</td>
<td>5.47 ± 0.97</td>
<td>44.8 ± 6.13</td>
<td>485</td>
</tr>
<tr>
<td></td>
<td>RSC02</td>
<td>1.06 ± 0.01 *</td>
<td>27.3 ± 4.28</td>
<td>262 ± 59.0 *</td>
<td></td>
</tr>
<tr>
<td>Heptanoic acid</td>
<td>LSBJ</td>
<td>0.93 ± 0.01</td>
<td>23.4 ± 0.48</td>
<td>198 ± 4.27</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>RSC02</td>
<td>1.02 ± 0.06</td>
<td>30.2 ± 2.28</td>
<td>319 ± 22.8 *</td>
<td></td>
</tr>
<tr>
<td>Sodium octanoate</td>
<td>LSBJ</td>
<td>1.22 ± 0.05</td>
<td>44.5 ± 8.68</td>
<td>543 ± 110</td>
<td>1.10</td>
</tr>
<tr>
<td></td>
<td>RSC02</td>
<td>1.03 ± 0.01 *</td>
<td>54.1 ± 1.06</td>
<td>549 ± 12.5</td>
<td></td>
</tr>
<tr>
<td>Decanoic acid</td>
<td>LSBJ</td>
<td>1.23 ± 0.05</td>
<td>29.3 ± 2.15</td>
<td>281 ± 152</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td>RSC02</td>
<td>1.49 ± 0.02 *</td>
<td>40.4 ± 1.30</td>
<td>603 ± 26.4 *</td>
<td></td>
</tr>
<tr>
<td>Dodecanoic acid</td>
<td>LSBJ</td>
<td>1.31 ± 0.17</td>
<td>23.5 ± 5.42</td>
<td>303 ± 38.4</td>
<td>7.26</td>
</tr>
<tr>
<td></td>
<td>RSC02</td>
<td>1.11 ± 0.03 *</td>
<td>29.3 ± 5.21</td>
<td>325 ± 64.0</td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\) All values are averages of biological triplicate experiments plus or minus the standard deviation about those averages.

\(^{b}\) Percent increase calculated as the increase in PHA concentration from RSC02 compared to LSBJ.
* Denotes statistically significant difference compared to LSBJ (Student’s t-test, two-tailed, \( \alpha = 0.05 \)).

### 2.4.2 Molecular weight comparison

To compare differences in physical properties of the polymers synthesized by *E. coli* LSBJ and RSC02, samples were analyzed by gel permeation chromatography (GPC) to determine the number average molecular weight (\( M_n \)), the weight average molecular weight (\( M_w \)), and the polydispersity (\( M_w/M_n \)) (Table 2.4). The polymers extracted from RSC02 had greater molecular weights than those from LSBJ for every polymer except poly(3-hydroxydodecanoate) (PHDD), which had a \( M_n \) of approximately 60 kDa and a \( M_w \) of approximately 172 kDa for both strains (Table 2.4). In addition, the polymers extracted from RSC02 had a greater degree of polydispersity, again with the exception of PHDD (Table 2.4).

**Table 2.4**: PHA molecular weight data.

<table>
<thead>
<tr>
<th>PHA(^a)</th>
<th>Strain</th>
<th>( M_w ) (kDa)</th>
<th>( M_n ) (kDa)</th>
<th>( M_w/M_n )</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHB</td>
<td>LSBJ</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>RSC02</td>
<td>390</td>
<td>117</td>
<td>3.3</td>
</tr>
<tr>
<td>PHV</td>
<td>LSBJ</td>
<td>18</td>
<td>16</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>RSC02</td>
<td>243</td>
<td>79</td>
<td>3.1</td>
</tr>
<tr>
<td>PHHx</td>
<td>LSBJ</td>
<td>134</td>
<td>77</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>RSC02</td>
<td>408</td>
<td>171</td>
<td>2.4</td>
</tr>
<tr>
<td>PHHp</td>
<td>LSBJ</td>
<td>219</td>
<td>106</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>RSC02</td>
<td>319</td>
<td>134</td>
<td>2.4</td>
</tr>
<tr>
<td>PHO</td>
<td>LSBJ</td>
<td>157</td>
<td>73</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>RSC02</td>
<td>285</td>
<td>99</td>
<td>2.9</td>
</tr>
<tr>
<td>PHD</td>
<td>LSBJ</td>
<td>145</td>
<td>50</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>RSC02</td>
<td>234</td>
<td>67</td>
<td>3.5</td>
</tr>
<tr>
<td>PHDD</td>
<td>LSBJ</td>
<td>173</td>
<td>58</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>RSC02</td>
<td>172</td>
<td>60</td>
<td>2.9</td>
</tr>
</tbody>
</table>

\(^a\) PHB, poly(3-hydroxybutyrate); PHV, poly(3-hydroxyvalerate); PHHx, poly(3-hydroxyhexanoate); PHHp, poly(3-hydroxyheptanoate); PHO, poly(3-hydroxyoctanoate); PHD, poly(3-hydroxydecanoate); PHDD, poly(3-hydroxydodecanoate); ND, not detected.
2.4.3 Growth profile of LSBJ and RSC02

We visually observed that RSC02 cultures appeared less optically dense than those of LSBJ during the first day of growth, which would be indicative of an increased lag time for this strain. To quantify this observation, we investigated the growth profiles of LSBJ and RSC02 under PHA biosynthesis conditions supplemented with either sodium butyrate or decanoic acid. There was both an increase in lag time and a decrease in the growth rate of RSC02 relative to LSBJ for both substrates (Fig. 2.3). Regardless of strain, when the fatty acid substrate was sodium butyrate the lag phase was 1 – 2 h shorter and the stationary phase was reached more rapidly (Fig. 2.3). Despite the increased lag phase duration and slightly slower growth rate, RSC02 reached the same culture density as LSBJ when supplemented with sodium butyrate (OD$_{600}$ ~3.5, 15 h), and reached a higher OD$_{600}$ when supplemented with decanoic acid of ~5.0 compared to ~3.6 in LSBJ by hour 17 (Fig. 2.3).
Figure 2.3: Growth profile of *E. coli* LSBJ and RSC02 during PHA-biosynthesis utilizing sodium butyrate (C4) or decanoic acid (C10). For both substrates, RSC02 displayed an extended lag time and slightly slower growth rate relative to LSBJ; however, RSC02 cultures reached approximately the same density as LSBJ upon reaching the stationary phase. For both strains, growth with sodium butyrate as the substrate caused a shorter lag phase than with decanoic acid substrate. All values are the averages of triplicate experiments plus or minus the standard deviation around those averages.

2.4.4 PHA yield at 24 vs 48 hours

To determine whether the increased lag phase in RSC02 had a negative effect on PHA yield earlier in the production cycle, small subsamples were removed from both LSBJ and RSC02 shake flasks after 24h and 48h during the growth profile experiments. Similar to the data shown in Table 2.3, LSBJ produced very little PHB at either 24 or 48 hours, and there was very little change between the two time points (Fig. 2.4). However, LSBJ yields of PHD increased from 22.6% to 31.7% of cell dry weight, an increase of approximately 40% (Fig. 2.4). RSC02 produced significantly more polymer than LSBJ regardless of the time or fatty acid substrate; PHB yield
increased from 8.2% to 12.3% from 24 to 48 h, while PHD yield increased by ~20% between time points from 37.2% to 45.0% (Fig. 2.4).

![Bar chart showing polymer yield for LSBJ and RSC02 at 24 h and 48 h, utilizing either sodium butyrate (C4) or decanoic acid (C10). LSBJ saw insignificant changes in PHB production between 24 and 48 h, while RSC02 increased slightly from 8.2% to 12.3% of cell dry weight. PHD yield from LSBJ increased from 22.6% to 31.7% between 24 and 48 h, an increase of ~40%, while PHD yield from RSC02 increased from 37.2% to 45.0%, an increase of ~20%. Regardless of the time, RSC02 produced more polymer than LSBJ in all cases. All values are the averages of triplicate experiments plus or minus the standard deviation about those averages.]

**Figure 2.4:** Comparison of PHA yield as a percentage of dry weight between LSBJ and RSC02 at 24 h and 48 h, utilizing either sodium butyrate (C4) or decanoic acid (C10). LSBJ saw insignificant changes in PHB production between 24 and 48 h, while RSC02 increased slightly from 8.2% to 12.3% of cell dry weight. PHD yield from LSBJ increased from 22.6% to 31.7% between 24 and 48 h, an increase of ~40%, while PHD yield from RSC02 increased from 37.2% to 45.0%, an increase of ~20%. Regardless of the time, RSC02 produced more polymer than LSBJ in all cases. All values are the averages of triplicate experiments plus or minus the standard deviation about those averages.

### 2.4.5 Relative gene expression of RSC02

To analyze the relative expression of fatty acid degradation genes, RNA was isolated from mid-exponential phase cultures of LSBJ and RSC02 and reverse transcribed into cDNA for qPCR. An appropriate OD<sub>600</sub> value (~ 0.6) for mid-exponential phase was determined using the previously defined growth profile (Fig. 2.3). All the genes analyzed were upregulated to some degree in RSC02; SCL fatty acid uptake genes atoA, atoE and atoD were all upregulated to an extremely large degree (>100-fold), while fadD, fadE, and fadL were all upregulated to a lesser degree (2-,

87
10-, and 31-fold) (Fig. 2.5). It is important to note that the expression values reported for the *ato* system may be exaggerated due to the low number of transcripts observed for LSBJ (as observed by a late-cycle emergence of the fluorescence during qPCR analysis).

![Figure 2.5: Relative gene expression of RSC02 compared to LSBJ, normalized to rpoD and measured as fold changes. The expression of genes related to fatty acid degradation was significantly increased in the RSC02 strain during mid-exponential growth phase (OD$_{600}$ of ~ 0.6). The SCL fatty acid degradation genes *atoA*, *atoE*, and *atoD* were massively upregulated by 103-fold, 119-fold, and 303-fold respectively. MCL fatty acid degradation genes *fadD*, *fadL*, and *fadE* were upregulated by 2-fold, 10-fold, and 31-fold respectively. Relative gene expression was calculated from qPCR fluorescence data using the LineRegPCR software (Ruijter et al. 2009) to calculate amplification efficiency and Cq values, and the Pfaffl method used to derive relative expression values with LSBJ as the calibrator (Fleige et al. 2006). All values are the averages of triplicate experiments plus or minus the standard deviation about those averages.

2.5 Discussion

The purpose of developing the mutant strains RSC02, RSC04, and RSC06 was to improve PHA biosynthesis by removing regulatory genes known to interact with components of the PHA biosynthesis pathway. Based on the evidence seen in Fig. 2.2, the singular deletion of *arcA* conferred a significant increase in the production of PHD compared to the parental LSBJ. A
reasonable explanation for this observation is that ArcA becomes active in E. coli LSBJ during the growth conditions utilized for polymer production and inhibits the transcription of fadL, fadD, and fadE, which are known targets of ArcA regulation (Table 2.1). In an arcA deletion mutant this inhibition cannot occur, likely resulting in higher basal transcription of these fad genes. The deletion of ompR and the double deletion of ompR/arcA provided no benefit to PHD biosynthesis, and no statistically significant differences were observed between these mutants and LSBJ (Fig. 2.2). One possibility for this apparent lack of effect is that OmpR regulatory target FadL is not active towards decanoic acid, which has been demonstrated previously (Black 1990). However, the similarity between the RSC06 double deletion mutant and the RSC04 mutant suggests another possibility; the loss of regulation by OmpR may yield a mildly toxic phenotype that inhibits cell growth and metabolism. This possibility is supported by both the observation that both RSC04 and RSC06 had significantly lower cell dry weights than either LSBJ and RSC02 (Table F1, Appendix F), and by previous reporting from Oshima et al. that showed a marked growth deficiency in E. coli ΔompR mutants (2002). These findings provided motivation for further characterization of the RSC02 mutant.

A variety of fatty acid substrates were used to characterize the differences in PHA homopolymer production between RSC02 and LSBJ. In nearly all cases, the deletion of arcA resulted in a significantly higher polymer yield (Table 2.3). Most surprisingly, the largest increases in polymer yield observed between RSC02 and LSBJ were for the two SCL polymers, PHB and PHV, as well as the shortest MCL polymer, PHHx (Table 2.3). This result is interesting considering that there are fewer steps involved in SCL fatty acid metabolism that are regulated by ArcA. Short chain fatty acids enter the β-oxidation cycle via enzymes derived from the ato operon, bypassing both FadL and FadD (Fig. 2.1). This operon has no known interaction with ArcA and
is instead regulated by the response regulator AtoC, which is present in both LSBJ and RSC02 as a constitutively expressed mutant that confers a high level of *atoDAEB* transcription (Spratt et al. 1981; Jenkins and Nunn 1987; Kyriakidis and Tiligada 2009). One possible explanation for this drastic difference in SCL PHA production is that secondary metabolite pools are different between the two strains; polyamines for example have been shown to have significant effects on the regulation of AtoC (Kyriakidis and Tiligada 2009), and previous studies have shown that aspects of polyamine metabolism are affected by the deletion of *arcA* (Partridge et al. 2006).

The differences in MCL PHA production between LSBJ and RSC02 are more varied than those observed for SCL polymers. No significant difference was found between strains when comparing the yields of poly(3-hydroxyoctanoate) (PHO) and PHDD; however, the yields of poly(3-hydroxyheptanoate) (PHHp) and PHD were significantly increased in RSC02 (Table 2.3). The most reasonable explanation for this is that there are numerous differences in the substrate specificities of the enzymes involved in the PHA biosynthesis pathway. The long chain fatty acid transporter FadL is predominantly active on fatty acids containing 16 or more carbons, and no binding has been observed for decanoic acid, therefore it is unlikely that changes in *fadL* expression would account for the differences we observed (Black 1990). In contrast, the acyl-CoA synthetase FadD has high activity towards 12 and 10 carbon fatty acids and only low activity for 8 and 6 carbon fatty acids (Iram and Cronan 2006; Ford and Way 2015), while the recombinant enzymes PhaJ4 and PhaC1(STQK) each have their own well-documented substrate specificities that could contribute to the observed variation without any direct regulation by ArcA (Tsuge et al. 2003; Matsumoto et al. 2005; Sato et al. 2011). However, a complete understanding of fatty acid flux through this PHA biosynthesis pathway cannot be achieved with the current lack of information regarding the enzymatic activity of the acyl-CoA dehydrogenase FadE.
The molecular weight data showed a great degree of variability between PHAs, as well as between LSBJ and RSC02, with polydispersity values ($M_w/M_n$) from 1.2 to 3.3 (Table 2.4). The molecular weight of polymers produced by RSC02 were higher for all PHA polymers produced except for PHDD, and in general the polydispersity indices were higher for RSC02-derived polymers as well. Both the PHO and PHD molecular-number-average-molecular weights ($M_n$) were substantially lower for LSBJ than in previous studies, while the molecular weight and polydispersity for PHDD was found to be significantly higher than previously reported (Liu et al. 2011; Tappel et al. 2012b). A reliable comparison of the molecular weights observed in this study to other studies is difficult due to the variety of growth conditions and pathways employed. However, a possible explanation for the differences observed between LSBJ and RSC02 is that increased basal expression of $\beta$-oxidation genes improves the supply of 3-hydroxy fatty acyl-CoA monomers to the PhaC1 (STQK) polymer synthase. The evidence summarized in Table 2.4 suggests that the molecular machinery in *E. coli* plays some role in determining the molecular weight of the polymers produced.

A comparison between the polymer yield data in Table 2.3 and the molecular weight data in Table 2.4 appears to support this explanation. The PHA homopolymers with the greatest increases in yield (PHB, PHV, and PHHx) similarly showed the greatest increases in molecular weight (with the partial exception of PHB, which was not extracted in a great enough quantity to detect by GPC). The PHHp and PHD obtained from RSC02, which also saw moderate improvement in terms of yield, was observed to have a higher molecular weight than that obtained from LSBJ, although to a lesser extent than the shorter chain length PHA homopolymers. Interestingly, PHO produced by RSC02 had a higher molecular weight despite there being no significant difference in yield, while PHDD was not significantly different in either of those
measurements. While it appears that improving the utilization of fatty acids also improves PHA molecular weight using this biosynthetic platform, these minor discrepancies with regards to PHO reveal that this relationship is more complex than that.

Another possible explanation for this increase in molecular weights could be that less ethanol is produced by RSC02 than LSBJ. It is typical for bacterial cultures grown into stationary phase in shake flasks to reach some level of microaerobiosis, leading to the production of fermentative byproducts such as ethanol (Gupta and Rao 2003; Losen et al. 2004). It was previously reported that the supplementation of ethanol in cultures of recombinant *E. coli* led to a decrease in the molecular weight of PHB due to a chain transfer reaction from PhaC to ethanol (Hiroe et al. 2013). In *E. coli*, the reversible enzyme AdhE is largely responsible for ethanol flux within the cell, allowing for both the biosynthesis and degradation of ethanol (Membrillo-Hernandez et al. 2000). It has been previously shown that an Δ*arcA* mutant has an improved ethanol tolerance compared to wild type, and it has been hypothesized that this tolerance is derived from increased expression of *adhE* along with enzymes involved in the tricarboxylic acid cycle (TCA), which could drive the breakdown of ethanol to acetyl-CoA (Goodarzi et al. 2010). There is also evidence in the literature showing that *adhE* expression is significantly higher in an *E. coli* Δ*arcA* mutant than in wild type under a range of microaerobic conditions (Shalel-Levanon et al. 2005). It is therefore possible that endogenously produced ethanol is reducing PHA molecular weights via chain termination in LSBJ, and that this effect could be mitigated by an improved flux of ethanol back to acetyl-CoA. However, further investigation of this possibility is required as there is currently no direct evidence to support this explanation.

We investigated our observation that RSC02 had slower growth than LSBJ by recording hourly culture densities, and found that while RSC02 had a significantly longer lag phase, the
growth rate was nearly identical to LSBJ, with cultures reaching similar final cell densities regardless of which fatty acid substrate was provided (Fig. 2.3). These observations match the evidence found in the Keio collection of single-gene knockouts, which showed that arcA is nonessential, an ΔarcA mutant grows only slightly slower than wild type, and reaches only marginally lower culture densities (Baba et al. 2006). Despite the slightly slower growth observed with RSC02, the strain is capable of producing significantly more polymer than LSBJ even after only 24 hours of growth (Fig. 2.4). This offers an advantage when using this strain for a large-scale continuous fermentation, and shows that the lengthened lag phase does not significantly impede PHA biosynthesis.

Our analysis of the qPCR results shows a clear increase in the expression of each of the fatty acid degradation genes tested (Fig. 2.5). Although the improvements to fadD, fadL, and fadE were expected and support our hypothesis, the magnitude of the increase in the three ato genes came as a surprise. As we mentioned previously, ArcA is not known to directly regulate expression of the ato system, and both LSBJ and RSC02 harbor a constitutively expressed mutant atoC gene (Spratt et al. 1981; Jenkins and Nunn 1987; Kyriakidis and Tiligada 2009). Comparing the drastic differences seen in SCL PHA yields between LSBJ and RSC02 provides strong evidence to suggest that the similarly drastic increases in gene expression are responsible for these effects (Table 2.3). These results support the idea that the ΔarcA mutation indirectly effects the expression of the ato system, possibly mediated by an altered polyamine metabolism.

The differences between the relative expression of fadD, fadL and fadE also raise some interesting observations. For example, fadD was only modestly up-regulated (2-fold) and its protein product has a high activity towards both 10 and 12 carbon fatty acid substrates; however, while PHD biosynthesis was significantly improved PHDD biosynthesis was not (Table 2.3, Fig. 2.5).
Similarly, \textit{fadL} expression was significantly increased in RSC02 (10-fold) which does have limited binding affinity for 12 carbon fatty acids (Black 1990), and yet this does not appear to improve PHDD biosynthesis either. These results suggest that the binding affinity of FadE may be an important limiting factor in PHA biosynthesis using this system, considering that \textit{fadE} was the most highly up-regulated of these three genes (31-fold) and yet only the yields of PHHx, PHHp, and PHD were significantly improved (\textbf{Table 2.3, Fig. 2.5}). While the binding affinity of FadE is not well-known, our results suggest that it may have low binding affinity towards octanoyl-CoA and dodecanoyl-CoA, although further investigation is needed to test that hypothesis.

One of the largest challenges still facing the PHA industry is the relatively low yield of polymer obtained, which contributes to the overall cost (Kaur 2015). The strain \textit{E. coli} RSC02 that was developed in this study offers a significant improvement to the previously reported strain, \textit{E. coli} LSBJ. The most significant improvements were seen in the biosynthesis of PHB, PHV, and PHHx, with modest increases observed in PHHp and PHD. These results are supported by the increased expression of \textit{atoA}, \textit{atoD}, and \textit{atoE} which correlates with improved PHB and PHV biosynthesis, and also the increased expression of \textit{fadD}, \textit{fadL}, and \textit{fadE} which may contribute to the increased biosynthesis of PHHx, PHHp, and PHD.

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Chapter 3: Increased production of the value-added biopolymers poly(R-3-hydroxyalkanoate) and poly(γ-glutamic acid) from hydrolyzed paper recycling waste fines

The work presented here was published in Frontiers in Bioengineering and Biotechnology (2019). Please see Appendix G for publication cover.

3.1 Abstract

Reject fines, a waste stream of short lignocellulosic fibers produced from paper linerboard recycling, are a cellulose-rich paper mill byproduct that can be hydrolyzed enzymatically into fermentable sugars. In this study, the use of hydrolyzed reject fines as a carbon source for bacterial biosynthesis of poly(R-3-hydroxyalkanoate) (PHA) and poly(γ-glutamic acid) (PGA) was investigated. Recombinant *Escherichia coli* harboring PHA biosynthesis genes were cultivated with purified sugars or crude hydrolysate to produce both poly(R-3-hydroxybutyrate) (PHB) homopolymer and medium chain length-containing copolymer (PHB-co-MCL). Wild-type *Bacillus licheniformis* WX-02 were cultivated with crude hydrolysate to produce PGA. Both PHB and short chain-length-co-medium chain-length (SCL-co-MCL) PHA yields from crude hydrolysate were a 2-fold improvement over purified sugars, and the MCL monomer fraction was decreased slightly in copolymers produced from crude hydrolysate. PGA yield from crude hydrolysate was similarly increased 2-fold. The results suggest that sugars from hydrolyzed reject fines are a viable carbon source for PHA and PGA biosynthesis. The use of crude hydrolysate is not only possible but beneficial for biopolymer production, eliminating the need for costly separation and purification techniques. This study demonstrates the potential to divert a lignocellulosic waste stream into valuable biomaterials, mitigating the environmental impacts of solid waste disposal.
3.2 Introduction

Paper waste fines are cellulose fibers that have become too short for incorporation into paper products due to repeated recycling and must therefore be rejected from this process. These rejected waste fines make up a significant proportion of the waste stream from paper mills, which is becoming increasingly difficult to landfill due to transportation costs and legislation (Laurijssen et al., 2010; Villanueva and Wenzel, 2007). Since reject fines are predominantly composed of cellulose, they can be readily hydrolyzed into monomeric sugars, making them an attractive waste stream for the production of value-added products, including biofuels, platform chemicals, and biopolymers such as polyhydroxyalkanoates (Bhuwal et al., 2014; Galbe and Zacchi, 2002; Min et al., 2015; Wang et al., 2013; Zhang, 2008). Waste fines from the recycling of old corrugated cartons (OCC) are particularly valuable as a source of fermentable sugars, as they are typically high in cellulose and low in lignin and other inhibitory chemicals and minerals compared with deinked paper pulp from other waste streams (Min et al., 2015; Saini et al., 2020).

Polyhydroxyalkanoates (PHAs) are a diverse class of bacterially produced polyesters known for their biodegradability and biocompatibility, which occur naturally as a form of carbon storage (Lee, 1996; Lu et al., 2009). The physical characteristics of PHAs are dependent on both monomeric composition and molecular weight, and they range from stiff and brittle crystalline materials to flexible and elastomeric amorphous polymers (Laycock et al., 2013). Poly(R-3-hydroxybutyrate) (PHB) is the most abundant PHA from both natural and anthropogenic sources; however, this material is of limited use due to its high brittleness. The copolymerization of 3HB with other monomers, particularly those of medium chain-length (6-14 carbons), can improve toughness and elasticity for a more versatile material (Noda et al., 2005). The large-scale production and utilization of PHAs is mainly limited by the production cost, a large portion of
which stems from the cost of the feedstock and which is high relative to the production costs for petroleum-based plastics with similar properties. One way to address these costs is to examine alternative inexpensive feedstocks, which has sparked interest in lignocellulosic waste streams as a cheap carbon source.

Poly(γ-glutamic acid) (PGA) is another biopolymer that has generated interest as a renewable material for a number of applications. PGA is biosynthesized naturally by a variety of *Bacillus* species, and is an edible, water-soluble, biodegradable, and anionic biopolymer (Bajaj and Singhal, 2011; Ogunleye et al., 2015). These properties make PGA suited for a variety of applications, including metal-ion binding and flocculation for wastewater treatment, composite materials for tissue engineering and drug delivery, and as a medicinal metal chelator for heavy metal removal (Inbaraj and Chen, 2012; Shih et al., 2001; Siao et al., 2009; Ye et al., 2006; Yokoi et al., 1996). Current research into improving PGA production for human use is focused on the metabolic engineering of various *Bacillus* species; notably, wild-type *B. licheniformis* WX-02 is capable of producing large amounts of PGA from glucose and glutamate and has been successfully engineered for enhanced biosynthesis (Cai et al., 2018, 2017).

The work presented in this study demonstrates the successful biosynthesis of both PHA and PGA biopolymers from crude hydrolyzed paper waste fines.

### 3.3 Methods

#### 3.3.1 Hydrolysate from waste fines

A recycled liner board mill provided waste fines from the screw-press sludge. The analysis of the waste fines is provided in Min et al., 2018. The enzymatic hydrolysis procedure was conducted as described by Min and Ramarao (2017). Enzymatic hydrolysis was conducted at 50°
C and with commercially available CTec2 enzymes (Novozymes USA) at a substrate consistency of 5%. All other details are described by Min et al., (2018) and Min and Ramarao (2017).

### 3.3.2 Media and cultivation

A complete list of strains and plasmids is shown in Table 3.1. All *E. coli* strains were grown on LB-Lennox (LB; composition per liter: 10 g tryptone, 5 g yeast extract, and 5 g sodium chloride, pH 7.0) purchased from Difco, with 15 g L\(^{-1}\) agar when needed. Glucose (Acros Organics) and xylose (Sigma Aldrich) were supplemented as carbon sources when noted, as well as purified or crude linerboard waste fines hydrolysate (Min et al., 2015). *Bacillus licheniformis* WX-02 were maintained using nutrient broth no. 2 (Oxoid) media. For PGA biosynthesis, *B. licheniformis* WX-02 was cultivated in the following PGA biosynthesis media (composition per liter): 90 g glucose (or 10% crude hydrolysate, v/v), 40 g sodium glutamate, 10 g sodium citrate trihydrate, 10 g sodium nitrate, 8 g ammonium chloride, 1 g potassium phosphate trihydrate, 1 g magnesium sulfate heptahydrate, 1 g zinc sulfate heptahydrate, 1 g calcium chloride, 0.15 g manganese sulfate monohydrate, pH 7.3 ± 0.1. *E. coli* strains were made chemically competent and transformed by heat shock following standard procedures (Sambrook and Russell, 2001), and selection was performed on LB agar (15 g L\(^{-1}\)) plates. The antibiotics kanamycin (50 mg L\(^{-1}\)) and ampicillin (100 mg L\(^{-1}\)) were added to media for selection and plasmid retention as appropriate. All liquid media cultures were cultivated using a rotary shaking incubator (New Brunswick Scientific).

### Table 3.1: Bacterial strains and plasmids.

<table>
<thead>
<tr>
<th>Strains/Plasmids</th>
<th>Relevant Characteristics</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>BW25113</td>
<td><em>E. coli</em>, Δ(araBAD)567, ΔlacZ4787::rrnB3, λ, rph-1, Δ(rhaBAD)568, hsdR514</td>
<td>Lessard et al. 1998, Datsenko and Wanner 2000</td>
</tr>
<tr>
<td>LSBJ</td>
<td><em>E. coli</em> LS5218, ΔfadB, ΔfadJ, atoC512 (Const), fadR601</td>
<td>Tappel et al. 2012</td>
</tr>
<tr>
<td><em>B. licheniformis</em></td>
<td><em>Bacillus licheniformis</em> WX-02, saline soil isolate.</td>
<td>Wei et al. 2010</td>
</tr>
<tr>
<td>pBBRSTQKAB</td>
<td>pBBR1MCS-2 derivative (lac promoter); phaC1(STQK), phaA, phaB</td>
<td>Nomura et al. 2004</td>
</tr>
<tr>
<td>pTrcGK</td>
<td>pTrc99a derivative (trc promoter); phaG, alkK</td>
<td>Wang et al. 2012</td>
</tr>
</tbody>
</table>
PHA biosynthesis methods were adapted from previous studies (Wang et al., 2012; Tappel et al., 2014). Individual colonies of transformed bacteria harboring PHA biosynthesis genes (pBBRSTQKAB or pBBRSTQKAB / pTrcGK) were used to inoculate separate 2 mL LB seed cultures, in triplicate for each strain. Seed cultures were grown for 16 h at 37 °C and 200 rpm and used to inoculate 100 mL of LB media in 500-mL baffled shake flasks (final concentration of 0.5%). Shake flasks were cultivated at 30 °C and 250 rpm rotary shaking for a total of 48 hrs. After reaching an OD$_{600}$ of 1.0, cultures were induced with isopropyl-$\beta$-D-thiogalactoside (IPTG) at a final concentration of 1 mM. Carbon supplements (glucose, xylose, or pure hydrolysate at 20 g L$^{-1}$; or crude hydrolysate at 8% v/v) were added 3 hours post-IPTG induction. Cells were collected by centrifugation at 3716 × g for 15 minutes, washed once with 45 mL of 35% ethanol and once with 45 mL of water, and dried via lyophilization.

PGA biosynthesis methods were adapted from a previous study (Cai et al., 2018). Individual colonies were used to inoculate separate 2 mL LB seed cultures, in triplicate, and incubated for 12 hours at 37 °C and 200 rpm. Seed cultures were used to inoculate 100 mL of PGA biosynthesis media in 500-mL baffled shake flasks (final concentration of 0.5%). Shake flasks were cultivated at 37 °C and 200 rpm for a total of 36 hours. To collect PGA, the pH of 10 mL of culture was adjusted to 2.0 using HCl (conc.) and centrifuged at 8000 × g for 10 minutes. The supernatant was collected and neutralized with NaOH (10M), then precipitated with 30 mL of 100% ethanol and mixed by vortex. The precipitate was collected by centrifugation at 8000 × g for 10 minutes, then dried via lyophilization to obtain a dry weight.
3.3.3 Analytical procedures

The sugar content of the crude hydrolysate was determined using $^1$H-NMR (Kiemle et al., 2003). A calibration curve was generated from the integration of $\alpha$ anomeric proton peaks of pure glucose and xylose at known concentrations relative to glucosamine added as an internal standard (5 g L$^{-1}$). Crude hydrolysate was diluted 10-fold, doped with the glucosamine standard, and the sugar concentrations calculated from the calibration curve. Inductively coupled plasma optical emission spectroscopy (ICP-OES) was used to analyze abundance of metal ions (Optima 5300 DV). Hydrolysate was diluted 10-fold and introduced to the ICP-OES instrument at a flow rate of 1.5 mL min$^{-1}$. Al, As, Ba, Cd, Co, Cu, Cr, Fe, Mo, P, Pb, S, and Zn were analyzed with an axial plasma view, while Ca, K, Mn, Mg, and Na were analyzed radially. Abundance was analyzed by measuring peak areas for each element compared to 4-point calibration curves of known standards.

The yields and repeating unit compositions of PHA polymers were determined using gas chromatography (GC), as previously described with slight modification (Braunegg et al., 1978; Scheel et al., 2016). ACS HPLC-grade chloroform and methanol were used for GC sample preparation. Lyophilized cells (15-20 mg) were suspended in 2 mL of a 15% (v/v) sulfuric acid solution in methanol and 2 mL of chloroform and heated at 100 °C for 140 min in a 10 mL pressure vial (Kimax). The samples were cooled to room temperature, and 1 mL of Nanopure filtered water and 500 μL of methyl octanoate standard (0.25% v/v) in chloroform were added and mixed by vortex. Aqueous and organic layers were separated by centrifugation for 5 minutes at 700 rpm (Marathon 6K, Fisher Scientific). The organic layer was passed through a 0.2 μm polytetrafluoroethylene (PTFE) filter using a vacuum manifold (Millex Samplicity) into 2 mL GC vials. Samples were injected and separated using a GC 2010 Gas Chromatograph with an AOC-20i autoinjector and a flame ionization detector. Shimadzu’s GCsolution software was used to
analyze the data. Statistical analyses were performed using the Data Analysis Toolpak for Microsoft Excel.

PGA was verified by \textsuperscript{1}H-NMR spectroscopy using a Bruker AVANCE III 600 MHz instrument. Spectra were processed with Bruker TopSpin v3.5pI2.

3.4 Results

3.4.1 Hydrolysate characterization

The concentration of glucose and xylose in the crude linerboard waste fines hydrolysate were calculated to be 98.5 g L\textsuperscript{-1} and 28.8 g L\textsuperscript{-1}, respectively (Fig. A4, Appendix A). The metal ion composition of the crude hydrolysate was determined to be: Ca (5486 ppm), Na (1853 ppm), S (21.0 ppm), Mg (20.0 ppm), K (13.0 ppm), Zn (4.70 ppm), Mn (4.44 ppm), Al (2.24 ppm), Ba (1.56 ppm), P (1.12 ppm), Fe (0.30 ppm), Pb (0.24 ppm), Cu (0.19 ppm), Co (40 ppb), As (32 ppb), Mo (17 ppb), Cd (10 ppb), and Cr (7 ppb).

3.4.2 PHA analysis

PHB and SCL-co-MCL PHA were synthesized in recombinant BW25113 and LSBJ using several different carbon sources. The yield of PHB biosynthesized by BW25113 and LSBJ when crude hydrolysate was the carbon source was 6.88 g L\textsuperscript{-1} and 7.65 g L\textsuperscript{-1}, respectively, which was greater than a 2-fold increase compared with any other pure carbon source analysed (Fig. 3.1A). These results were determined to be statistically significant using a Student’s T-test (two-tailed, \( \alpha = 0.05 \)) comparing yields from each pure carbon source to that of the hydrolysate. PHB yields from pure carbon sources were not significantly different between BW25113 and LSBJ, except for the mixed glucose/xylose which led to yields of 1.18 g L\textsuperscript{-1} and 3.13 g L\textsuperscript{-1}, respectively.

The yields of SCL-co-MCL biosynthesized by BW25113 and LSBJ displayed similar trends to PHB homopolymer production; with crude hydrolysate, PHA yields were 6.82 g L\textsuperscript{-1} and
6.26 g L\(^{-1}\), respectively, which was slightly less than a 2-fold increase compared to the other carbon sources (Fig. 3.1B). These results were determined to be statistically significant using a Student’s T-test (two-tailed, \(\alpha = 0.05\)) comparing yields from each pure carbon source to that of the hydrolysate. In LSBJ, none of the SCL-co-MCL yields from pure carbons were significantly different. However, in BW25113 the utilization of xylose led to significantly higher yields (3.21 g L\(^{-1}\)) compared to glucose and the mixed glucose/xylose (Fig. 3.1B).

**Figure 3.1:** PHA produced by *E. coli* strains BW25113 and LSBJ harboring (A) pBBRSTQKAB or (B) pBBRSTQKAB and pTrcGK. Yields of PHB in g L\(^{-1}\) for each carbon source are shown for both strains in panel (A), while yields of PHB-co-MCL are shown in panel (B). Data shown are averages and standard deviations of 3 biological replicates. Hydrolysate denotes the crude hydrolysate (white bar), Glu/Xyl denotes purified hydrolysate sugars (grey striped bar), and Glucose and Xylose are store-bought pure sugars (black and solid grey bars, respectively).

The 3HB monomer fractions were substantially different between BW25113 and LSBJ, with the former strain producing copolymers with greater than 99.5% 3HB regardless of carbon source (Fig. 3.2). LSBJ produced copolymers with greater variability in their monomer content, though still dominated by 3HB monomers which ranged from 95.1% to 98.4% (Fig. 3.2). Due to
the high variation observed in copolymer composition from LSBJ, only PHAs produced from glucose and the mixed glucose/xylose were found to be significantly different between LSBJ and BW25113 using a Student’s T-test (two-tailed, α = 0.05). The MCL monomer composition followed similar trends between carbon sources, but not between strains; polymers produced by BW25113 contained no observable 3-hydroxyoctanoate (3HO) monomers, whereas 3HO constituted a significant percentage of the MCL fraction of PHA produced by LSBJ (Fig. 3.3). 3-hydroxydecanoate (3HD) was the other dominant MCL monomer in PHA produced by LSBJ, with the combined 3HO and 3HD fractions making up greater than 87% of the total MCL fraction. The MCL monomer compositions were compared between each pure carbon source and the hydrolysate separately for both LSBJ and BW25113 using a two-factor ANOVA with replication (α = 0.05), and each comparison was found to be statistically significant with the exception of xylose from the LSBJ strain.

**Figure 3.2:** 3HB monomer content of PHB-co-MCL produced by *E. coli* strains BW25113 and LSBJ harboring pBBRSTQKAB (*phaC, phaA, phaB*) and pTrcGK (*phaG, alkK*). Data shown are averages and standard deviations of 3 biological replicates. Hydrolysate denotes the crude hydrolysate (white bar), Glu/Xyl denotes purified hydrolysate sugars (grey striped bar), and Glucose and Xylose are store-bought pure sugars (black and solid grey bars, respectively).
3.4.3 PGA analysis

PGA was synthesized by *Bacillus licheniformis* WX-02 from either pure glucose or the crude hydrolysate. PGA yields from these two carbon supplements were 3.25 g L\(^{-1}\) and 6.46 g L\(^{-1}\) (standard deviations of 0.32 and 0.90), respectively, and were determined to be significantly different using a Student’s T-test (two-tailed, \(\alpha = 0.05\)). The identity of the PGA was confirmed by \(^1\)H-NMR (Fig. A5, Appendix A). A minor impurity was observed in the 1H-NMR spectrum (3.08–2.88 ppm), which was confirmed to be unconnected to the polymer backbone by COSY-NMR (Fig. A6, Appendix A).

3.5 Discussion

In this study there was a marked increase in both PHA and PGA biopolymer yields when substituting the crude hydrolysate for the pure sugar carbon sources. In comparison with previous
studies, PHB and SCL-co-MCL PHA yields from crude hydrolysate were significantly improved. Using similar methodologies and an unrelated *E. coli* strain (JM109), Nomura *et al.* achieved a yield of 2.31 g L\(^{-1}\) of PHB from pure glucose (Nomura *et al.*, 2004). Similarly, SCL-co-MCL polymers were previously produced with a yield of 3.49 g L\(^{-1}\) using the same growth conditions and *E. coli* LS5218, the parental strain of LSBJ (Tappel *et al.*, 2014). For the PGA production experiment, our results are an improvement over early studies that use similar media and growth methods, where researchers observed a yield of 2.16 g L\(^{-1}\) (Wei *et al.*, 2010). However, there have been much greater improvements in yield by optimizing media formulations and genetically modifying WX-02 to improve ATP supply, with recent yields as high as 43.81 g L\(^{-1}\) (Cai *et al.*, 2018, 2017; Wei *et al.*, 2010).

Due to the complex composition of the crude hydrolysate, it is difficult to pinpoint and investigate specific hypotheses for the observed increase in biopolymer yield. One possible explanation for these results is the abundance of several important metal ions in the hydrolysate. The most abundant metal in the hydrolysate was calcium which was present at physiologically relevant concentrations (~10 mM in hydrolysate supplemented shake flasks) (Holland *et al.*, 1999). Although the role of calcium in prokaryotes is not completely understood, it has been implicated in processes such as cell division, chemotaxis, and regulation of mechanosensitive ion channels (Booth, 2014; Domínguez *et al.*, 2015; Kung *et al.*, 2010; Martins *et al.*, 2011; Norris *et al.*, 1996). Interestingly, there is also evidence of non-proteinaceous PHB and polyphosphate acting as voltage-gated calcium channels in *E. coli* as a strategy to maintain calcium homeostasis, and the increased PHB yield observed in this study could be partially attributed to that (Das *et al.*, 1997; Reusch *et al.*, 1995). However, the extraction and analysis methods in this study cannot differentiate between transmembrane PHB and intracellular granules, so this is merely speculation.
Mg$^{2+}$ and K$^+$ are both vital to bacterial survival, and the presence of these two ions in the hydrolysate may have enhanced bacterial growth (Epstein, 2003; Romani and Scarpa, 2000). A recent study found evidence that peptide-based media may be Mg$^{2+}$-limited, and that *E. coli* grown on tryptone-based media supplemented with glucose were unable to completely utilize that glucose unless supplemented with Mg$^{2+}$ (Christensen et al., 2017). However, this would not explain the enhancement of PGA biosynthesis which was carried out in a defined medium with abundant Mg$^{2+}$. Transition metal cofactors such as Fe, Zn, Mn, and Cu are also beneficial for bacterial growth in modest concentrations, and the trace amounts present in the hydrolysate could have also contributed to improved biomass and biopolymer yields (Hood and Skaar, 2012). Although heavy metals such as Pb, As, and Cd are toxic to bacteria, they were not present in high enough concentrations to inhibit bacterial growth (Mitra et al., 1975; Neumann and Leimkühler, 2008; Peng et al., 2007).

The concentration of glucose and other sugars in bacterial media is an important parameter to consider, and under-feeding or over-feeding can significantly alter metabolism (Stephanopoulos et al., 1998). Although 20 g L$^{-1}$ of glucose is often used in PHA experiments, there is evidence that this concentration is above the optimum for sustained bacterial growth (Christensen et al., 2017; Shang et al., 2003). In fed-batch cultures of *Ralstonia eutropha*, PHB yields were highest when glucose was maintained at 9 g L$^{-1}$ and a decrease in biomass and PHB yield was observed as glucose concentration increased (Shang et al., 2003). Other studies have found evidence that carbon or nitrogen limitation can increase PHB yields in recombinant *E. coli* (Wang et al., 2009). This may have contributed to the increased biopolymer yields observed in this study, as the crude hydrolysate was supplemented at lower sugar concentrations than pure sugars (7.30 g L$^{-1}$ glucose and 2.13 g L$^{-1}$ xylose for PHA experiments, 8.95 g L$^{-1}$ glucose and 2.62 g L$^{-1}$ xylose for PGA experiments).
experiments). Additionally, acetate was present in the hydrolysate at 1 g L\(^{-1}\) and has been previously shown to be utilized by \(E. \ coli\) LS5218 derivatives and incorporated into PHB (Salamanca-Cardona et al., 2016, 2014). However, this amount of acetate would only contribute a maximum theoretical yield of 0.865 g L\(^{-1}\) PHB, which does not account for the total increase of PHB in cells grown on hydrolysate as opposed to pure sugars (Fig. 3.1).

The copolymerization of PHB with MCL 3HA monomers often gives desirable material properties by reducing the crystallinity and brittleness of the resulting polymers, even with MCL fractions as low as 6 mol% (Matsusaki et al., 2000; Sudesh et al., 2000). The SCL-co-MCL PHAs produced by LSBJ in this study only incorporated a small amount of MCL monomers, and almost no MCL monomers were incorporated by BW25113 regardless of the carbon source (Fig. 3.2 & 3.3). There are several potential reasons for the low MCL incorporation; these MCL monomers are scavenged from fatty acid biosynthesis by the transacylase activity of PhaG, a putative 3-hydroxyacyl-ACP-CoA acyltransferase (Fiedler et al., 2000; Rehm et al., 2001). Free 3-hydroxy fatty acids can also be incorporated through the 3-hydroxyacyl-CoA ligase activity of AlkK (Wang et al., 2012). The reduced concentration of sugars from crude hydrolysate is likely to slow the growth rate of the cultures and shift metabolism away from fatty acid biosynthesis, resulting in less availability of 3-hydroxyacyl compounds (Li and Cronan, 1993; Takamura and Nomura, 1988). In \(E. \ coli\) LSBJ, the transcriptional regulator FadR, which both activates fatty acid biosynthesis and represses β-oxidation, is non-functioning (DiRusso et al., 1992; Iram and Cronan, 2005). This may explain why LSBJ was able to incorporate a greater percent of MCL monomers than BW25113, which has a fully functioning fatty acid biosynthesis pathway and is less likely to have large pools of 3-hydroxyacyl-ACP available.
In this study we have successfully biosynthesized PHB, SCL-co-MCL PHA, and PGA using sugars derived from the hydrolysis of linerboard recycling waste fines. Although sugars obtained from this hydrolysis can be purified and do indeed result in PHA biosynthesis, this can be a costly and time-consuming process and generates a low-concentration sugar solution that must be concentrated for bacterial fermentation. The work here has shown that the crude waste fines hydrolysate is a good source of lignocellulosic sugars for value-added biopolymer production.

3.6 References


polyhydroxyalkanoate copolymer production by coexpression of genetically engineered 3-ketoacyl-acyl-carrier-protein synthase III (fabH) and polyhydroxyalkanoate synthesis genes. Biomacromolecules 5, 1457–1464. https://doi.org/10.1021/bm049959v


Chapter 4: Optimizing a fed-batch high-density fermentation process for medium chain-length poly(3-hydroxyalkanoates) in *Escherichia coli*

The work presented here is being prepared for submission to Frontiers in Bioengineering and Biotechnology.

4.1 Abstract

Production of medium chain-length (MCL) poly(3-hydroxyalkanoates) (PHA) of homopolymeric composition via high-density fed-batch fermentation was investigated in β-oxidation deficient *Escherichia coli* LSBJ. These homopolymeric PHAs exhibit attractive material properties such as increased flexibility and elasticity relative to PHB; however, these polymers are difficult to biosynthesize in native PHA-producing organisms, and there is a paucity of research towards developing high-density cultivation methods while retaining compositional control. In this study, we developed and optimized a fed-batch fermentation process in a stirred tank reactor, beginning with the biosynthesis of poly(3-hydroxydecanoate) (PHD) from decanoic acid by recombinant *E. coli* LSBJ with glucose as an energy source for growth. Bacteria were cultured in two phases, a biomass accumulation phase (37°C, pH 7.0) and a PHA biosynthesis phase (30°C, pH 8.0). Through iterative optimizations of semi-defined media composition and glucose feed rate, 6.0 g of decanoic acid was converted to PHD with an 87.5% yield (4.54 g L\(^{-1}\)). Stepwise increases in the amount of decanoic acid fed during the fermentation correlated with an increase in PHD, resulting in a final decanoic acid feed of 25 g converted to PHD at a yield of 89.4% (20.1 g L\(^{-1}\), 0.42 g L\(^{-1}\) h\(^{-1}\)), at which point foaming became uncontrollable. Hexanoic acid, octanoic acid, 10-undecenoic acid, and 10-bromodecanoic acid were all individually supplemented at 20 g each and successfully polymerized with yields ranging from 66.8 to 99.0% (9.24 to 18.2 g L\(^{-1}\)). Using this bioreactor strategy, co-fatty acid feeds of octanoic acid/decanoic acid and octanoic acid/10-azidodecanoic
acid (8:2 mol ratio each) resulted in the production of their respective copolymers at nearly the same ratio and at high yield, demonstrating that these methods can be used to control PHA copolymer composition.

4.2 Introduction

High-density fermentation of Escherichia coli has been studied for the last 50 years in an effort to achieve maximal cell densities (~200 g L$^{-1}$ dry cell weight), frequently to attain high volumetric productivity (g L$^{-1}$ h$^{-1}$) of a heterologously-expressed product (Shiloach and Fass, 2005). While this is often a product such as a protein or antibiotic, these techniques have also been employed to produce biopolymers such as poly(hydroxyalkanoates) (PHAs). PHAs are a broad class of bacterially-derived polyesters prized for their biodegradability and biocompatibility, which can be produced from renewable carbon sources such as sugars and lipids (Lu et al., 2009). There are two dominant types of PHA characterized by the number of carbons present in each monomer, short chain-length (SCL) PHA containing 3-5 carbons, and medium chain-length (MCL) PHA containing 6-14 carbons. The most commonly discussed PHAs in the literature are poly[(R)-3-hydroxybutyrate] (PHB) and SCL copolymers of PHB with 3-hydroxyvalerate, which exhibit stiff and brittle material properties with a high Young’s modulus (i.e. the measured stiffness of a material) (Lu et al., 2009), while MCL PHAs and SCL-co-MCL copolymers are the subject of more recent studies due to their more viscoelastic properties (Rai et al., 2011; Tappel et al., 2012a).

Control over the monomeric composition of MCL PHAs can be achieved by culturing β-oxidation deficient (ΔfadBJ) E. coli LSBJ harboring the PHA biosynthesis genes phaJ4 and phaC1(STQK) in the presence of fatty acid substrates as previously described by our laboratory (Tappel et al., 2012a, 2012b). Controlling the monomer composition confers control over the
material properties, which can be further customized by the incorporation of functionalized fatty acids that enable post-production chemical modifications (Levine et al., 2015; Pinto et al., 2016). One of the main limitations of this MCL PHA biosynthesis platform is the low polymer yields obtained; the first reported MCL PHA biosynthesis in *E. coli* LSBJ reported yields from shake flask cultivations of approximately 0.26 – 0.4 g L\(^{-1}\) (Tappel et al., 2012b), which were later improved slightly by the deletion of the *arcA* transcriptional regulator to 0.26 – 0.6 g L\(^{-1}\) (Scheel et al., 2016), and most recently improved to 5.44 g L\(^{-1}\) by utilizing glucose as a co-substrate, doubling the culture duration, and heterologously expressing an acyl-CoA synthetase from *Pseudomonas putida* (Mohd Fadzil et al., 2018).

Cultivating PHA-producing bacteria to a high-density in stirred tank reactors (STR, bioreactors) rather than shake flasks has been shown to greatly enhance volumetric productivity, and is a necessary step to scaling up production (Blunt et al., 2018; Koller, 2018; Lee et al., 1999). However, the most common products created using these high-density fermentation techniques are SCL PHAs and MCL ter- and quadri-polymers with uncontrolled monomer composition, with only a few reports of successful MCL biosynthesis with controlled compositions. One notable exception is the biosynthesis of near-homopolymeric poly(3-hydroxydecanoate) (PHD) reported by Gao et al., who obtained a yield of 11.8 g L\(^{-1}\) (0.41 g L\(^{-1}\) h\(^{-1}\)) from β-oxidation deficient *Pseudomonas putida* KT2440 (Gao et al., 2018).

The goal of this study was to optimize a fed-batch fermentation process for the high-density cultivation of recombinant *E. coli* LSBJ to produce MCL PHA polymers with controlled composition from fatty acid substrates. PHA biosynthesis from fatty acids is mostly decoupled from primary metabolism; exogenous fatty acids are imported and converted into 3-hydroxyacyl-CoA via three steps of a modified β-oxidation pathway and are polymerized by the PHA synthase
PhaC1(STQK), while glucose catabolism drives cell growth and provides ATP and reducing equivalents (Fig. 5.1). Catabolite repression induced by the presence of glucose is one regulatory network expected to bridge the gap between primary metabolism and β-oxidation. Fatty acid metabolism is tightly regulated in *E. coli* and is subject to catabolite repression through the activity of the cyclic AMP receptor protein – cyclic AMP complex (CRP-cAMP), a global transcriptional regulator that activates transcription of *fad* genes in response to elevated cAMP levels in the absence of glucose (see Chapter 1 for more information on CRP); this activation is lost when glucose is present in sufficient concentrations (Deutscher, 2008; Feng and Cronan, 2012; Fic et al., 2009; Iram and Cronan, 2006).

The work presented in this study demonstrates the first successful biosynthesis of MCL PHAs in *E. coli* LSBJ via high-density fermentation, and a subsequent process optimization to increase PHA yields and volumetric productivity.
Figure 4.1: Simultaneous glucose consumption and PHA biosynthesis in *E. coli* LSBJ. Glucose is metabolized solely for cell growth, generating ATP and reducing equivalents via glycolysis and the tricarboxylic acid (TCA) cycle. Extracellular fatty acids (R = 1-9 carbon) cross the outer membrane by diffusion and are simultaneously transported and activated by the acyl-CoA synthetase FadD (Kameda and Nunn, 1981; Lepore et al., 2011). Acyl-CoAs are converted to enoyl-CoAs by the acyl-CoA dehydrogenase FadE and are unable to proceed by native β-oxidation due to the absence of FadB and FadJ (Tappel et al., 2012b). Recombinant enzymes PhaJ4 and PhaC1(STQK), an R-specific enoyl-CoA hydratase and an engineered PHA synthase respectively, are conferred by the pBBR-CIJ4SII plasmid and complete the PHA biosynthesis pathway (Table 4.1). MCL PHA biosynthesis from fatty acids is largely uncoupled from cell growth; however, both *fadD* and *fadE* expression are activated by the binding of the cyclic AMP receptor protein – cyclic AMP (CRP-cAMP) complex (Feng and Cronan, 2012). A high ATP concentration inversely lowers cAMP concentration, reducing the amount of complexed CRP and potentially decreasing the expression of *fadD* and *fadE* and preventing PHA biosynthesis. ATP shown in this figure is a product of the intermediates NADH, GTP, and FADH2 (with the exception of some ATP generated by glycolytic enzymes).
4.3 Methods

4.3.1 Media and inoculum preparation

A complete list of strains, plasmids, and primers used for this study can be found in Table 4.1. Unless otherwise specified, all strains were maintained in LB-Lennox (LB; composition per liter: 10 g tryptone, 5 g yeast extract, and 5 g NaCl, pH 7.0) purchased from Difco, and the antibiotics kanamycin (50 mg L\(^{-1}\)) and ampicillin (100 mg L\(^{-1}\)) were added to media as necessary. When necessary, Bacto agar (Difco) was added to media at a concentration of 15 g L\(^{-1}\) to make plates.

The fatty acids hexanoic acid (Sigma Aldrich), sodium octanoate (Sigma Aldrich), decanoic acid (Alfa Aesar), 10-undecenoic acid (Sigma Aldrich), 10-bromodecanoic acid (A.K. Scientific), and 10-azidodecanoate (synthesized as described below) were supplemented as substrates for polymer production as noted. Fatty acid feed solutions for bioreactor fermentations were brought to pH 8.0 by the addition of NaOH (10 M) to form the respective conjugate base and either autoclaved (saturated fatty acids) or filter-sterilized (functionalized fatty acids) prior to addition to the bioreactor.
<table>
<thead>
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<th><strong>Source or reference</strong></th>
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</tr>
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<td>Scheel et al., 2016</td>
</tr>
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</tr>
<tr>
<td>LSBJ CRP*</td>
<td>crp*(I112L, T127I, A144T) LSBJ</td>
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<td>SM06</td>
<td>Δdld, ΔarcA, Δaas LSBJ CRP*</td>
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**Plasmids**

- **pKD46**: λ Red recombine expression plasmid; expresses exo, β, and γ genes from λ phage; ParaB promoter; araC; ApR; temperature sensitive replicon. Datsenko and Wanner, 2000
- **pBRR-CI4SII**: pBRR1MCS-2 derivative, phaI4, phaC1 (STQK), KmR. Tappel et al. 2012
- **pZS411**: pTrc99a derivative, pct (Megasphaera elsdenii), ApR. This study
- **pBRL690**: pBRR-CI4SII derivative, tac promoter. This study
- **pRAS02**: pJet1.2/blunt derivative, native crp (E. coli MG1655). This study
- **pRAS05**: pJet1.2/blunt derivative, engineered crp*. This study
- **pTrcDAEB**: pTrc99a derivative, atoDAEB operon (E. coli MG1655). This study

**Primers**

<table>
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</tr>
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</tr>
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</tr>
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</table>

*a* Underlined sequences are homologous to the gene to be deleted.

*b* Forward and reverse primers are denoted with an F or R, respectively.

*c* Lowercase base-pairs indicate codons subjected to site-directed mutagenesis.
4.3.2 E. coli engineering and plasmid construction

The dld, arcA, and aas genes were deleted using homologous recombination via the λ Red recombinase protocol described by Datsenko and Wanner (2000). Knockout cassettes were generated by PCR (PrimeSTAR HS polymerase, Takara) using gene-specific primers to amplify the kanamycin resistance marker from pKD13, gel-purified, and concentrated by ethanol precipitation. Knockout cassettes were introduced to parental strains harboring pKD46 by electroporation (1500V, 5m; BTX ECM 399), recovered in 1 mL LB at 30 °C and 250 rpm for 1 h, and plated on LB agar supplemented with kanamycin at 37 °C for 24 h. For the dld knockout, colonies were patched onto minimal media agar plates (22 mM KH$_2$PO$_4$, 42 mM Na$_2$HPO$_4$, 8.6 mM NaCl, 2.0 mM MgSO$_4$, 18.9 mM NH$_4$Cl, 15 g L$^{-1}$ agar, pH 7.0) that were supplemented with 25 mM D-lactate. Colonies that did not grow on the D-lactate minimal agar plates were considered putative dld::Km$^R$ mutants. Successful gene replacement for the arcA::Km$^R$ and aas::Km$^R$ mutants was confirmed by loci screening using PCR. The temperature-sensitive pKD46 plasmid was removed by growth for 12 hours at 42 °C. Antibiotic markers were removed by expression of FLP recombinase from the pCP20 plasmid, which was confirmed by the loss of kanamycin resistance and loci screening by PCR. The temperature-sensitive pCP20 plasmid was removed by growth for 12 hours at 42 °C. Successful mutants were prepared as 30% glycerol stocks and stored at -80 °C.

The crp* mutation, a group of mutations that exhibit reduced dependence on cAMP for DNA binding and subsequent transcriptional regulation (Guidi-Rontani et al., 1981; Khankal et al., 2009), was introduced to E. coli LSBJ by first deleting the native crp gene and then replacing it with the mutated crp* gene. Native crp was removed following the same λ Red recombinase protocol described above. The crp* gene was generated from the native E. coli MG1655 crp gene.
using the Q5® Site-Directed Mutagenesis Kit (New England Biolabs) following manufacturers recommendations. In brief, native *crp* was PCR amplified from MG1655 genomic DNA using the native.*crp*.F/native.*crp*.R primers and blunt ligated into pJet1.2/blunt to generate pRAS02. This plasmid was subjected to three successive rounds of site-directed mutagenesis using the *crp*M1, *crp*M2, and *crp*M3 primer sets to make the following substitutions, respectively: I112L, T127I, and A144T. The final *crp* gene harboring all three mutations, designated pRAS05, was verified by Sanger sequencing (GENEWIZ, NJ).

The *crp* gene was PCR amplified from pRAS05 using the native.*crp*.F/native.*crp*.R primers. The PCR mixture was treated with Dpn I and the desired ~700 bp *crp* product was gel purified. The purified *crp* was concentrated via ethanol precipitation and subsequently electroporated into LSBJ Δ*crp* cells harboring the λ Red plasmid pKD46. Cells were recovered in 1 mL of LB at 30 °C and 250 rpm for 1 h, and 0.5 mL of recovery culture was harvested by centrifugation and suspended in 0.2 mL of sterile water. Cell suspension (0.1 mL) was plated onto minimal medium agar consisting of 22 mM KH₂PO₄, 42 mM Na₂HPO₄, 8.6 mM NaCl, 2.0 mM MgSO₄, 20 mM pyruvate (carbon source), and 20 mM L-tryptophan (nitrogen source). This selection method was chosen because *crp* is required for growth on L-tryptophan as a nitrogen source in the presence of pyruvate as a carbon source (Isaacs et al., 1994). Plates were incubated at 37 °C for 48 h, and colonies visible after 48 h were patched onto LB agar plates and subsequently screened via Sanger sequencing to verify the presence of the *crp* gene. The LSBJ CRP* mutant was prepared as a 30% glycerol stock and stored at -80 °C.

The pZS411 plasmid, harboring the *pct* gene necessary for lactyl-CoA formation from lactate, was constructed by excising the *pct* gene from the pTV118NpctC1AB(STQK) plasmid (Taguchi et al., 2008) using 5` and 3` EcoRI sites and ligating into pTrc99a following standard
cloning procedures (Sambrook and Russell, 2001). The pTrcDAEB plasmid was constructed by amplifying the *atoDAEB* operon using the *atoDAEB.F/R* primer set and ligating this fragment into the blunt cloning vector pJet1.2/blunt following manufacturer’s specifications. The operon was then excised with 5´ *Sac*I and 3´ *Kpn*I, purified by gel electrophoresis, and ligated into pTrc99a. The pBRL690 plasmid was constructed by mutating the *lac* promoter of pBBRC1J4SII into the *tac* promoter by site-directed mutagenesis of the -35 region using the BL621.F/R primer set and the Q5® site-directed mutagenesis kit.

4.3.3 Shake flask production

PHA production in shake flasks was performed as described in Chapter 2 (Scheel et al., 2016). In brief, chemically competent *E. coli* LSBJ was transformed with MCL PHA biosynthesis plasmid pBBR-C1J4SII by heat-shock following standard procedures (Sambrook and Russell, 2001). Transformants were grown on LB-agar plates, and single colonies were used to inoculate three separate 2.5 mL LB seed cultures. Seed cultures were grown for 16 h at 37 °C and 200 rpm rotary shaking, and 500 μL were used to inoculate 100 mL of growth media (LB, 4.0 gL⁻¹ Brij-35, 10 mM decanoic acid, 8 mM sodium phosphate dodecahydrate, kanamycin, pH 7.0) in 500-mL baffled shake flasks. Cultures were grown for 12, 24, 36, or 48 h at 30°C and 220 rpm, then harvested by centrifugation as previously described (Tappel et al., 2012b). Before harvesting, the pH of each culture was measured (Accumet® pH meter, Fisher Scientific) immediately after removal from the incubator.

4.3.4 Low-density fed-batch production

A summary of all bioreactor trials outlining the *E. coli* strains, plasmids, and conditions can be found in Table 4.2. All fed-batch fermentations were performed in a 2-L vessel with a BioFlo 310 benchtop bioreactor system (New Brunswick Scientific) using conditions and media
adapted from Pfeifer et al. (2002). A schematic of this bioreactor assembly is shown in Fig. F5 (Appendix F). For initial experiments, the assembled bioreactor containing 750 mL of defined medium (F1 Salts; 0.4 g L\(^{-1}\) (NH\(_4\))\(_2\)SO\(_4\), 1.5 g L\(^{-1}\) KH\(_2\)PO\(_4\), and 4.35 g L\(^{-1}\) K\(_2\)HPO\(_4\), pH 7.0) was autoclaved at 121 °C for 35 min. Prior to inoculation, 7.5 g glucose, 1.5 g yeast extract, 0.12 g MgSO\(_4\), 1 mL of 0.05 g L\(^{-1}\) kanamycin sulfate, and 0.75 mL of trace metal solution were added aseptically. Trace metal solution contained 5 g L\(^{-1}\) NaCl, 1 g L\(^{-1}\) ZnSO\(_4\) \cdot 7H\(_2\)O, 4 g L\(^{-1}\) MnCl\(_2\) \cdot 4H\(_2\)O, 4.75 g L\(^{-1}\) FeCl\(_3\), 0.4 g L\(^{-1}\) CuSO\(_4\) \cdot 5H\(_2\)O, 0.58 g L\(^{-1}\) H\(_3\)BO\(_3\), 0.5 g L\(^{-1}\) NaMoO\(_4\) \cdot 2H\(_2\)O, and 8 mL L\(^{-1}\) concentrated H\(_2\)SO\(_4\). The bioreactor was inoculated with 40 mL of bacterial seed culture, which was grown in LB for 16 h at 37 °C and 200 rpm rotary shaking, pelleted at 3,716 \(\times\) g for 10 min, and resuspended in 2 mL PBS buffer before sterile transfer to the bioreactor vessel.

Fermentation was carried out in two phases, a biomass accumulation phase (Phase 1) and a polymer biosynthesis phase (Phase 2). The first phase of fermentation was performed at 37 °C with an initial agitation rate of 200 rpm and an aeration rate of 5.0 L min\(^{-1}\), and pH was maintained at 7.0 by automated addition of concentrated NH\(_4\)OH (17M). Dissolved oxygen was maintained at 50% air saturation controlled by agitation and air (compressed atmospheric) sparging cascades (200 to 950 rpm, then 5.0 to 20.0 L min\(^{-1}\) air flow). Foaming was controlled by automated addition of Antifoam B (J.T. Baker, 10% v/v unless otherwise noted). Once the initial glucose was consumed (as evidenced by a sudden increase in dissolved oxygen), a glucose feed solution (500 mL, filter-sterilized) consisting of 430 g L\(^{-1}\) glucose, 3.9 g L\(^{-1}\) MgSO\(_4\), 110 g L\(^{-1}\) (NH\(_4\))\(_2\)SO\(_4\), and 50 mg L\(^{-1}\) kanamycin was supplied at a rate of 14.5 mL min\(^{-1}\) with a separate peristaltic pump. OD\(_{600}\) measurements were made on 1 mL culture aliquots each hour using a Genesys 10S UV-Vis spectrophotometer (Thermo Scientific).
After 12 h of growth in Phase 1, Phase 2 was initiated by decreasing the fermentation temperature to 30 °C, increasing the pH setpoint to 8.0 (with the exception of preliminary experiments, Table 4.2), decreasing the dissolved oxygen setpoint to 30% air saturation, and beginning the addition of a fatty acid feed solution supplied at a rate of 5.5 mL min\(^{-1}\) (except where otherwise noted for preliminary experiments) with another peristaltic pump. For Trial 1, D-lactate (11 g) was added alongside octanoic acid as a bulk supplement. Glucose feed addition during the second phase was either maintained at 14.5 mL min\(^{-1}\) or reduced to 9.7 mL min\(^{-1}\) where noted. Culture samples (10 mL) were removed at 24 and 48 h and stored at -80 °C for later quantitative PHA analysis. The remaining culture at 48 h was harvested as described below.

**4.3.5 High-density fed-batch production**

High-density fermentations were carried out in the same 2-L vessel, using media adapted from Lau et al. (2004). The F1 Salts medium was replaced with 750 mL of defined medium consisting of 0.4 g L\(^{-1}\) (NH\(_4\))\(_2\)SO\(_4\), 4.16 g L\(^{-1}\) KH\(_2\)PO\(_4\), and 11.95 g L\(^{-1}\) K\(_2\)HPO\(_4\). Prior to inoculation, 7.5 g glucose, 1.5 g yeast extract, 0.12 g MgSO\(_4\), 1 mL of 0.05 g L\(^{-1}\) kanamycin sulfate, and 2.25 mL of trace metal solution were added aseptically. The amount of yeast extract was increased to 3.0 g for trial 15 and for each subsequent trial (Table 4.2). The amount of (NH\(_4\))\(_2\)SO\(_4\) in the glucose feed was reduced to 6 g L\(^{-1}\), and 15 mL of trace metal solution was added to the feed as well. All other bioreactor conditions were the same as for the low-density production process.

Fatty acid feed solutions initially contained 40 g L\(^{-1}\) decanoic acid (6 g in 150 mL H\(_2\)O, pH 8.0), and were raised in subsequent trials as shown in Table 4.2. To maintain a constant volume of the combined glucose and fatty acid feeds, as fatty acid feed volumes increased (due to a maximum decanoic acid solubility of 100 g L\(^{-1}\) at pH 8.0) the glucose feed volume was decreased by the same magnitude while holding the mass of feed components constant. To account for this
change in glucose feed concentration, the addition rate was reduced proportionally so that the glucose \( h^{-1} \) was kept constant. Antifoam concentration was increased to 20% for Trials 18 and 24 to combat excessive foaming.

**Table 4.2: Summary of Bioreactor Conditions**

<table>
<thead>
<tr>
<th>Trial</th>
<th>Strain</th>
<th>Plasmid</th>
<th>Glucose Feed Rate (mL h(^{-1}))</th>
<th>Fatty Acid Feed (^{1}); Total Mass (g)</th>
<th>Fatty Acid Feed Rate (mL h(^{-1}))</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SM02</td>
<td>pBBRC1J4SII/pZS411</td>
<td>14.5</td>
<td>C8; 0.8</td>
<td>Bulk</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>SM04</td>
<td>pBBRC1J4SII</td>
<td>14.5</td>
<td>C8; 6</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>SM06</td>
<td>pBBRC1J4SII</td>
<td>14.5 / 9.7</td>
<td>C8; 1.0</td>
<td>Bulk</td>
<td>7</td>
</tr>
<tr>
<td>4</td>
<td>SM06</td>
<td>pBBRC1J4SII</td>
<td>14.5 / 9.7</td>
<td>C8; 1.5</td>
<td>Bulk</td>
<td>7</td>
</tr>
<tr>
<td>5</td>
<td>SM06</td>
<td>pBBRC1J4SII</td>
<td>14.5 / 9.7</td>
<td>C8; 6</td>
<td>8.3</td>
<td>7</td>
</tr>
<tr>
<td>6</td>
<td>SM06</td>
<td>pBBRC1J4SII</td>
<td>14.5 / 9.7</td>
<td>C8; 6</td>
<td>5.5</td>
<td>7</td>
</tr>
<tr>
<td>7(^{4})</td>
<td>SM06</td>
<td>pBRL690</td>
<td>14.5 / 9.7</td>
<td>C8; 6</td>
<td>5.5</td>
<td>7</td>
</tr>
<tr>
<td>8(^{4})</td>
<td>LSBJ</td>
<td>pBBRC1J4SII / pTrcDAEB</td>
<td>14.5</td>
<td>C10; 6</td>
<td>5.5</td>
<td>7</td>
</tr>
<tr>
<td>9</td>
<td>LSBJ</td>
<td>pBBRC1J4SII</td>
<td>14.5</td>
<td>C10; 6</td>
<td>5.5</td>
<td>7 / 8</td>
</tr>
<tr>
<td>10</td>
<td>RSC02</td>
<td>pBBRC1J4SII</td>
<td>14.5</td>
<td>C10; 6</td>
<td>5.5</td>
<td>7 / 8</td>
</tr>
<tr>
<td>11</td>
<td>LSBJ CRP(^{*})</td>
<td>pBBRC1J4SII</td>
<td>14.5</td>
<td>C10; 6</td>
<td>5.5</td>
<td>7 / 8</td>
</tr>
<tr>
<td>12</td>
<td>LSBJ</td>
<td>pBBRC1J4SII</td>
<td>14.5 / 9.7</td>
<td>C10; 6</td>
<td>5.5</td>
<td>7 / 8</td>
</tr>
<tr>
<td>13</td>
<td>LSBJ</td>
<td>pBBRC1J4SII</td>
<td>14.5 / 9.7</td>
<td>C10; 6</td>
<td>5.5</td>
<td>7 / 8</td>
</tr>
<tr>
<td>14</td>
<td>LSBJ</td>
<td>pBBRC1J4SII</td>
<td>14.5 / 9.7</td>
<td>C10; 12</td>
<td>5.5</td>
<td>7 / 8</td>
</tr>
<tr>
<td>15(^{2})</td>
<td>LSBJ</td>
<td>pBBRC1J4SII</td>
<td>14.5 / 9.7</td>
<td>C10; 12</td>
<td>5.5</td>
<td>7 / 8</td>
</tr>
<tr>
<td>16</td>
<td>LSBJ</td>
<td>pBBRC1J4SII</td>
<td>14.5 / 9.7</td>
<td>C10; 15</td>
<td>5.5</td>
<td>7 / 8</td>
</tr>
<tr>
<td>17</td>
<td>LSBJ</td>
<td>pBBRC1J4SII</td>
<td>12.9 / 8.7</td>
<td>C10; 20</td>
<td>6.5</td>
<td>7 / 8</td>
</tr>
<tr>
<td>18</td>
<td>LSBJ</td>
<td>pBBRC1J4SII</td>
<td>11.4 / 7.7</td>
<td>C10; 25</td>
<td>7.5</td>
<td>7 / 8</td>
</tr>
<tr>
<td>19</td>
<td>LSBJ</td>
<td>pBBRC1J4SII</td>
<td>12.9 / 8.7</td>
<td>C6; 20</td>
<td>6.5</td>
<td>7 / 8</td>
</tr>
<tr>
<td>20</td>
<td>LSBJ</td>
<td>pBBRC1J4SII</td>
<td>12.9 / 8.7</td>
<td>C8; 20</td>
<td>6.5</td>
<td>7 / 8</td>
</tr>
<tr>
<td>21</td>
<td>LSBJ</td>
<td>pBBRC1J4SII</td>
<td>12.9 / 8.7</td>
<td>C11Δ10; 20</td>
<td>6.5</td>
<td>7 / 8</td>
</tr>
<tr>
<td>22</td>
<td>LSBJ</td>
<td>pBBRC1J4SII</td>
<td>12.9 / 8.7</td>
<td>C10Br; 20</td>
<td>6.5</td>
<td>7 / 8</td>
</tr>
<tr>
<td>23</td>
<td>LSBJ</td>
<td>pBBRC1J4SII</td>
<td>12.9 / 8.7</td>
<td>C8/C10; 15.4/4</td>
<td>6.5</td>
<td>7 / 8</td>
</tr>
<tr>
<td>24</td>
<td>LSBJ</td>
<td>pBBRC1J4SII</td>
<td>12.9 / 8.7</td>
<td>C8/C10N(_{3}); 15.4/4.95</td>
<td>6.5</td>
<td>7 / 8</td>
</tr>
</tbody>
</table>

\(^{1}\) Trials 1 – 8 are preliminary experiments which did not result in polymer production. Trials 1 – 12 were performed following low-density fermentation methods, while trials 13 – 24 were performed following high-density fermentation methods. Trials 9 – 17 were performed in duplicate as two consecutive bioreactor fermentations, with the exception of trial 14.

\(^{2}\) Yeast extract in culture medium was doubled to 3.0 g total for trial 15, and this amount was maintained for each subsequent trial.

\(^{3}\) Fatty acids are abbreviated as follows: octanoic acid (C8), decanoic acid (C10), hexanoic acid (C6), 10-undecenoic acid (C11Δ10), 10-bromodecanoic acid (C10Br), 10-azidodecanoic acid (C10N\(_{3}\)). Feed volume was held at 150 mL for Trials 2-16, increased to 200 mL for Trial 17 and 250 mL for Trial 18. Trials 19-24 were reduced back to 200 mL.

\(^{4}\) Isopropyl β-D-1-thiogalactopyranoside (IPTG) added to Trials 7 and 8 at a final concentration of 0.5 mM to induce pBRL690 and pTrcDAEB expression, respectively.
4.3.6 PHA quantification

Shake flask cultures were harvested as described in Chapter 3 (Scheel et al., 2019). In brief, cells were collected by centrifugation at 3716 × g for 15 minutes, washed once with 45 mL of 35% ethanol and once with 45 mL of Nanopure filtered water, frozen at -80 °C, and dried via lyophilization.

The 10 mL culture samples from bioreactor fermentations were thawed and pelleted by centrifugation at 7,000 × g for 15 min. The supernatant was reserved and frozen at -80 °C for lyophilization and later analysis. Cell pellets were washed once with 40% ethanol (40 mL) and once with Nanopure filtered water (40 mL), pelleting with the same centrifugation conditions after each wash. Pellets were then resuspended in 5 mL Nanopure filtered water, frozen at -80 °C, and dried via lyophilization.

The PHA yields and repeating unit compositions were analyzed by GC as described in Chapter 3 (Scheel et al., 2019). In brief, lyophilized cells (10-15 mg) were suspended in 2 mL of a 15% (v/v) sulfuric acid solution in methanol and 2 mL of chloroform and heated at 100 °C for 140 min in a 10 mL pressure vial (Kimax). The samples were cooled to room temperature, and 1 mL of Nanopure filtered water and 500 μL of methyl octanoate standard (0.25% v/v) in chloroform were added and mixed by vortex. Aqueous and organic layers were separated by centrifugation for 5 minutes at 700 rpm (Marathon 6K, Fisher Scientific). The organic layer was passed through a 0.2 μm polytetrafluoroethylene (PTFE) filter using a vacuum manifold (Millex Samplicity®) into 2 mL GC vials. Samples were injected and separated using a GC 2010 Gas Chromatograph (Shimadzu) with an AOC-20i autoinjector and a flame ionization detector. Shimadzu’s GCsolution software was used to analyze the data based on 3-hydroxyacyl methyl ester standard curves (except for 3-hydroxy-10-azidodecanoate monomers, which is described below).
4.3.7 10-azidodecanoic acid synthesis

The 10-azidodecanoic acid substrate was synthesized following previously described methods (Riva et al., 2014), and is summarized in Scheme 4.3.1. Sodium azide (15.01 g, 231 mmol) was added to a solution of 10-bromodecanoic acid (1, 40.00 g, 159 mmol) in dimethyl sulfoxide (1000 mL). The mixture was stirred at room temperature for 24 hours and then water (2000 mL) was added. The aqueous solution was extracted with ethyl acetate (4 x 700 mL) and then washed with half-saturated brine (6 x 500mL) and brine (2 x 400mL) and dried over sodium sulfate. The sodium sulfate was filtered off and ethyl acetate was removed in vacuo to yield the azide (2, 32.84 g, 94%).

![Scheme 4.3.1: Synthesis of 10-azidodecanoic acid.](image)

4.3.8 PHA purification and molecular weight analysis

Bioreactor cultures were harvested after 48 h by dividing the volume equally among 800-mL centrifuge buckets and centrifuging at 3,716 \times g for 45 min. Cell pellets were washed once with 40% ethanol (approximately 400 mL) and once with Nanopure filtered water (400 mL), pelleting with the same centrifugation conditions after each wash. Final cell pellets were transferred to a tared 250-mL centrifuge tube, frozen at -80 °C, and dried via lyophilization.

PHA was isolated from dried cells by Soxhlet extraction. Approximately 5-10 g of cells were transferred to 80 × 25 mm cellulose extraction thimbles (Whatman) and refluxed with 150 mL chloroform for 6 h. Crude polymer was concentrated by rotary evaporation and purified by
dropwise addition to stirring methanol (180 mL, 4°C) without exceeding a 1:10 ratio of chloroform to methanol. The methanol solution was centrifuged at 7,000 × g and 4°C for 20 min to collect any suspended polymer. This methanol purification process was repeated twice, and purified PHA was dissolved in chloroform and passed through a syringe filter (0.45 μm PTFE) before drying in vacuo.

The weight average (Mₘ) and number average (Mₙ) molecular weights for each sample were determined by gel permeation chromatography (GPC) as described in Chapter 2 (Scheel et al., 2016). In brief, PHA solutions of approximately 5.0 g L⁻¹ were prepared by dissolution in chloroform and passed through a syringe filter (0.45 μm PTFE). Samples were injected (50 μL) into a Shimadzu LC-20AD liquid chromatograph equipped with a Shimadzu SIL-20A autosampler, a Shimadzu CTO-20A column oven, and a Shimadzu RID-10A refractive index detector. Samples were passed through an 8 x 50 mm styrenedivinylbenzene (SDV) guard column (5 μm particles; Polymer Standards Service) and an 8 x 300 mm SDV analytical column (5 μm particles; mixed bed porosity; max molecular weight 1E6 Da; Polymer Standards Service product sda0830051lim). The column oven was maintained at 40°C with a 1 mL min⁻¹ mobile phase of chloroform. Molecular weight standards of polystyrene with a narrow polydispersity index were used for calibration. LCsolution software (Shimadzu) was used for data analysis.

The presence of functional groups on applicable PHAs was verified by ¹H-NMR spectroscopy using either a Bruker AVANCE III 600 MHz or Bruker AVANCE III HD 800 MHz instrument as noted. ¹H-NMR was also used to confirm PHA production from the first successful bioreactor fermentation (Trial 9, Table 4.2). Spectra were processed with Bruker TopSpin v3.5pl2.
4.3.9 Calculations

PHA yields (g L\(^{-1}\)) were calculated from the weight percent obtained using GC calibration curves as described above and the dried cell weight of 10.0 mL samples, dividing by the sample volume:

\[
PHA \text{ yield (g L}^{-1}\text{)} = \left(\frac{\text{Weight }\% \times \text{ Dried Cell Weight (g)}}{0.010 L}\right)
\]

The total PHA accumulated for each bioreactor fermentation was calculated by multiplying the PHA yield by the final culture volume from the bioreactor:

\[
Total \ PHA \ (g) = PHA \ \text{yield (g L}^{-1}\text{)} \times Final \ \text{volume (L)}
\]

Maximum theoretical yields of PHA were calculated from the total mass of fatty acid substrate available, multiplied by the molecular weight ratio of diradical monomeric equivalents to fatty acid precursor, assuming complete 1:1 conversion:

\[
Theoretical \ (g) = Fatty \ acid \ substrate \ (g) \times \left(\frac{MW_{\text{diradical monomer (g mol}^{-1})}}{MW_{\text{fatty acid (g mol}^{-1})}}\right)
\]

The mole fraction (\(\chi\)) of 3-hydroxy-10-azidodecanoate (DN\(_3\)) monomers in the PHODN\(_3\) copolymer was calculated via \(^1\)H-NMR (Appendix A). Peak integrations (A), calibrated to the three protons of the terminal methyl group on 3-hydroxyoctanoate (3HO) monomers (\(\delta 0.88\)), were used to determine the ratio of the DN\(_3\) protons in the alpha position (with respect to the azido group, \(\delta 3.2, 2\text{H}\)) to the sum of the integrations of both chemical shifts (Sperling, 2015):

\[
\chi_{\text{DN}_3} = \frac{A_{3.2}}{\frac{A_{3.2}}{2} + \frac{A_{0.88}}{2}}
\]
The PHODN₃ yield (g L⁻¹) for trial 24 (Table 4.2) was calculated by first obtaining the yield of the 3HO fraction using GC as described above. This was multiplied by the ratio of the mole fractions of DN₃ and 3HO to find the yield of the DN₃ fraction, and added to the yield of 3HO:

\[
PHODN_3 \text{ yield (g L}^{-1}\text{)} = 3HO \text{ (g L}^{-1}\text{)} \times \frac{MW_{DN_3} \times \chi_{DN_3}}{MW_{3HO} \times \chi_{3HO}} + 3HO \text{ (g L}^{-1}\text{)}
\]

4.4 Results

4.4.1 Preliminary trials and the effect of pH

The goal of this investigation was to enhance the production of MCL PHA by developing high density fermentation methods for our engineered production platform, *E. coli* LSBJ. Previous attempts by this lab have been unsuccessful using LSBJ (unpublished data). Preliminary trials (trials 1-8, Table 4.2) sought to address this by generating several further mutations hypothesized to eliminate negative regulation of the PHA biosynthesis pathway. The mutant strains LSBJ CRP*, SM02, SM04, and SM06 were developed and cultured by high-density fermentation in an attempt to produce either poly(3-hydroxyoctanoate), poly(3-hydroxydecanoate), or poly(lactate-co-3-hydroxyoctanoate) (PHO, PHD, PLA-co-PHO; Fig. 4.2A). Despite some improvement in biomass accumulation, no observable polymer was produced in any of these experiments.

Based on previous observations that LSBJ would not produce PHA during bioreactor fermentation unless the pH was left uncontrolled, LSBJ was cultured in baffled shake flasks supplemented with decanoic acid using typical PHA biosynthesis conditions (Chapter 2), and both pH and PHA yield were measured every 12 h during growth (Fig. 4.2B). The pH of culture media increased substantially over 48 h to a final pH of 8.8. PHA was present after 12 h at 6.01% of dry cell weight, and more than tripled to 19.7% by 24 h while the pH increased to 8.5. The amount of
PHA continued to increase at a pH between 8.5 and 8.8 for the remainder of the experiment. These results suggest a correlation between an alkaline pH and PHA biosynthesis from fatty acids.

Based on our findings from the shake flask experiment, LSBJ harboring pBBRC1J4SII was cultured in the bioreactor and supplemented with decanoic acid, and upon initiating Phase 2 the pH was increased to 8.0 and maintained there for the duration of the fermentation. PHD was observed by GC analysis at a concentration of 0.16 g L\(^{-1}\), which was confirmed by NMR spectroscopy on purified polymers obtained by Soxhlet extraction due to the presence of characteristic chemical shifts at 5.15 and 2.5 ppm (Fig. 4.2C).
Figure 4.2: Summary of initial bioreactor experiments leading to successful PHD biosynthesis. (A) Results of preliminary fermentations that did not result in polymer accumulation. (B) Culture pH and PHA accumulation in LSBJ harboring pBBRC1J4SII over 48 h growth in shake flasks, sampling every 12 h. PHA was measured as a percent of dry cell weight. Data shown is the average of triplicate cultures; errors bars represent minus 1 standard deviation. (C) Photograph and $^1$H NMR (600 MHz) spectrum of PHD extracted from LSBJ-pBBRC1J4SII bioreactor fermentation (Trial 9). Diagnostic chemical shift for PHA at δ 5.17 – 5.13 ppm (pentet, 1H); 2.59 – 2.45 (multiplet, 2H), 1.61 – 1.47 (multiplet, 2H), 1.33 – 1.18 (multiplet, 10H), 0.8 – 0.9 (triplet, 3H). Integration indicative of residual fatty acid.
4.4.2 Optimization of PHD production

After successfully producing PHD in trial 9, mutant strains RSC02 (ΔarcA) and LSBJ CRP* were analyzed to determine the effects of removing these transcriptional regulators (Trials 10 & 11, Table 4.2). Little difference was observed between LSBJ, RSC02, and LSBJ CRP*; 0.48 g L$^{-1}$, 0.39 g L$^{-1}$, and 0.44 g L$^{-1}$ of PHD were produced by each strain after 48 h, respectively, with no observable difference between 24 h and 48 h, and high variation between duplicate fermentations (Fig. 4.3B). Dry biomass yield decreased for both mutants relative to LSBJ, reflecting a higher weight percent of PHD, and biomass decreased between 24 h and 48 h for all three strains (Fig. 4.3A). Total polymer accumulation after 48 h was low relative to the maximum theoretical yield, ranging from 5.6 to 8.3% with high variation between duplicate fermentations (Fig. 4.3C).

Next, we reduced the rate of glucose feed addition during Phase 2 to 9.7 mL h$^{-1}$ and cultured LSBJ harboring pBBRC1J4SII to determine the effect on PHD biosynthesis (LSBJ-2, Fig. 4.3). Biomass yield increased by approximately 50% at 24 h relative to LSBJ (29.6 g L$^{-1}$); biomass still decreased between 24 h and 48 h to a final concentration of 21.3 g L$^{-1}$ (Fig. 4.3A). PHD accumulation increased considerably to 2.48 g L$^{-1}$ after 48 h, with no observable difference between 24 h and 48 h (Fig. 4.3B). Total polymer accumulation after 48 h was 2.60 g, a yield of 43.8 % (Fig. 4.3C).

With bioreactor fermentations continuing to show a decrease of biomass and unchanged PHA accumulation between 24 h and 48 h, believed to be due to culture death, the bioreactor medium was changed to that described in high-density fed-batch production to support greater growth of LSBJ harboring pBBRC1J4SII (LSBJ-3, Fig. 4.3). Biomass yield increased relative to LSBJ-2 to 41.3 and 52.0 g L$^{-1}$ at 24 and 48 h, respectively (Fig. 4.3A). PHD accumulated at a
concentration of 2.27 g L\(^{-1}\) by 24 h, which doubled to 4.54 g L\(^{-1}\) by 48 h (Fig. 4.3B). Total polymer accumulation after 48 h was 5.12 g, a yield of 87.5% (Fig. 4.3C).

With PHD yields approaching the theoretical maximum, we increased the amount of decanoic acid added to the feed solution from 6.0 to 12.0 g (LSBJ-3 (12g), Fig. 4.3). This resulted in increased PHD yields of 3.87 and 6.18 g L\(^{-1}\) at 24 and 48 h, respectively, with a total yield of 7.35 g, or 62.0% of the theoretical maximum (Fig. 4.3BC). Biomass accumulation decreased slightly relative to LSBJ-2 in response to the additional fatty acid (Fig. 4.3A). To further support cell growth and PHA biosynthesis, the amount of yeast extract added to the culture medium was increased from 1.5 to 3.0 g (LSBJ-4, Fig. 4.3). This again resulted in increased PHD yields, up to 5.38 and 10.1 g L\(^{-1}\) at 24 h and 48 h, bringing the total yield to 10.6 g (89.7%). As the amount of decanoic acid was increased from 12 g to 25 g over subsequent trials, PHD accumulation continued to increase as well until reaching 20.1 g L\(^{-1}\) at 48 h, a total yield of 22.1 g (89.4%), at which point excessive foaming became an issue and the optimization process was halted (Fig. 4.3BC).

Purified polymers from each optimization experiment were analyzed by GPC to determine the number average molecular weight (\(M_n\)), weight average molecular weight (\(M_w\)), and polydispersity index (PDI, \(M_w/M_n\)) (Table 4.3). \(M_n\) values ranged from 28.6 to 64.3 kDa with no discernable trend and a high degree of variation. The polydispersity index was similarly variable, ranging from 2.3 to 3.9.
Figure 4.3: Optimization of PHD biosynthesis. (A) Biomass) and (B) PHD accumulation (g L^{-1}) at 24 (light grey) and 48 h (dark grey) from bioreactor fermentations. (C) Total PHD yield in grams (dark grey), displayed as an overlay of the maximum theoretical yield (light grey). All data shown is the mean of duplicate trials, plus 1 standard deviation about that mean, except for where n = 1.
Table 4.3: PHD Molecular Weight from Optimization Experiments.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>$M_n$ (kDa)</th>
<th>$M_w$ (kDa)</th>
<th>PDI ($M_w/M_n$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSBJ</td>
<td>28.6 ± 13.9</td>
<td>81.5 ± 54.1</td>
<td>2.7 ± 0.6</td>
</tr>
<tr>
<td>RSC02</td>
<td>64.3 ± 25.4</td>
<td>157 ± 23.7</td>
<td>2.6 ± 0.6</td>
</tr>
<tr>
<td>LSBJ CRP*</td>
<td>40.2 ± 6.4</td>
<td>127 ± 4.4</td>
<td>3.2 ± 0.4</td>
</tr>
<tr>
<td>LSBJ-2</td>
<td>31.6 ± 9.8</td>
<td>80.4 ± 28.7</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td>LSBJ-3</td>
<td>35.9 ± 4.8</td>
<td>81.0 ± 9.5</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td>LSBJ-4 (12g)</td>
<td>44.7 ± 4.7</td>
<td>125 ± 4.4</td>
<td>2.8 ± 0.4</td>
</tr>
<tr>
<td>LSBJ-4 (15g)</td>
<td>37.3 ± 0.1</td>
<td>117 ± 0.6</td>
<td>3.1 ± 0.0</td>
</tr>
<tr>
<td>LSBJ-4 (20g)</td>
<td>37.3 ± 4.8</td>
<td>103 ± 26.0</td>
<td>2.8 ± 0.4</td>
</tr>
<tr>
<td>LSBJ-4 (25g)</td>
<td>42.0 ± 3.7</td>
<td>164 ± 43.7</td>
<td>3.9 ± 0.7</td>
</tr>
</tbody>
</table>

4.4.3 Production of alternative MCL PHA

To further investigate the capabilities of the optimized high-density fermentation process, LSBJ harboring pBBRC1J4SII was supplemented with two other saturated MCL fatty acids in place of decanoic acid: hexanoic acid and octanoic acid (Trials 19 & 20, Table 4.2). Feed rates and fatty acid concentrations (20 g in 200 mL) were kept the same as Trial 17 to avoid the foaming issue observed at higher decanoic acid concentrations (Trial 18). High biomass accumulation was observed for both trials, similar to biomass yields obtained for Trial 17 (Fig. 4.4A). Both fatty acids were successfully converted into their corresponding polymers, PHHx and PHO, with yields of 18.2 and 17.3 g L\(^{-1}\) after 48 h, respectively, and followed a similar trend of increased PHA accumulation between 24 h and 48 h (Fig. 4.4B). Total polymer accumulation approached the maximum theoretical yield for both PHHx and PHO (99.0% and 90.8%) (Fig. 4.4C).
molecular weights of the resulting polymers were higher than those obtained for PHD, while the PDI remained consistent (Table 4.4).

Biosynthesis of functionalized PHAs, desirable materials that are more challenging to produce, was also attempted using the optimized high-density fermentation process (Trial 21 & 22, Table 4.2). Utilizing the same culture conditions and inoculum, 10-undecenoic acid and 10-bromodecanoic acid were supplemented to produce their corresponding polymers, poly(3-hydroxy-10-undecenoate) (PHU) and poly(3-hydroxy-10-bromodecanoate) (PHDBr) (Fig. 4.4). PHU yield was lower at 48 h compared to other polymers at 9.24 g L\(^{-1}\), although due to excessive foaming and subsequent antifoam addition the final culture volume was high and the total polymer accumulation was 13.2 g (66.8% of theoretical, Fig. 4.4BC). Similarly, 10-bromodecanoic acid caused excessive foaming and resulted in a lower yield and higher final volume, although to a lesser extent; PHDBr yield at 48 h was 14.3 g L\(^{-1}\) with a total accumulation of 18.0 g (90.7%). The \(M_n\) of PHU was similar to that of PHD at 39.1 kDa, while the \(M_n\) of PHDBr was low at 21.6 kDa (Table 4.4).

Biosynthesis of copolymers was also demonstrated using the high-density fermentation process by supplementing the feed with two fatty acids simultaneously. Octanoic acid was first mixed with decanoic acid (Trial 23), then with 10-azidodecanoic acid (Trial 24), both with 80 mol% octanoic acid (0.58 M total fatty acid). The copolymers poly(3-hydroxyoctanoate-co-3-hydroxydecanoate) (PHOD) and poly(3-hydroxyoctanoate-co-3-hydroxy-10-azidodecanoate) (PHODN\(_3\)) were both produced successfully, with yields at 48 h of 16.7 g L\(^{-1}\) and 18.7 g L\(^{-1}\) and total accumulation of 16.7 (97.4%) and 17.8 g (97.9%), respectively (Fig. 4.4BC). The \(M_n\) of PHOD was 105 kDa, and the \(M_n\) of PHODN\(_3\) was 47.1 kDa (Table 4.4).
Figure 4.4: Alternative MCL PHA biosynthesis. (A) Biomass) and (B) PHD accumulation (g L\(^{-1}\)) at 24 (light grey) and 48 h (dark grey) from bioreactor fermentations. (C) Total PHD yield in grams (dark grey), displayed as an overlay of the maximum theoretical yield (light grey). Trials were not performed with replicates and demonstrate proof-of-concept for expanding the possible substrates for the optimized high-density fermentation process.
Table 4.4: Alternative MCL PHA Molecular Weights

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Mn (kDa)</th>
<th>Mw (kDa)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHHx</td>
<td>117.8</td>
<td>334.9</td>
<td>2.8</td>
</tr>
<tr>
<td>PHO</td>
<td>66.3</td>
<td>192.3</td>
<td>2.9</td>
</tr>
<tr>
<td>PHU</td>
<td>39.1</td>
<td>132.9</td>
<td>3.4</td>
</tr>
<tr>
<td>PHDBr</td>
<td>21.6</td>
<td>70.5</td>
<td>3.3</td>
</tr>
<tr>
<td>PHOD</td>
<td>104.7</td>
<td>286.7</td>
<td>2.7</td>
</tr>
<tr>
<td>PHODN3</td>
<td>47.1</td>
<td>149.0</td>
<td>3.1</td>
</tr>
</tbody>
</table>

4.5 Discussion

The goal of this study was to develop a fed-batch high-density fermentation process capable of producing MCL PHA with controlled monomer compositions at a higher titer than that previously achieved in shake flasks. Based on the evidence in Fig. 4.3 and Fig. 4.4b, this process offers a significant improvement over previous shake flask studies, and a variety of MCL PHA homopolymers and copolymers were made successfully (Scheel et al., 2016; Tappel et al., 2012b).

In initial experiments with high-density fermentation of LSBJ, including those published here as well as unpublished data from our research group, we were unable to detect any evidence of PHA biosynthesis. Preliminary experiments sought to address this through a series of genetic engineering experiments, beginning with the creation of LSBJ CRP*. In LSBJ, which has a wild-type CRP, catabolite repression plays an important role in regulating primary and secondary metabolism (Franchini et al., 2015; Perrenoud and Sauer, 2005). In the bioreactor fermentation, which used glucose as the carbon source, we hypothesized that low levels of CRP-cAMP were preventing the expression of β-oxidation genes necessary for PHA biosynthesis, and that
conferring the CRP* mutation to LSBJ would alleviate this repression (Clark, 1981; Khankal et al., 2009; Wade et al., 2001). This was not found to be the case in initial experiments (data not shown), and further mutations were made to this strain in the subsequent preliminary experiments (Trials 1-8, Table 4.2, Fig. 4.2A).

Production of PLA-co-PHO, a novel copolymer that has generated research interest, was attempted in Trial 1 in conjunction with an ongoing project in the Nomura Research Group. Deletion of the dld gene from LSBJ CRP*, which encodes for D-lactate dehydrogenase and has been shown to prevent the accumulation of PLA in E. coli (Taguchi et al., 2008), did not result in any detectable PLA-co-PHO. Although attempts to incorporate lactate monomers were abandoned in subsequent trials, the Δdld mutant (SM02) was further engineered by deletion of the arcA gene. Deletion of arcA was previously shown to upregulate expression of β-oxidation genes and enhance MCL PHA biosynthesis, and we hypothesized that the removal of arcA combined with the CRP* mutation (SM04) would enable PHA biosynthesis during bioreactor fermentation (Scheel et al., 2016). Again, this was not supported by the evidence, with no detectable polymer observed (Trial 2).

We next hypothesized that the exogenous octanoic acid was being directed towards fatty acid biosynthesis rather than degradation, and that this could be prevented by deletion of the aas gene encoding for acyl-acyl carrier protein (acyl-ACP) synthetase. Acyl-ACP synthetase has a high affinity towards octanoate, and coupled with the increased membrane biosynthesis necessary for rapid culture growth during high-density fermentation it is possible that the octanoic acid was routed away from β-oxidation (Black and DiRusso, 2003; Ray and Cronan, 1976). However, the aas-deficient strain SM06 was unable to produce PHO from octanoic acid (Trial 3). Increasing the amount of octanoic acid available, both in bulk addition and continuous feeding strategies with
SM06, likewise did not result in polymer accumulation (Trial 4-6). A stronger promoter, generated by converting the lac promoter of pBBRC1J4SII into the tac promoter by mutagenesis of the -35 region, was hypothesized to enable PHA biosynthesis by increasing expression of phaJ4 and phaC1 (Trial 7). This did not work, however this is unsurprising as those two genes are under the control of a low-expressing constitutive Cupriavidus necator promoter inserted before the lac promoter of pBBRC1J4SII, and PHA biosynthesis has been demonstrated with this system numerous times during growth in shake flasks (Tappel et al., 2012b; Wang et al., 2012). Overexpression of the atoDAEB operon, which was previously shown to occur in the arcA-deficient LSBJ strain RSC02 (Scheel et al., 2016), was also unsuccessful in enabling PHA biosynthesis (Trial 8).

The effect of increasing the culture pH to 8.0 during Phase 2 is simultaneously the most profound and confounding result from this study (Trial 9). It was essential to the production of MCL PHA from exogenous fatty acids in LSBJ (Fig. 4.2C); maintaining the pH at 7.0 prevented PHA accumulation in all preliminary trials (Fig. 4.2A), as well as numerous trials not shown here. It is well-documented that growing E. coli to stationary phase in LB (and other tryptone-based media) commonly results in alkalinization, which explains the results of Fig. 4.2B and could explain why shake flask production is successful at producing MCL PHA. The literature does not offer any clear connections between MCL fatty acid uptake and catabolism at an alkaline pH, although there is a paucity of research in this area relative to growth conditions at a neutral pH (Maurer et al., 2005; Padan et al., 2005; Stancik et al., 2002; Yohannes et al., 2004). Despite no clear connections, several aspects of E. coli pH homeostasis in alkaline environments may have an indirect effect on PHA biosynthesis. Numerous transport systems are up-regulated to direct the H+ flow into the cell, and FadD does transport H+ concomitantly with fatty acids (Weimar et al.,
2002). No evidence of differential expression for *fadD* has been observed, however transcriptome studies can not capture proteins that are regulated at the activity level rather than expression, which is common for many Na\(^+/H^+\) antiporters (Maurer et al., 2005; Padan et al., 2005). Another contributing factor could be the repression of the flagellar biosynthesis and chemotaxis regulons, which are down-regulated extensively (47 out of 50 genes, pH 8.7) at an alkaline pH (Maurer et al., 2005). Repression of this energy-intensive process could have freed up cellular resources such as ATP for PHA biosynthesis. Further research into fatty acid uptake and metabolism would be necessary to determine the direct effects of pH on PHA biosynthesis, which falls far beyond the scope of this study.

Although successful, the PHD yield in Trial 9 did not offer an improvement over typical shake flask production. To test the earlier hypotheses of low CRP-cAMP inhibiting biosynthesis and repression by ArcA, we repeated the conditions used in Trial 9 with both RSC02 (Trial 10) and LSBJ CRP\(^*\) (Trial 11). No benefit to PHD yield was observed for either Trial; PHD weight percent was improved slightly, but this was offset by a reduced biomass (Fig. 4.3AB).

Reducing the rate of glucose addition during Phase 2 (Trial 12) increased the yield of PHD to 2.48 g L\(^{-1}\), greater than a 5-fold improvement (LSBJ-2, Fig. 4.3B). One explanation for this is the overabundance of glucose in previous trials and the subsequent increase in overflow metabolism. Overflow metabolism in *E. coli* occurs during high levels of aerobic glucose consumption, and results in the excretion of acetate which can become toxic and growth-limiting (Vemuri et al., 2006). This is particularly pronounced in *E. coli* K-12 strains, which LSBJ is derived from, due to an inactive or low-activity glyoxylate shunt (Noronha et al., 2000; Rand et al., 2017; Walle and Shiloach, 1998). An increase in biomass of approximately 50% provides additional evidence to support this explanation, however this biomass increase is not enough to
account for the magnitude of the increase in PHD yield (Fig. 4.3A). Another effect of the reduced glucose feed rate could be an increase in the cAMP pool resulting from increased adenylate cyclase activity due to decreased glucose transport (Notley-McRobb et al., 1997). This in turn would increase CRP-cAMP abundance and could lead to upregulation of *fadD* and *fadE*, resulting in enhanced PHD biosynthesis.

One issue that needed to be addressed was the apparent death of the bacterial culture during Phase 2, as evidenced by a reduction in biomass and lack of PHA accumulation between 24 and 48 h (Fig. 4.3AB), and by a marked decrease in oxygen consumption as early as 13 h until DO% remained nearly saturated (Fig. D1-6, Appendix D). Previous research has indicated that high ammonia concentrations during high-density fermentation inhibit the growth of *E. coli* and limit secondary metabolism, which could explain the results observed in this study (Lau et al., 2004; Riesenberg et al., 1991; Shiloach and Fass, 2005). Reformulating the culture medium and glucose feed based on low ammonia alternatives described by Lau et al. (2004) appeared to eliminate the issue of culture death (Trial 13); biomass and PHD yield increased between 24 and 48 h (Fig. 4.3AB), oxygen demand slowly increased over the entire fermentation (Fig. D7, Appendix D), and total PHD accumulation at 48 h reached 87.5% of the theoretical yield (Fig. 4.3C). In addition to drastically reducing the ammonium concentration, the new formulation contained more phosphate to support a higher cell density and more trace metals to ensure adequate cofactor abundance (Herbert et al., 1965; Neidhardt et al., 1974).

With a media formulation capable of supporting high-density fermentation and the majority of fatty acid substrate being converted to polymer, the next logical step was the optimization of the fatty acid feed; specifically, increasing the amount and rate of decanoic acid addition (Trials 14-18). Progressively increasing the amount of decanoic acid added to totals of 12 g, 15 g, 20 g,
and 25 g (addition rates of 0.44, 0.55, 0.65, and 0.75 g h⁻¹, respectively) correlated with an increase in PHD yield, culminating in a final titer of 20.1 g L⁻¹ (Fig. 4.3B). However, adding 25 g of decanoic acid resulted in excessive foaming likely as a result of exogenous decanoate behaving as a surfactant, and replication of this feeding strategy proved to be difficult due to uncontrollable foaming and loss of culture. Alternative strategies exist to control foam in an industrial bioprocess setting, such as mechanical disruption or ultrasound, but these are incompatible with the small benchtop vessel used in this study (Delvigne and Lecomte, 2009; Routledge, 2012). As such the optimization of the fatty acid feed was halted, and analysis of previous metabolic engineering strategies (CRP*, ΔarcA, etc.) was deemed unnecessary due to the physical nature of the foaming limitation. It is also important to note that the amount of yeast extract added to the initial culture medium was doubled to 3.0 g for Trial 15 and for each subsequent Trial. This was in response to a decrease in % yield when decanoic acid was increased to 12 g, believed to be due to an increased fatty acid toxicity. Fadzil et al. has previously shown that increasing the concentration of yeast extract for E. coli LSBJ cultures utilizing co-carbon sources (glucose and decanoic acid) increases their tolerance towards fatty acids and enhances MCL PHA biosynthesis (2018). Our results support this observation; increasing the concentration of yeast extract led to improved biomass, PHD yield, and decanoic acid utilization (89.7% yield) (LSBJ-4 (12g), Fig. 4.3).

The optimized high-density fermentation process developed here was also shown to be flexible with regard to fatty acid substrate; hexanoic acid, octanoic acid, 10-undecenoic acid, 10-bromodecanoic acid, and 10-azidodecanoic acid were all successfully polymerized in addition to decanoic acid (Fig. 4.4), with confirmation by NMR for the functionalized PHU, PHDBr, and PHODN₃ (Appendix A). Each alternative MCL PHA tested was produced at yields of 9.24 g L⁻¹ or higher and with no detectable co-monomers present except for the intentional copolymers
PHOD and PHODN₃. These copolymers contained monomer compositions closely resembling the mol% of the feed solution (81.8 and 80.4% 3HO, respectively), which is unsurprising given the high fatty acid utilization observed (approximately 97% for both) (Fig. 4.4C). This control over monomer composition is one of the primary benefits to using *E. coli* LSBJ as the production host, particularly over a wide range of fatty acid substrates (Tappel et al., 2012b). Although numerous high-density cultivation methods have been reported using native producers such as *Pseudomonas* species, only PHD has previously been produced at near-homopolymeric compositions and high titers, and there are no reported bioprocesses for the production of PHHx, PHO, PHU, or PHDBr homopolymers to the best of our knowledge (Follonier et al., 2015; Gao et al., 2018; Jiang et al., 2013; Oliveira et al., 2020; Sun et al., 2007).

The molecular weights varied for the PHAs produced, and the PDIs ranged from 2.3 to 3.9 (Table 4.3 & 4.4). The molecular weights (Mₙ) of PHD showed considerable variation, ranging between 28.6 and 64.3 kDa. This is comparable and slightly less than previous work performed in shake flasks, where Mₙ values of 61.5 to 111 kDa were observed (Mohd Fadzil et al., 2018; Tappel et al., 2012b). Similarly, the Mₙ value of PHU was slightly less than previously observed (39.1 vs. 60 kDa) (Tappel et al., 2012a), while the Mₙ of PHHx was higher than previous reports (118 vs. 77 kDa) (Scheel et al., 2016). Overall molecular weights are comparable to those from similar studies, however with the high degree of variation and lack of replicates for alternative MCL PHAs only limited comparisons can be made.

The high-density fermentation process described here was developed to allow for MCL PHA biosynthesis with controlled monomer composition at a higher yield than shake flask production can accommodate. The productivity shown here of PHD biosynthesis from 25 g decanoic acid (0.42 g L⁻¹ h⁻¹) rivals that of the highest published productivity for nearly
homopolymeric PHD (0.41 g L$^{-1}$ h$^{-1}$), although this work had issues with reproducibility (Gao et al., 2018). High MCL PHA yields were demonstrated from a variety of fatty acid substrates, both saturated and functionalized, showing that this is a versatile process capable of producing unique and valuable materials.

4.6 References


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Chapter 5: Progress towards a poly(3-hydroxyoctanoate-co-3-hydroxy-10-azidodecanoate) nanoparticle drug delivery system

5.1 Abstract

This chapter describes progress towards the production of nanoparticles made from azide-containing poly(3-hydroxyalkanote) copolymers (Azido PHAs), with the purpose of developing a targeted drug-delivery system. Two azido PHAs, poly(3-hydroxybutyrate-co-3-hydroxy-10-azidodecanoate) (PHBDN₃) and poly(3-hydroxyoctanoate-co-3-hydroxy-10-azidodecanoate) (PHODN₃), were biosynthesized in recombinant Escherichia coli RSC02 harboring PHA biosynthesis genes. Bacterial endotoxin was removed from isolated polymers by a combination of sodium hydroxide treatment and methanol precipitation, which were effective individually and in combination to reduce endotoxin below 0.01 endotoxin units per mg (EU mg⁻¹). Polymer molecular weights were largely unaffected by the sodium hydroxide treatment. Nanoparticles were iteratively produced by single emulsion or nanoprecipitation techniques to determine the most effective method for generating nanoparticles <100 nm in diameter. The ideal production method was nanoprecipitation of PHODN₃ dissolved in 15% ethanol/acetone solution and precipitated in Nanopure filtered water, which yielded particles with an average diameter of 70.6 nm. With this process, paclitaxel was encapsulated by PHODN₃ with an encapsulation efficiency as high as 80.8% when 1% w/w paclitaxel was administered, with a decrease in efficiency as the concentration of paclitaxel was increased. PLGA nanoparticles were prepared by the same process, wherein encapsulation efficiencies under 14.3% were achieved. Nanoparticle uptake kinetics and cytotoxicity experiments must still be performed with human cancer cells.
5.2 Introduction

Polymeric nanoparticles have received considerable attention in recent years as potential drug-delivery systems, particularly for use in targeted chemotherapy treatments (Hans and Lowman, 2002; Hillaireau and Couvreur, 2009; Lee et al., 2017). Nanoparticles are generally defined as spherical, solid particles approximately 100 nm in size. They have been produced using a variety of polymers, though most commonly with poly(lactide-co-glycolide) (PLGA) due to its biocompatibility and biodegradability into common metabolites (lactate and glycolate), commercial availability, and its approval by both the U.S. Food and Drug Administration (FDA) and European Medicine Agency (EMA) for use in therapeutic devices (extensively reviewed by Danhier et al., 2012).

Numerous nanoparticle targeting strategies have been investigated as a way to selectively administer drugs to target cells and reduce systemic toxicity, most commonly by the conjugation of targeting moieties to the exterior particle surface (Yu et al., 2012). These strategies include; antibodies such as anti-prostate stem cell antigen antibody and mutant anti-epidermal growth factor receptor antibody (Hadjipanayis et al., 2010; Ling et al., 2011), peptides and proteins like transferrin (Petrelli et al., 2010; Sarris et al., 2000), and small molecules such as riboflavin and folic acid (Foraker et al., 2003; Kennedy et al., 2003). Folic acid in particular has generated significant research interest due to its low cost and high availability, and more importantly because of the high folate-receptor expression observed in many types of cancerous cells (Bahrami et al., 2015). Folate receptor-α (FR-α) has a high binding affinity for folate and induces uptake by endocytosis when bound, and folate-conjugated nanoparticles have been shown to be similarly transported, offering a direct route for nanoparticle internalization (Paulos et al., 2004; Zhao et al., 2014).
Poly[(R)-3-hydroxyalkanoates] (PHAs) are another class of biodegradable, biocompatible polymers that are promising biomedical materials (Rai et al., 2011). These bacterially-derived polyesters have been investigated as a potential nanoparticle drug-delivery platform, with limited success; the predominant PHAs employed for nanoparticle synthesis included the short chain-length (SCL) PHA poly(3-hydroxybutyrate) and its copolymers containing 3-hydroxyvalerate, which were characterized by low drug encapsulation efficiency, large particle size (>160 nm), and poor drug retention (Poletto et al., 2008; Pramual et al., 2016; Xiong et al., 2010). These SCL PHAs also suffer from chemical intractability, making them difficult to functionalize with targeting moieties. Additionally, PHAs biosynthesized via gram-negative bacteria contain lipopolysaccharides (LPS, endotoxin), a component of the outer membrane of gram-negative bacteria that can induce a severe inflammatory response in humans known as endotoxic shock (Calvano and Coyle, 2012; Rietschel et al., 1994). Endotoxins are persistent, requiring additional purification steps to remove them from PHAs (Furrer et al., 2007; Lee et al., 1999).

Recently, a number of medium chain length (MCL) PHAs have been biosynthesized containing terminal alkene, alkyne, azide, and bromo functional groups (Nkrumah-Agyeefi and Scholz, 2017; Pinto et al., 2016; Tappel et al., 2012b, 2012a). These functional PHAs are produced in both native-producing (e.g. Pseudomonas sp.) and recombinant bacteria (e.g. Escherichia coli) from functionalized fatty acid substrates, which enter the β-oxidation pathway and are converted to 3-hydroxyacyl-CoA esters, and subsequently polymerized by a PHA synthase. These functional groups confer significant chemical reactivity to the polymer and are notably amenable to the so-called “Click” reactions, which are desirable due to their selectivity, high yield, stereospecificity, and benign reaction conditions (Kolb et al., 2001). In particular, the strain-promoted azide-alkyne cycloaddition (SPAAC) reaction has seen significant utilization as a conjugation method for
nanomedicine, owing to its simple reaction conditions (room temperature, aqueous solvents) and the omission of toxic copper-catalysts, summarized in Scheme 5.1 (Dommerholt et al., 2016).

Scheme 5.1: General strain-promoted alkyne-azide cycloaddition (SPAAC) reaction. For our studies, $R_1$ denotes the alkyl side-chain of azido-PHA monomers and $R_2$ denotes a di-benzyl substitution on the cyclooctyne ring (dibenzylcyclooctyne, DBCO).

The goal of this study was to utilize newly characterized copolymers poly(3-hydroxybutyrate-co-3-hydroxy-10-azidodecanoate) (PHBDN$_3$) and poly(3-hydroxyoctanoate-co-3-hydroxy-10-azidodecanoate) (PHODN$_3$) to develop a targeted nanoparticle drug delivery system based on folate receptor-mediated endocytosis and the model chemotherapeutic paclitaxel (Fig. 5.1) (Pinto et al., 2016).

**Figure 5.1:** Proposed synthesis of targeted nanoparticles for drug delivery. Azide-containing PHA copolymers (Azido PHA) will be used to encapsulate the chemotherapeutic agent paclitaxel (PTX, yellow dots). Folic acid analogs will be conjugated to surface-exposed azides (red dots) via strain-promoted azide-alkyne cycloaddition (SPAAC).
5.3 Methods

5.3.1 Bulk polymer biosynthesis

Azido-PHAs were biosynthesized as previously described (Pinto et al., 2016). In brief, *E. coli* RSC02 was transformed with pBBRC1J4SII by heat shock following standard procedures (Sambrook and Russell, 2001). A 50-mL seed culture (LB-Lennox, composition per liter: 10 g tryptone, 5 g yeast extract, 5 g sodium chloride) supplemented with 50 μg mL⁻¹ kanamycin sulfate was inoculated with LSBJ-pBBRC1J4SII and grown for 16 h at 37°C and 200 rpm rotary shaking. Growth media (6 × 400 mL LB in 2-L flasks) was supplemented with synthetic 10-azidodecanoic acid (2 mM) and either sodium octanoate or sodium butyrate (8 mM), along with kanamycin sulfate (50 μg mL⁻¹). The surfactant Brij-35 (4.0 g L⁻¹, Sigma-Aldrich) was added to ensure dissolution of the fatty acids. Growth flasks were inoculated with 0.5% (v/v) seed culture and grown for 48 h at 30°C and 250 rpm. Bacteria were harvested by centrifugation at 3,716 × g for 15 min in 800-mL buckets, washed once with 200-mL 40% ethanol and once with 200-mL Nanopure filtered water. Cell pellets were transferred to 50-mL centrifuge tubes and resuspended 5-15 mL Nanopure water, frozen, and dried *via* lyophilization.

Polymer was isolated from dried cells by Soxhlet extraction with 250 mL chloroform (HPLC Grade, Fisher Scientific) for 6 h, with the extract then concentrated by rotary evaporation. Polymer was purified *via* non-solvent precipitation by drop-wise addition to cold methanol (4°C) with moderate stirring, at a 1:10 ratio of chloroform:methanol. Precipitated polymer was collected by centrifugation at 8000 × g and 4°C for 20 min. Polymers were subjected to three rounds of methanol purification, then dried *in vacuo* and stored at 4°C until needed. Polymer was produced using these procedures as necessary to generate more material.
5.3.2 Endotoxin removal and analysis

Growth media for azido PHA endotoxin analysis experiments was composed of; LB, Brij-35 (4.0 gL\(^{-1}\)), kanamycin (50 mg L\(^{-1}\)), and the fatty acids 10-azidodecanoic acid (2 mM) and either sodium octanoate or sodium butyrate (8 mM). *E. coli* RSC02 was transformed with the pBBR-C1J4SII plasmid by heat shock, and grown on an LB-agar plate at 37°C for 16 hours with kanamycin selection. Individual colonies were picked to inoculate 5 mL LB seed cultures, in triplicate, and grown for 16 h at 37°C and 200 rpm rotary shaking. Seed cultures were used to inoculate triplicate groups of 6 baffled shake flasks each containing 100 mL of growth media at a final seed concentration of 0.5%, for a total of 18 flasks per copolymer. Flasks were cultured for 48 h at 30°C and 250 rpm.

Cells were harvested by centrifugation at 3,716 × g, washed once with 40 mL of 45% ethanol and then once with 40 mL of Nanopure water. Each 600 mL triplicate group was divided in half to have a 3 × 100 mL control group and a 3 × 100 mL sodium hydroxide (NaOH) treatment group. The control groups were resuspended in 5 mL of endotoxin-free water (Fisher Scientific) after harvest, frozen, and dried via lyophilization. The NaOH groups were resuspended in 35 mL of NaOH (0.2 M) in endotoxin-free water and incubated for 5 h at 37°C with 80 rpm reciprocal shaking, in a protocol adapted from Lee *et al.* (1999). The cell suspension was neutralized with endotoxin-free hydrochloric acid (HCl, 2 M), harvested by centrifugation at 6000 × g for 20 min, and washed once with endotoxin-free water before being frozen and dried via lyophilization.

From this point forward, all glassware was heat-treated at 250° for 1 h to destroy endotoxin, prior to coming in contact with polymer. Polymer from both the control and NaOH treated cells was isolated by Soxhlet extraction (150 mL chloroform, 6 h reflux), then transferred to tared 20-mL scintillation vials. A sample of this crude polymer was set aside (~20 mg) using flame-treated
steel implements, with the remainder being purified by non-solvent precipitation in cold methanol (4°C) with moderate stirring, at a 1:10 ratio of chloroform:methanol. The precipitated polymer was collected by centrifugation at 8000 × g and 4°C for 20 min, re-dissolved in chloroform, and transferred to tared scintillation vials. Each polymer underwent 3 cycles of methanol purification, with 20 mg samples removed each time for endotoxin testing. All samples were dried in vacuo to remove residual solvents before weighing and recorded masses were used for calculations.

Endotoxin testing was conducted using the Pierce™ LAL Chromogenic Endotoxin Quantitation Kit following the manufacturers recommended procedure. Endotoxin-free water was added to the dried polymer samples at a concentration of 40 μL mg⁻¹ of polymer and incubated at 37°C with 200 rpm rotary shaking for 24 h, adapted from previously described methods (Furrer et al., 2007; Lee et al., 1999). This water was used for the endotoxin assay (50 μL per sample). The absorbance was measured at 410 nm using a Synergy™ H4 microplate reader (BioTek), and endotoxin was quantified as endotoxin units (EU) using a calibration curve of known endotoxin concentrations.

5.3.3 Molecular weight determination

Both weight average (M_w) and number average (M_n) molecular weights were determined using gel permeation chromatography as described previously (Scheel et al., 2016). Polymer samples were dissolved in chloroform to an approximate concentration of 1.0 mg mL⁻¹ and passed through a 0.45 μm PTFE syringe filter. Samples were injected at a volume of 50 μL into a Shimadzu LC-20AD liquid chromatograph equipped with a Shimadzu SIL-20A autosampler, a Shimadzu CTO-20A column oven, and a Shimadzu RID-10A refractive index detector. Samples were passed through an 8 × 50 mm styrenedivinylbenzene (S-DVB) guard column (5 μm particles; Polymer Standards Service) and an 8 × 300 mm S-DVB analytical column (5 μm particles; mixed
bed porosity; max molecular weight $1 \times 10^6$ Da; Polymer Standards Service product sda083005lim). The column oven was maintained at 40 °C, and chloroform was used as the mobile phase at 1 mL min$^{-1}$. Polystyrene molecular weight standards with a narrow polydispersity index were employed for calibration. Data analysis was performed using Shimadzu LCSolution software.

5.3.4 Production of PHA nanoparticles

Numerous nanoparticle production strategies were tested using a variety of conditions, including two techniques (single emulsion-solvent evaporation and nanoprecipitation), two different surfactants, seven different organic solvent mixtures, and two different aqueous:organic ratios, which is summarized in Appendix B. The method that produced the smallest average diameter and the most stable nanoparticles (least aggregation) was used for subsequent paclitaxel encapsulation and conjugation experiments; this method is described in detail below.

Polymer solutions of PHODN$_3$ or 50:50 poly(DL-lactide-co-glycolide) (PLGA, purchased from DURECT) were prepared via nanoprecipitation by dissolusion in 85:15 acetone:ethanol to a 2.8 mg mL$^{-1}$ final concentration. The drug paclitaxel (PTX, LC Laboratories) was also dissolved in 85:15 acetone:ethanol to final concentrations of 0.028, 0.14, and 0.28 mg mL$^{-1}$, corresponding to 1%, 5%, and 10% w/w of the polymer employed. In quadruplicate, the PTX and polymer solutions were combined in equal volumes to produce 20 mL solutions, followed by slow drop-wise addition (~1 mL min$^{-1}$ by addition funnel) to a beaker containing vigorously stirred deionized water (20 mL; prefiltred through a 0.20 μm PES syringe filter). Paclitaxel-free nanoparticles were produced by combining the polymer solution with pure 85:15 acetone:ethanol and following the same procedure. The resulting solution was left to stir uncovered at room temperature for 24 h. The resulting aqueous heterogeneous suspension was purified, first through a plug of cotton, and
then by gel filtration chromatography (GFC). Aqueous nanoparticle suspensions were loaded (2.5 mL aliquots) onto PD-10 desalting columns containing 8.3 mL of Sephadex G-25 medium (GE Healthcare Life Sciences) and eluted by gravity with 3.5 mL Nanopure filtered water following manufacturer specifications.

Nanoparticle sizes were determined by dynamic light scattering (DLS) with a Zetasizer Nano ZS (Malvern) equipped with a 4.0 mW He-Ne laser (λ = 633 nm), operated at room temperature, and at a scattering angle of 173°. Polystyrene cells with 10 mm optical paths were used for DLS experiments, with ~1 mL sample volume and a concentration of polymer nanoparticle suspensions of 0.50–1.0 mg mL⁻¹. Each sample was analyzed in triplicate. Following DLS analysis, samples were either frozen and lyophilized for paclitaxel quantification or used in subsequent conjugation experiments.

5.3.5 Quantification of paclitaxel by HPLC

High performance liquid chromatography (HPLC) was performed with a Shimadzu LC2010A HT HPLC system equipped with a reverse phase Zorbax Eclipse XDB-C18 (4.6 x 15 mm, particle size 5 μm, Agilent) analytical column and a UV detector. Two mobile phases were employed, mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile). Lyophilized nanoparticle samples were dissolved in 1 mL of acetonitrile, injected (5 μL), and analyzed by isocratic elution consisting of 55% B over 10 min with a flow rate of 1 mL min⁻¹ while monitoring at 227 nm. Column oven was maintained at 40°C. Paclitaxel was quantified using Shimadzu’s LabSolutions LC software based on calibration curves generated with pure paclitaxel. Encapsulation efficiency (EE) was calculated as shown here:

\[
Encapsulation\ efficiency\ %\ (EE) = \left(\frac{\text{Mass drug loaded (mg)}}{\text{Mass of drug added to solution (mg)}}\right) \times 100
\]
5.3.6 Conjugation of PEG to PHODN₃ and PLGA nanoparticles

Dibenzocyclooctyne-poly(ethylene glycol) (DBCO-PEG, 5kDa, purchased from Nanocs) was conjugated to 5% of the azido moieties on PHODN₃ via the SPAAC reaction. The mol% of PHODN₃ was calculated by ¹H-NMR as described in Chapter 4. PHA was dissolved in 1.0 mL chloroform at a concentration of 100 mg mL⁻¹ and combined with 2.0 mL of DBCO-PEG solution (27 mg mL⁻¹). The reaction was carried out at room temperature for 1 h, and successful conjugation was determined by ¹H-NMR by the appearance of a multiplet peak at δ 4.5-4.0 corresponding to the two protons alpha to the triazole (Fig. A11, Appendix A). The resulting pegylated-polymer (PHODN₃-PEG) was dried in vacuo and used for nanoparticle production as described above.

PLGA was conjugated to PEG (5 kDa, Sigma Aldrich) as described by El-Gogary et al., with minor adaptations (2014). In brief, PLGA (500 mg, 0.12 mmol), N-hydroxysuccinimide (4 eq, Acros Organics), and N,N’-dicyclohexylcarbodiimide (4 eq, Acros Organics) were dissolved in 1.2 mL dry dichloromethane at room temperature. This solution was filtered (0.45 μm PTFE, Restek) and added to 1.05 eq PEG and stirred overnight. N,N-diisopropylethylamine (10 eq, Acros Organics) was added, and the solution was stirred for 48 h. The product was precipitated with cold diethyl ether and collected by centrifugation for 30 min at 3500 rpm. PLGA-PEG was verified by ¹H-NMR based on characteristic peaks at δ 5.3, 4.9, and 1.46 (PLGA) and δ 3.3 (PEG), as well as its high solubility in dimethyl sulfoxide (DMSO).

5.4 Results

5.4.1 Endotoxin removal and molecular weight analysis

The endotoxin content of PHBDN₃ and PHODN₃ was measured using a Limulus amoebocyte lysate chromogenic assay (LAL). Treatment with 0.2M NaOH was effective at removing nearly all endotoxin from both PHBDN₃ and PHODN₃, reducing the measured
endotoxin units (EU) per mg by 96% and 98%, respectively (Fig. 5.2A). Polymer molecular weight ($M_n$) remained relatively unchanged between the NaOH and control groups (Fig. 5.2B). Both polymers responded similarly to the treatment; endotoxin was drastically reduced in treated groups and molecular weights were unaffected.

![Figure 5.2](image)

**Figure 5.2:** (A) Endotoxin present (EU mg$^{-1}$) in crude PHBDN$_3$ (dark gray) and PHODN$_3$ (striped) as determined by the LAL chromogenic endpoint assay. The sodium hydroxide treatment (0.2 M NaOH) removed 0.22 EU mg$^{-1}$ (~96%) of detectable endotoxin compared to the untreated (Control) PHBDN$_3$, and 0.19 EU mg$^{-1}$ (~98%) was removed from PHODN$_3$. (B) Average molecular weight ($M_n$) of crude polymer samples as determined by GPC. Values shown in both (A) and (B) are an average of triplicate experiments, plus or minus the standard deviation around those averages.

The two polymers responded differently to successive methanol purifications. The endotoxin measured from the PHBDN$_3$ control group was reduced by 97% after the first methanol purification down to the same level as the NaOH group, while endotoxin measured from the PHODN$_3$ control remained unchanged until the final round of purification (Fig. 5.3AB). An
increase in endotoxin was observed for PHODN$_3$ after the second round of purification, which is believed to be due to external contamination (Fig. 5.3B). A minor decrease in $M_n$ was observed for both polymers in the NaOH treatment group, which was only observed after methanol purification (Fig. 5.3CD). However, molecular weights increased slightly by the third round of purification for the PHODN$_3$ control and PHBDN$_3$ NaOH groups. No data was collected for the thrice purified PHBDN$_3$ control, as the polymer samples became insoluble in chloroform.

**Figure 5.3:** Change in endotoxin content (EU mg$^{-1}$) and molecular weight ($M_n$) over successive methanol (MeOH) purifications for PHBDN$_3$ (A, C) and PHODN$_3$ (B, D). An increase in endotoxin for NaOH-treated PHODN$_3$ (light gray diamonds) was observed after the second methanol purification, believed to be due to contamination from an external source. Endotoxin content of control PHODN$_3$ (dark gray squares) remained high for the first 2 methanol purifications before decreasing dramatically. All values are an average of triplicate experiments, plus or minus the standard deviation around those averages.
5.4.2 Comparison of nanoparticle production methods

Numerous methodologies were tested to determine a reproducible production process for uniform nanoparticles <100 nm in diameter. More information on the initial methods used to generate nanoparticles can be found in Appendix B, and a summary of the nanoparticle sizes produced is shown in **Fig. 5.4**. Initial experiments with PHBDN₃ formed large aggregates (data not shown), and the remainder of the experiments were performed exclusively with PHODN₃. Particles produced by single emulsion in sodium dodecyl sulfate (SDS, 4.4 mg mL⁻¹) were much smaller than those produced in poly(vinyl alcohol) (PVA, 4.4 mg mL⁻¹) at 160.7 nm (**Fig. 5.4A**), and SDS was used in subsequent experiments. Varying the duration of ultrasonication had little effect on particle size (**Fig. 5.4B**) and reducing the polymer concentration to 15 or 7.6 mg mL⁻¹ only resulted in minor decreases (**Fig. 5.4C**). Particles produced by the nanoprecipitation method varied widely depending on the organic solvent system used, and most organic solvents resulted in particles of a large range (**Fig. 5.4D**). Particles were centrifuged and washed to remove residual SDS, which resulted in the precipitation of larger particles but not smaller ones, depending on the organic solvent used. The acetone/ethanol and dimethylformamide (DMF) solvent systems were chosen for further analysis (**Fig. 5.5**). Longer centrifugation times enabled the precipitation of larger particles produced using DMF, however the smaller particles remained in suspension, and the acetone/ethanol mixture produced smaller particles overall. Replacing the SDS solution with pure water and using acetone/ethanol as the organic solvent generated the smallest particles yet, and with the narrowest polydispersity. No particles could be precipitated by centrifugation, however the removal of surfactant eliminates the need for a wash step.
Figure 5.4: Survey of PHODN₃ nanoparticle production methods by size. Particles produced by single emulsion via ultrasonication (A-C) were selected for smallest size using an iterative approach. (A) The surfactants poly(vinyl alcohol) (PVA) and sodium dodecyl sulfate (SDS) were compared first, and SDS selected for subsequent experiments. (B) Effect of varying ultrasonication durations, 50 sec duration used in prior and subsequent experiments. (C) Effect of PHODN₃ concentration on size. Particles produced by nanoprecipitation (D) were compared according to organic solvent system and by the ratio of organic solvent to aqueous SDS (4.4 mg mL⁻¹) solution (1:1 or 1:10). Particles in panel D were measured first by DLS after 24 h (SDS, striped) and after pelleting by centrifugation and resuspending in water (Pellet, light gray). Particles suspended in the supernatant after centrifugation were also analyzed (Supernatant, dark gray). Abbreviations: tetrahydrofuran (THF), acetone (ace), ethanol (EtOH), dimethyl sulfoxide (DMSO), dimethylformamide (DMF). Both Ace/DMSO and Ace/EtOH contained 85% acetone (v/v).
**Figure 5.5**: Size comparison of PHODN$_3$ nanoparticles produced by nanoprecipitation. Polymers dissolved in DMF and acetone/ethanol (Ace/EtOH, 85% acetone v/v) were added dropwise to aqueous SDS solutions (4.4 mg mL$^{-1}$) and centrifuged after 24 h. Particle size was measured by DLS for both the supernatant and the resuspended pellet. Polymers dissolved in acetone/ethanol (Ace/EtOH, 85% acetone v/v) were also added dropwise to pure water, which produced uniform particles measuring 70.6 nm and no pelleted particles. Data shown are the average of triplicate experiments, plus or minus the standard deviation around those averages.

5.4.3 Paclitaxel encapsulation efficiency

PHODN$_3$ and PLGA nanoparticles, along with their pegylated counterparts, were compared with regard to their ability to encapsulate PTX at three loading concentrations (Fig. 5.6). PHODN$_3$ was far more effective at encapsulating PTX at all three concentrations, and un-pegylated PHODN$_3$ encapsulated approximately 80% of PTX when it was combined at 1% (w/w). All polymers tested were less effective at encapsulating PTX as the loading concentration increased.
Figure 5.6: Encapsulation efficiency of polymeric nanoparticles. Particles were prepared using the nanoprecipitation method and varying concentrations of paclitaxel (PTX). Data shown are the average of quadruplicate experiments, plus or minus the standard deviation around those averages.

5.5 Discussion

The NaOH treatment was very effective at reducing endotoxin toxin levels, bringing these values to below 0.01 EU mg\(^{-1}\), a decrease of approximately 97% compared to the control (Fig. 5.2A). At this concentration of endotoxin, up to 71.4 mg of PHBDN\(_3\) or 125 mg of PHODN\(_3\) per mL could be administered intravenously under the FDA guidelines of 0.5 EU mL\(^{-1}\), with a maximum of 2.8 g or 5.0 g per dose, respectively (20 EU/device limit) (U.S. Food and Drug Administration, 2016). However, additional purification is necessary to remove other hydrophobic contaminants such as lipids and proteins, which can be achieved by precipitation in a non-solvent such as methanol (Sevastianov et al., 2003). Precipitation in methanol appeared to remove endotoxin quite readily from the PHBDN\(_3\) control group, which was reduced to the same EU mg\(^{-1}\) as the NaOH group with a single purification step (Fig. 5.3A). This phenomenon was not observed with PHODN\(_3\); the endotoxin was only removed from the control after the final purification (Fig. 5.3B). It is unclear why endotoxin remained associated with PHODN\(_3\) through
several rounds of purification; it is possible that the lipid A component of LPS formed stronger hydrophobic interactions with the longer side chains of 3-hydroxyoctanoate monomers in comparison with the short side chains of 3-hydroxybutyrate, although this speculation is based solely on the structural characteristics of LPS (Rietschel et al., 1994).

As a polyester, PHAs can be susceptible to backbone cleavage under basic conditions. To determine the effects of our 0.2 M NaOH treatment, we used GPC to analyze the polymer molecular weights with and without treatment (Fig. 5.2B). We found that there was no change in molecular weight observed for the crude polymer after treatment. As the polymers were successively purified by methanol precipitation, the molecular weights of the PHODN₃ control group increased incrementally, likely as a result of the loss of lower molecular weight species (Fig. 5.3A). A similar trend was observed for the PHBDN₃ NaOH group (Fig. 5.3C). Based on the inconsequential changes observed in the molecular weight post-NaOH treatment, and the efficacy of both methods for removing endotoxin, both purification methods were deemed viable options for low-endotoxin nanoparticle production.

One of the key characteristics of nanoparticle drug-delivery platforms is their size. The circulation time (Ahmed et al., 2005; Hoshyar et al., 2016), rate and mechanism (diffusion, endocytosis, or phagocytosis) of cellular uptake and/or expulsion (Lu et al., 2009; Peruzynska et al., 2015), and intracellular localization (Wu et al., 2019) of nanoparticles have all been shown to be highly dependent on size, with differences observed for sizes in a range as small as 20 nm. Optimal size depends on the target cell type and the desired route of internalization, however most of these studies concluded that nanoparticles of approximately 90-100 nm show desirable internalization efficiency. Over numerous iterative experiments comparing nanoparticle production methods, we were able to determine the ideal conditions for producing PHODN₃
nanoparticles of a consistent, uniform size (Fig. 5.4 & 5.5). Nanoprecipitation of our polymer into pure water not only resulted in a desirable particle size, it also eliminated time-consuming surfactant removal steps and the use of poorly scalable techniques such as ultrasonication and centrifugation.

The PHODN$_3$ far outperformed the PLGA, regarded as the gold standard for polymeric nanoparticles, in its ability to encapsulate paclitaxel in this study (Fig. 5.6). PLGA has been shown to be capable of higher efficiency with respect to PTX encapsulation, with efficiencies of approximately 70-75% reported (Betancourt et al., 2009; Danhier et al., 2009; Wang et al., 2011). These studies differed in the techniques used to prepare nanoparticles (single emulsion, double emulsion, and nanoprecipitation), the organic and aqueous solutions employed, and the use of polymer blends, demonstrating that drug encapsulation is dependent on multiple factors. These studies also produced nanoparticles larger than the sizes achieved here with PHODN$_3$, ranging from 112-240 nm, which as described above is an important factor in cell uptake. Further characterization of our nanoparticles with regard to cellular uptake and drug release is needed to determine their efficacy.

The data presented in this chapter is largely preliminary, setting the foundation for a new polymeric drug delivery system. The next steps needed are to evaluate the shelf-life stability of PTX-loaded PHODN$_3$ nanoparticles and the release of PTX over time, the in vitro cellular uptake kinetics of folate-conjugated nanoparticles by human cancer cells, and the cytotoxicity of these nanoparticles. Currently, work is underway assessing cellular uptake of folate-labelled nanoparticles by human breast cancer cells, which will be analyzed by live-culture fluorescence microscopy (IncuCyte®, Sartorius) with fluorophore-conjugated nanoparticles. Once cell uptake kinetics have been established, cell viability after exposure to paclitaxel-loaded nanoparticles will
be assessed to determine the cytotoxicity, and these experiments are in the preliminary stages of study design.

5.6 References


Chapter 6: Conclusions and future outlook

6.1 Discussion

The most significant finding of this dissertation is that Escherichia coli LSBJ is able to produce homopolymeric MCL PHAs from fatty acid substrate at yields approaching the theoretical maximum using an optimized fed-batch, high-density fermentation process. Although other recent works using engineered Pseudomonas putida KT2440 have achieved similar yields for near-homopolymeric PHD (highest to date; 11.8 g L\(^{-1}\), productivity of 0.41 g L\(^{-1}\) h\(^{-1}\)), none have succeeded in expanding this compositional control to other MCL PHAs (Gao et al., 2018; Oliveira et al., 2020). Furthermore, no other described processes have successfully produced PHAs from functionalized fatty acids (10-undecenoic acid, 10-bromodecanoic, and 10-azidodecanoic) at the yields demonstrated in Chapter 4. This has the considerable potential to expand the production of PHAs for biomedical materials, which require tightly controlled material properties and can benefit greatly from chemically reactive moieties (Hazer et al., 2012; Kai and Loh, 2014; Rai et al., 2011).

Removal of the arcA transcriptional regulator from E. coli LSBJ was shown to enhance both SCL and MCL PHA biosynthesis from the respective fatty acid substrates (Chapter 2). This is believed to have occurred due to the increased expression of the β-oxidation genes *fadD*, *fadE*, and *fadL* in the mutant strain, which saw relative increases of 2-fold, 31-fold, and 10-fold compared to E. coli LSBJ, respectively (Scheel et al., 2016). Interestingly, the expression of the *atoDAEB* operon (implicated in SCL fatty acid uptake and activation) was greatly upregulated despite no previously reported interactions between the ArcA and Ato systems (Cho et al., 2006; Theodorou et al., 2011, 2006). These results suggest that ArcA-mediated transcriptional regulation plays a role in PHA biosynthesis in E. coli, and that crosstalk between ArcA and SCL fatty acid
metabolism may be more complex than previously assumed. Removal of the OmpR regulator was also shown to deleteriously affect PHA biosynthesis, likely due to a toxic phenotype.

The use of lignocellulosic waste substrates as bacterial feedstock has experienced significant interest in recent years due to growing concerns about environmental sustainability (H. Isikgor and Remzi Becer, 2015; Zhang, 2008). One such substrate, enzymatically hydrolyzed paper mill waste fines, was a surprisingly effective feedstock for the biosynthesis of PHB and PHB-co-MCL PHAs in E. coli LSBJ, discussed in Chapter 3 (Min et al., 2015; Scheel et al., 2019). Both PHB and PHB-co-MCL PHA yields increased by approximately 100% when pure sugar substrates were replaced with hydrolysate, although the MCL monomer content was reduced.

Progress towards the nanoparticle drug delivery system discussed in Chapter 5 is still underway, with promising preliminary results presented therein. The field of nanomedicine, of which nanoparticle drug delivery is a subset, is rapidly expanding but limited in scope to a narrow selection of materials including PLGA and PEG (Danhier et al., 2012; Lee et al., 2017; Santander-Ortega et al., 2010). Previous studies investigating PHB and PHBV nanoparticles found them to be inferior materials compared to PLGA and PLGA-based blends, which is why the objective of Chapter 5 was to produce superior particles using functional MCL PHAs (Pinto et al., 2016; Poletto et al., 2008; Pramual et al., 2016). Future experiments are needed to evaluate the shelf-life stability of PTX-loaded PHODN3 nanoparticles and the release of PTX over time, the in vitro cellular uptake kinetics of folate-conjugated nanoparticles by human cancer cells, and the cytotoxicity of these nanoparticles. The results of these experiments will ultimately determine the efficacy of this novel drug-delivery platform.

Limitations in the production of MCL PHAs are largely due to expensive feed stocks, limited bioprocess productivity, and difficulty in controlling the monomeric composition to
achieve desired material properties (Blunt et al., 2019; Hazer et al., 2012; Rai et al., 2011). The work presented here has made some advancements in this field, although the current dependence on expensive fatty acid substrates limits the applicability to higher-performance biomedical materials rather than more common consumer plastics (Kai and Loh, 2014; Sparks and Scholz, 2009). Further modification to the bioprocess described in Chapter 4 to allow the production of SCL monomers such as 3HB or 4HB from glycolytic metabolites, while simultaneously incorporating desirable MCL monomers from fatty acid substrates, would go a long way towards expanding the availability of these novel materials.

6.2 References


Appendix A: Nuclear Magnetic Resonance Spectroscopy Data

The data presented in this appendix are from nuclear magnetic resonance (NMR) experiments performed to characterize PHAs and other small molecules as described in each appropriate chapter. Unless otherwise specified, $^1$H NMR spectra were recorded on a Bruker AVANCE III 600 MHz instrument, and were calibrated using residual undeuterated solvents as internal reference (chloroform, $\delta = 7.26$ ppm; water, $\delta = 4.79$ ppm). Chemical shifts ($\delta$) are reported in parts per million (ppm); NMR peak multiplicities are denoted by the following abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, sext = sextet, dd = doublet of doublets, dt = doublet of triplets, m = multiplet, br = broad. Spectra were processed with Bruker TopSpin v3.5pI2.

Fig. A1: $^1$H-NMR (600 MHz, CDCl3) spectrum of poly(R-3-hydroxybutyrate) (PHB) extracted from E. coli RSC02; $\delta$ 5.28-5.23 (sext, 1H), 2.62-2.45 (m, 2H), 1.28-1.25 (d, 3H).
**Fig. A.2:** 1H-NMR (600 MHz, CDCl3) spectrum of poly(R-3-hydroxyvalerate) extracted from E. coli RSC02; δ 5.17-5.13 (p, 1H), 2.59-2.50 (m, 2H), 1.66-1.59 (m, 2H), 0.91-0.88 (t, 3H). The asterisk (*) at δ 1.53 denotes a water impurity (Fulmer et al., 2010).

**Fig. A3:** 1H-NMR (600 MHz, CDCl3) spectrum of poly(R-3-hydroxyhexanoate) extracted from E. coli RSC02; δ 5.21-5.18 (p, 1H), 2.59-2.48 (m, 2H), 1.60-1.54 (m, 2H), 1.35-1.27 (m, 2H), 0.91 (t, 3H). The asterisk (*) at δ 1.53 denotes a water impurity (Fulmer et al., 2010).
**Fig. A4:** 1H-NMR (600 MHz, 50% D2O) of crude hydrolysate in a 1:10 dilution with glucosamine as an internal standard; δ 5.45 (d, glucosamine α-anomeric H), δ 5.24-5.23 (d, glucose α-anomeric H), δ 5.20-5.19 (d, xylose α-anomeric proton), δ 4.95-4.94 (d, glucosamine β-anomeric H), δ 4.65-4.64 (d, glucose β-anomeric H), δ 4.57 (d, xylose β-anomeric H), δ 1.97 (s, 3H from acetate).

**Fig. A5:** 1H-NMR (600 MHz, D2O) of poly(γ-glutamic acid) (PGA); δ 4.41 (m, 1H), δ 3.08-2.88 (dd, unknown H), δ 2.46 (m, 2H), δ 2.11-1.99 (doublet of broad multiplets, 2H).
**Fig. A6:** COSY spectra (600 MHz, D2O) of poly(γ-glutamic acid). Determined that the unknown shift at δ 3.08-2.88 (dd, Fig. A.5) was not connected to the polymer.
Fig. A7: 1H-NMR (600 MHz, CDCl3) spectrum of poly(R-3-hydroxy-10-undecenoate) extracted from E. coli LSBJ after bioreactor fermentation; δ 5.83-5.76 (m, 1H), 5.21-5.18 (p, 1H), 5.00-4.92 (dd, 2H), 2.59-2.48 (m, 2H), 2.05-2.01 (q, 2H), 1.60-1.54 (m, 2H), 1.38-1.28 (m, 8H).

Fig. A8: 1H-NMR (600 MHz, CDCl3) spectrum of poly(R-3-hydroxy-10-bromodecanoate) extracted from E. coli LSBJ after bioreactor fermentation; δ 5.21-5.18 (p, 1H), 3.37 (t, 2H), 2.59-2.48 (m, 2H), 1.84-1.82 (p, 2H), 1.60-1.54 (m, 2H), 1.35-1.27 (m, 2H), 1.28 (m, 6H). The asterisk (*) at δ 3.43 denotes a methanol impurity (Fulmer et al., 2010).
Fig. A9: $^1$H-NMR (800 MHz, CDCl$_3$) spectrum of poly(R-3-hydroxyoctanoate-co-3-hydroxy-10-azidodecanoate) extracted from E. coli LSBJ after 24 h bioreactor fermentation; δ 5.19-5.17 (p, 1H), 3.26-3.24 (t, 1H), 2.58-2.48 (m, 2H), 1.59-1.58 (m, 2H), 1.36-1.25 (m, 6H/8H), 0.89-0.87 (t, 3H).

Fig. A10: $^1$H-NMR (800 MHz, CDCl$_3$) spectrum of poly(R-3-hydroxyoctanoate-co-3-hydroxy-10-azidodecanoate) extracted from E. coli LSBJ after 48 h bioreactor fermentation; δ 5.19-5.17 (p, 1H), 3.26-3.24 (t, 1H), 2.58-2.48 (m, 2H), 1.59-1.58 (m, 2H), 1.36-1.25 (m, 6H/8H), 0.89-0.87 (t, 3H).
Fig. A11: $^1$H-NMR (600 MHz, DMSO-d$_6$) spectrum of poly(R-3-hydroxyoctanoate-co-3-hydroxy-10-azidodecanoate)-graft-dibenzocyclooctyne-poly(ethylene glycol methyl ether), prepared by strain-promoted azide-alkyne cycloaddition as described in Chapter 5.

References

Appendix B: Nanoparticle Methods Development

This appendix contains supplemental information on the methodologies used to produce nanoparticles in Figure 5.4 and Figure 5.5.

Nanoparticle production by single emulsion

Nanoparticles produced for Figure 5.4ABC were produced using a single emulsion technique adapted from (Budhian et al., 2007; Mainardes and Evangelista, 2005). Poly[(R)-3-hydroxyoctanoate-co-(R)-3-hydroxy-10-azidodecanoate] (PHODN₃) was dissolved in 0.5 mL chloroform, then added to 1 mL of surfactant solution (4.44 mg mL⁻¹, sodium dodecylsulfate or poly[vinyl alcohol]) while continuously mixing vigorously by vortex. This solution was then sonicated using a Branson Analog Sonifier 250A fitted with a 1/8” microtip at an output of 4 for 50 seconds (unless otherwise noted), with 10 second on/off pulses. The test tube containing the emulsion was kept in an ice bath during the off cycles.

The sonicated emulsion solution was transferred to 45 mL of surfactant solution (SDS or PVA, the same as the initial solution) and stirred for 3 hours to evaporate the organic solvent. The resulting particles were analyzed by DLS, without the centrifugation and cleaning procedure utilized for later methods.

Nanoparticle production by nanoprecipitation

Nanoparticles prepared by nanoprecipitation followed a similar procedure as that described in Chapter 5, but on a smaller scale and with additional cleaning steps (Chang et al., 2009; Cheng et al., 2007). PHODN₃ was dissolved in one of several organic solvent systems: tetrahydrofuran (THF), dioxane, acetone, 15% dimethyl sulfoxide (DMSO) in acetone, 15% ethanol in acetone, and dimethylformamide. Polymer solutions were added dropwise to rapidly stirring SDS solutions (4.44 mg mL⁻¹) in one of two ratios; for a 1:1 ratio, 2 mL of 1 mg mL⁻¹ polymer solution was added
to 2 mL SDS solution, and for a 1:10 ratio 1 mL of 1 mg mL\(^{-1}\) polymer solution was added to 10 mL SDS. The combined solutions were stirred for 24 hours to evaporate the organic solvents.

To remove the SDS from the nanoparticles, 1 mL of nanoparticle solution was transferred to a 1.5 mL microcentrifuge tube and centrifuged for 30 min at 16,100 \(\times\) g (Eppendorf 5415R). The supernatant was carefully removed by aspiration and analyzed by DLS, while the pelleted nanoparticles were resuspended in 1 mL Nanopure water and centrifuged again. This supernatant was discarded, and the pellets resuspended in 1 mL water for DLS analysis. For 1:10 samples, 4 aliquots were centrifuged, resuspended in 0.25 mL, and combined to obtain more nanoparticles. Both the initial supernatant and the cleaned nanoparticle solutions were filtered through 0.2 um Nylon filters prior to DLS.

References


https://doi.org/10.1016/j.ijpharm.2004.11.027
Appendix C: Gel permeation chromatography data

The weight average (Mw) and number average (Mn) molecular weights for each sample were determined by gel permeation chromatography (GPC) as described previously (Pinto et al., 2016). Briefly, PHA solutions of approximately 1.0 g L\(^{-1}\) were prepared by dissolution in chloroform and passed through a syringe filter (0.45 μm PTFE). Samples were injected (50 μL) into a Shimadzu LC-20AD liquid chromatograph equipped with a Shimadzu SIL-20A autosampler, a Shimadzu CTO-20A column oven, and a Shimadzu RID-10A refractive index detector. Samples were passed through an 8 x 50 mm styrenedivinylbenzene (SDV) guard column (5 μm particles; Polymer Standards Service) and an 8 x 300 mm SDV analytical column (5 μm particles; mixed bed porosity; max molecular weight 1E6 Da; Polymer Standards Service product sda0830051lim). The column oven was maintained at 40°C with a 1 mL min\(^{-1}\) mobile phase of chloroform. Molecular weight standards of polystyrene with a narrow polydispersity index were used for calibration. Shimadzu’s LCsolution software was used to analyze the data, and these sample chromatograms were generated using RStudio. Figs. C1-C7 show data generated from experiments in Chapter 2, Figs. C8-C19 were generated from experiments in Chapter 4.

Fig. C1: GPC chromatogram of purified poly(3-hydroxybutyrate) (C4) produced by RSC02, plotted as normalized intensity vs retention time. No data available for LSBJ.
**Fig. C2:** GPC chromatogram of purified poly(3-hydroxyvalerate) (C5) produced by LSBJ (A) and RSC02 (B), plotted as normalized intensity vs retention time.

**Fig. C3:** GPC chromatogram of purified poly(3-hydroxyhexanoate) (C6) produced by LSBJ (A) and RSC02 (B), plotted as normalized intensity vs retention time.
Fig. C4: GPC chromatogram of purified poly(3-hydroxyheptanoate) (C7) produced by LSBJ (A) and RSC02 (B), plotted as normalized intensity vs retention time.

Fig. C5: GPC chromatogram of purified poly(3-hydroxyoctanoate) (C8) produced by LSBJ (A) and RSC02 (B), plotted as normalized intensity vs retention time.
**Fig. C6:** GPC chromatogram of purified poly(3-hydroxydecanoate) (C10) produced by LSBJ (A) and RSC02 (B), plotted as normalized intensity vs retention time.

**Fig. C7:** GPC chromatogram of purified poly(3-hydroxydodecanoate) (C12) produced by LSBJ (A) and RSC02 (B), plotted as normalized intensity vs retention time.
Fig. C8: GPC chromatogram of purified poly(3-hydroxydecanoate) produced by LSBJ via fed-batch fermentation (Trial 9A). Data plotted as normalized intensity vs retention time.

Fig. C9: GPC chromatogram of purified poly(3-hydroxydecanoate) produced by RSC02 via fed-batch fermentation (Trial 10B). Data plotted as normalized intensity vs retention time.
Fig. C10: GPC chromatogram of purified poly(3-hydroxydecanoate) produced by LSBJ via fed-batch fermentation (Trial 12A). Data plotted as normalized intensity vs retention time.

Fig. C11: GPC chromatogram of purified poly(3-hydroxydecanoate) produced by LSBJ via fed-batch fermentation (Trial 13A). Data plotted as normalized intensity vs retention time.
Fig. C12: GPC chromatogram of purified poly(3-hydroxydecanoate) produced by LSBJ via fed-batch fermentation (Trial 15A). Data plotted as normalized intensity vs retention time.

Fig. C13: GPC chromatogram of purified poly(3-hydroxydecanoate) produced by LSBJ via fed-batch fermentation (Trial 16A). Data plotted as normalized intensity vs retention time.
**Fig. C14:** GPC chromatogram of purified poly(3-hydroxydecanoate) produced by LSBJ via fed-batch fermentation (Trial 17A). Data plotted as normalized intensity vs retention time.

**Fig. C15:** GPC chromatogram of purified poly(3-hydroxydecanoate) produced by LSBJ via fed-batch fermentation (Trial 18A). Data plotted as normalized intensity vs retention time.
Fig. C16: GPC chromatogram of purified poly(3-hydroxyhexanoate) produced by LSBJ via fed-batch fermentation (Trial 19A). Data plotted as normalized intensity vs retention time.

Fig. C17: GPC chromatogram of purified poly(3-hydroxyoctanoate) produced by LSBJ via fed-batch fermentation (Trial 20A). Data plotted as normalized intensity vs retention time.
Fig. C18: GPC chromatogram of purified poly(3-hydroxy-10-undecenoate) produced by LSBJ via fed-batch fermentation (Trial 21A). Data plotted as normalized intensity vs retention time.

Fig. C19: GPC chromatogram of purified poly(3-hydroxy-10-bromodecanoate) produced by LSBJ via fed-batch fermentation (Trial 22A). Data plotted as normalized intensity vs retention time.
References

Appendix D: Bioreactor Run Parameters

The representative data presented in this appendix are from the dissolved oxygen (DO), pH, temperature (T), agitation speed, and air sparging rate which were monitored and recorded every 30 seconds during each fermentation. One replicate per Trial (starting with 9) is shown.

**Fig. D1:** Trial 9A. Bioreactor fermentation of *E. coli* LSBJ harboring pBBRC1J4SII, with 6 g decanoic acid added at 0.22 g h\(^{-1}\).
Fig. D2: Trial 9B. Bioreactor fermentation of *E. coli* LSBJ harboring pBBRC1J4SII, with 6 g decanoic acid added at 0.22 g h⁻¹.
Fig. D3: Trial 10A. Bioreactor fermentation of *E. coli* RSC02 harboring pBBRC1J4SII, with 6 g decanoic acid added at 0.22 g h⁻¹.
Fig. D4: Trial 11A. Bioreactor fermentation of *E. coli* LSBJ CRP* harboring pBBRC1J4SII, with 6 g decanoic acid added at 0.22 g h⁻¹.
Fig. D5: Trial 12A. Bioreactor fermentation of *E. coli* LSBJ harboring pBBRC1J4SII, with 6 g decanoic acid added at 0.22 g h$^{-1}$. Rate of glucose feed slowed to 9.7 mL h$^{-1}$ during phase 2.
Fig. D6: Trial 13A. Bioreactor fermentation of *E. coli* LSBJ harboring pBBRC1J4SII, with 6 g decanoic acid added at 0.22 g h\(^{-1}\). Media formulation changed to that described in Chapter 4, *high-density fed-batch production*. Rate of glucose feed slowed to 9.7 mL h\(^{-1}\) during phase 2. First run where culture survived for the entire 48 h (O\(_2\) demand slowly increased between 13 and 48 h).
Fig. D7: Trial 14. Bioreactor fermentation of *E. coli* LSBJ harboring pBBRC1J4SII, with 12 g decanoic acid added at 0.44 g h\(^{-1}\). Media described in Chapter 4, *high-density fed-batch production*. Rate of glucose feed slowed to 9.7 mL h\(^{-1}\) during phase 2.
Fig. D8: Trial 15A. Bioreactor fermentation of *E. coli* LSBJ harboring pBBRC1J4SII, with 12 g decanoic acid added at 0.44 g h\(^{-1}\). Media described in Chapter 4, *high-density fed-batch production*, with 3.0 g of yeast extract. Rate of glucose feed slowed to 9.7 mL h\(^{-1}\) during phase 2.
Fig. D9: Trial 16A. Bioreactor fermentation of *E. coli* LSBJ harboring pBBRClJ4SII, with 15 g decanoic acid added at 0.55 g h\(^{-1}\). Media described in Chapter 4, *high-density fed-batch production*, with 3.0 g of yeast extract. Rate of glucose feed slowed to 9.7 mL h\(^{-1}\) during phase 2.
Fig. D10: Trial 17A. Bioreactor fermentation of *E. coli* LSBJ harboring pBBRC1J4SII, with 20 g decanoic acid added at 0.65 g h\(^{-1}\). Media described in Chapter 4, *high-density fed-batch production*, with 3.0 g of yeast extract. Initial rate of glucose feed reduced to 12.9 mL h\(^{-1}\), slowed to 8.7 mL h\(^{-1}\) during phase 2.
Fig. D11: Trial 18. Bioreactor fermentation of *E. coli* LSBJ harboring pBBRC1J4SII, with 25 g decanoic acid added at 0.75 g h$^{-1}$. Media described in Chapter 4, *high-density fed-batch production*, with 3.0 g of yeast extract. Initial rate of glucose feed reduced to 11.4 mL h$^{-1}$, slowed to 7.7 mL h$^{-1}$ during phase 2.
Fig. D12: Trial 19. Bioreactor fermentation of *E. coli* LSBJ harboring pBBRC1J4SII, with 20 g hexanoic acid added at 0.65 g h\(^{-1}\). Media described in Chapter 4, *high-density fed-batch production*, with 3.0 g of yeast extract. Initial rate of glucose feed reduced to 12.9 mL h\(^{-1}\), slowed to 8.7 mL h\(^{-1}\) during phase 2.
Fig. D13: Trial 20. Bioreactor fermentation of *E. coli* LSBJ harboring pBBRC1J4SII, with 20 g octanoic acid added at 0.65 g h\(^{-1}\). Media described in Chapter 4, *high-density fed-batch production*, with 3.0 g of yeast extract. Initial rate of glucose feed reduced to 12.9 mL h\(^{-1}\), slowed to 8.7 mL h\(^{-1}\) during phase 2.
Fig. D14: Trial 21. Bioreactor fermentation of *E. coli* LSBJ harboring pBBRC1J4SII, with 20 g 10-undecenoic acid added at 0.65 g h\(^{-1}\). Media described in Chapter 4, *high-density fed-batch production*, with 3.0 g of yeast extract. Initial rate of glucose feed reduced to 12.9 mL h\(^{-1}\), slowed to 8.7 mL h\(^{-1}\) during phase 2.
Fig. D15: Trial 22. Bioreactor fermentation of *E. coli* LSBJ harboring pBBRC1J4SII, with 20 g 10-bromodecanoic acid added at 0.65 g h\(^{-1}\). Media described in Chapter 4, *high-density fed-batch production*, with 3.0 g of yeast extract. Initial rate of glucose feed reduced to 12.9 mL h\(^{-1}\), slowed to 8.7 mL h\(^{-1}\) during phase 2.
Fig. D16: Trial 23. Bioreactor fermentation of *E. coli* LSBJ harboring pBBRC1J4SII, with 15.4 g octanoic acid and 4 g decanoic acid added at 0.632 total g h\(^{-1}\). Media described in Chapter 4, *high-density fed-batch production*, with 3.0 g of yeast extract. Initial rate of glucose feed reduced to 12.9 mL h\(^{-1}\), slowed to 8.7 mL h\(^{-1}\) during phase 2.
Fig. D17: Trial 24. Bioreactor fermentation of *E. coli* LSBJ harboring pBBRC1J4SII, with 15.4 g octanoic acid and 4.95 g 10-azidodecanoic acid added at 0.663 total g h\(^{-1}\). Media described in *Chapter 4, high-density fed-batch production*, with 3.0 g of yeast extract. Initial rate of glucose feed reduced to 12.9 mL h\(^{-1}\), slowed to 8.7 mL h\(^{-1}\) during phase 2.
Appendix E: HPLC Sample Chromatograms

The data presented in this appendix are a sample high performance liquid chromatography (HPLC) chromatogram of paclitaxel and the standard curve generated for paclitaxel quantification. HPLC was performed as described in Chapter 5. Paclitaxel absorbance was measured at 227 nm.

Fig. E1: Calibration curve generated for the quantification of paclitaxel.

\[ y = 1.985,106.5255x \]
\[ R^2 = 0.9999 \]
Fig. E2: Sample HPLC chromatogram of paclitaxel encapsulated in PHODN3 nanoparticle (dissolved in acetonitrile). Photocopied from lab notebook due to campus shutdown (COVID-19).
Appendix F: Additional Supplemental Data

This section contains additional supplemental data published with Scheel et al. 2016 (Chapter 2), as well as GC chromatograms for poly[(R)-3-hydroxy-10-bromodecanoate] (PHDBr) and a schematic of the bioreactor used for experiments in Chapter 4.

Table F1: Poly(3-hydroxydecanoate) yield comparison between LSBJ, RSC02, RSC04, and RSC06

<table>
<thead>
<tr>
<th>Strain</th>
<th>CDWa (g L⁻¹)</th>
<th>PHAa (wt%)</th>
<th>PHA Concentrationa (mg L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSBJ</td>
<td>1.13 ± 0.02</td>
<td>17.4 ± 3.7</td>
<td>196 ± 40</td>
</tr>
<tr>
<td>RSC02</td>
<td>1.33 ± 0.04*</td>
<td>26.4 ± 4.7*</td>
<td>353 ± 77*</td>
</tr>
<tr>
<td>RSC04</td>
<td>0.93 ± 0.00*</td>
<td>16.5 ± 1.9</td>
<td>154 ± 18</td>
</tr>
<tr>
<td>RSC06</td>
<td>0.99 ± 0.02*</td>
<td>17.9 ± 3.0</td>
<td>179 ± 31</td>
</tr>
</tbody>
</table>

a All values are averages of biological triplicate experiments plus or minus the standard deviation about those averages.

* Denotes statistically significant difference compared to LSBJ (Student’s t-test, two-tailed, α = 0.05).

Fig. F1: Sample GC chromatogram of purified PHDBr subjected to methanolysis as described in Chapter 4. Several peaks were generated by the polymer, possibly due to side reactions from methanolysis. Broad peak at ~28 – 30 min is likely the 3-hydroxy-10-bromodecanoate methyl ester; however, this region is often populated by background peaks from methanolyzed cell components and the peak at 25 min was calibrated as a proxy for the polymer.
Fig. F2: Sample GC chromatogram of control lyophilized cells (polymer-free, 12h fermentation) subjected to methanolysis. Note 2 peaks at ~28.5 min; these background peaks grow with increased fermentation time and are the reason for choosing the peak at 25 min for PHDBr calibration.

Fig. F3: Sample GC chromatogram of methanolized cells from Trial 22 at 48h (PHDBr production, Table 4.2).
Fig. F4: Calibration curve generated for quantification of methanolyzed PHDBr.

\[
Y = aX + b \\
a = 1.574496 \times 10^{-4} \\
b = 0.0 \\
R^2 = 0.9918222 \\
R = 0.9959027
\]

Fig. F5: Schematic of the New Brunswick BioFlo 310 2L bioreactor vessel used in Chapter 4. Four sensors were installed to measure dissolved oxygen (DO), pH, temperature (Temp), and the culture level (for foam detection). Glucose and fatty acid feeds, as well as concentrated ammonium hydroxide and 10% anti-foam B in water, were added by peristaltic pumps as described in Chapter 4.
Appendix G: Peer-reviewed publication covers

Deletion of the pflA gene in *Escherichia coli* LS5218 and its effects on the production of polyhydroxyalkanoates using beechwood xylan as a feedstock

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Keywords: polyhydroxyalkanoates, xylanases, recombinant *Escherichia coli*, xylan, biomass, hemicellulose, acetate, pyruvate, formate, lysine

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Abstract

Engineering of microorganisms to directly utilize plant biomass as a feedstock for the biosynthesis of value-added products such as bioplastics is the aim of consolidated bioprocessing. In previous research, we successfully engineered *E. coli* LS5218 to produce polyhydroxyalkanoates (PHAs) from xylan. In this study we report further genetic modifications to *Escherichia coli* LS5218 in order to increase the lactic acid (LA) fraction in poly(lactic acid-co-hydroxyalkanoate) (P(LA-co-HA)) copolymers. Deletion of the pflA gene resulted in increased content of LA repeating units in the copolymers by over 3-fold compared with the wild type; however, this increase was offset by reduced yields in cell mass. Additionally, when acetate was used as a feedstock, LA monomer incorporation reached 18.5 (mol%), which suggests that acetate can be used as a feedstock for the production of P(LA-co-HA) copolymers by *E. coli*.

Introduction

Consolidated bioprocessing (CBP) is a proposed approach to engineer microorganisms that are capable of utilizing hemicellulosic biomass for the biosynthesis of value-added products in a single microbial event. Previously we reported the first ever biosynthesis of polyhydroxyalkanoates (PHAs) by *E. coli* from hemicellulosic biomass (i.e., beechwood xylan) in an effort to obtain an *E. coli* CBP system for the production of bioplastics. *E. coli* was engineered to express acetyl-CoA synthetase and acetyl-CoA carboxylase from *Bacillus halodurans* in order to produce fatty acid synthase (FAS) with enhanced fatty acid production and xylanolytic enzymes from *Cellvibrio* in order to degrade the xylan backbone. The resulting strain was used to produce PHAs using beechwood xylan as a feedstock. The PHA produced by this strain contained a high LA content, which was attributed to the presence of the xylanase enzyme. However, the low cell mass yield observed in this study suggested that further genetic modifications were needed to increase the LA fraction in the copolymer.

In this study, we report further genetic modifications to the *E. coli* strain in order to increase the LA fraction in the copolymer. Deletion of the pflA gene resulted in increased content of LA repeating units in the copolymers by over 3-fold compared with the wild type; however, this increase was offset by reduced yields in cell mass. Additionally, when acetate was used as a feedstock, LA monomer incorporation reached 18.5 (mol%), which suggests that acetate can be used as a feedstock for the production of P(LA-co-HA) copolymers by *E. coli*.
The metabolism of (R)-3-hydroxybutyrate is regulated by the enhancer-binding protein PA2005 and the alternative sigma factor RpoN in Pseudomonas aeruginosa PAO1

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A variety of soil-dwelling bacteria produce polyhydroxybutyrate (PHB), which serves as a source of energy and carbon under nutrient deprivation. Bacteria belonging to the genus Pseudomonas do not generally produce PHB but are capable of using the PHB degradation product (R)-3-hydroxybutyrate ((R)-3-HB) as a growth substrate. Essential to this utilization is the NAD⁺-dependent dehydrogenase BdhA that converts (R)-3-HB into acetoacetate, a molecule that readily enters central metabolism. Apart from the numerous studies that focused on the biochemical characterization of BdhA, there was nothing known about the assimilation of (R)-3-HB in Pseudomonas, including the genetic regulation of bdhA expression. This study aimed to define the regulatory factors that govern or dictate the expression of the bdhA gene and (R)-3-HB assimilation in Pseudomonas aeruginosa PAO1. Importantly, expression of the bdhA gene was found to be specifically induced by (R)-3-HB in a manner dependent on the alternative sigma factor RpoN and the enhancer-binding protein PA2005. This mode of regulation was essential for the utilization of (R)-3-HB as a sole source of energy and carbon. However, non-induced levels of bdhA expression were sufficient for P. aeruginosa PAO1 to grow on (±)-1,3-butanol, which is catabolized through an (R)-3-HB intermediate. Because this is, we believe, the first report of an enhancer-binding protein that responds to (R)-3-HB, PA2005 was named HboR for (R)-3-hydroxybutyrate catabolism regulator.

INTRODUCTION

Bacteria of the genus Pseudomonas are renowned for their versatile metabolism in that they can assimilate and breakdown a wide assortment of compounds to meet their nutritional demands. One compound that is not often affiliated with Pseudomonas metabolism is (R)-3-hydroxybutyrate ((R)-3-HB). (R)-3-HB is commonly recognized for its role as a reserve body produced by mammalian cells when carbohydrate availability is limiting (Akram, 2015). However, there are a number of bacteria that biosynthesize CoA derivatives of (R)-3-HB and other (R)-3-hydroxy acids, which are polymerized into macromolecular structures called polyhydroxyalkanoates (PHAs) (Anderson & Dawes, 1996; Lu et al., 2009). Under starvation conditions, the PHA granule is degraded into its 3-hydroxy acid components, which can be used as sources of carbon and energy (Henderson & Handrick, 2002; Preussmann et al., 1998).

Pseudomonas species do not biosynthesize nor incorporate (R)-3-HB into their PHA reserves (Hardman et al., 1989; Timm & Steinbüchel, 1990). Nonetheless, these bacteria possess an NAD⁺-dependent dehydrogenase (BdhA) that converts (R)-3-HB into acetoacetate, thereby allowing these bacteria to use (R)-3-HB as a growth substrate (Feller et al., 2006; Liu et al., 2006; Montesinos et al., 2010). BdhA dehydrogenases have been biochemically characterized for some species of Pseudomonas, including P. putida (Feller et al., 2006; Petrakanta et al., 2007), P. syringae (Zuo et al., 2006; Nakajima et al., 2005) and

Abbreviations: EMISA, electro mobility shift assay; EBP, enhancer binding protein; PHA, polyhydroxyalkanoate; P3H, polyhydroxypropionate; 3HB, (3-hydroxypropionate); 3HB-CoA, 3-hydroxypropionyl-CoA.

A supplementary figure is available with the online Supplementary Material.

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Soil microbial community toxic response to atrazine and its residues under atrazine and lead contamination

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Abstract Intensive use of atrazine and extensive dispersal of lead (Pb) have occurred in farmland with chemical agriculture development. However, the toxicological effect of their presence on soil microorganisms remains unknown. The objective of this study was to investigate the impacts of atrazine or Pb on the soil microbiota, soil net nitrogen mineralization, and atrazine residues over a 28-day microcosm incubation. The Shannon–Wiener diversity index, typical microbial species, and Neighbor-joining tree of typical species from sequencing denaturing gradient gel electrophoresis (DGGE) bands were determined across periodical sampling times. The results showed that the existence of atrazine or Pb (especially high concentration) in soils reduced microbial diversity (the lower H value is 2.23) compared to the control (H = 2.59) after a 28-day incubation. The species richness reduced little (from 17–19 species to 16–17 species) over the research time. But soil microbial community was significantly affected by the incubation time after the exposure to atrazine or Pb. The combination of atrazine and Pb had a significant inhibition effect on soil net nitrogen nitrification. Atrazine and Pb significantly stimulated soil cumulative net nitrogen mineralization and nitrification. Pb (300 and 600 mg kg⁻¹) accelerated the level of atrazine dissipation. The exposure might stimulate the significant growth of the autotrophic soil degraders which may use atrazine as C source and accelerate the dissipation of atrazine in soils.

Keywords Microbiota · Denaturing gradient gel electrophoresis (DGGE) · N mineralization · Herbicide · Heavy metal

Introduction

As one of the most widely applied pesticides, atrazine is applied to control pre- and post-emergence broadleaf and grassy weeds in maize and other crops (Li et al. 2008a; Kazian et al. 2008; Monard et al. 2008). The continuous use of this herbicide has resulted in soil and ground water contamination (Venticinque et al. 2013; Singh and Castonguay 2014), and may change the soil biological characteristics resulting in alterations in soil ecosystem functions. It has been earlier reported that atrazine significantly shifted the microbial community structure and function (Soghrad et al. 2003). Britzma et al. (2010) reported that atrazine amendment altered the bacterial community structure with the appearance of specific bands in the DGGE gels, and Tontola et al. (2013a) found that soil enzyme activities were reduced significantly with atrazine application but rapidly recovered. They also observed an inhibitory effect on microbial diversity using Biolog EcoPlate™. Our previous study indicated that the application of atrazine had various effects on soil enzyme...
Enhancing poly(3-hydroxyalkanoate) production in *Escherichia coli* by the removal of the regulatory gene *arcA*

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### Abstract

Recombinant *Escherichia coli* is a desirable platform for the production of many biologically important compounds including poly(3-hydroxyalkanoate), a class of naturally occurring biodegradable polymers with promising biomedical and material applications. Although the controlled production of desirable polymers is possible with the utilization of fatty acid feedstocks, a central challenge to the biosynthetic route is the improvement of the relatively low polymer yield, a necessary factor of decreasing the production costs. In this study we sought to address this challenge by deleting *arcA* and nicR, two global regulators with the capacity to inhibit the uptake and activation of exogenous fatty acids. We found that polymer yields in a *ΔarcA* mutant increased significantly with respect to the parental strain. In the parental strain, PHA yields were very low but improved 64-fold in the *ΔarcA* mutant (1.92 ± 0.12 mg L⁻¹). The *ΔarcA* mutant also allowed for modest increases in some medium chain-length polymer yields while weight average molecular weights improved by approximately 1.5-fold to 12-fold depending on the fatty acid substrate utilized. These results were supported by an analysis of differential gene expression, which showed that the key genes *fadB*, *fadD*, and *fadA* encoding fatty acid degradation enzymes were all upregulated by 2-, 11-, and 31-fold in an *arcA* mutant, respectively. Additionally, the short chain fatty acid uptake genes *pdoA*, *pdOD*, and *pdoC* were upregulated by 102-, 119-, and 253-fold, respectively; though these values are somewhat inflated due to low expression in the parental strain. Overall, this study demonstrates that *arcA* is an important target to improve PHA production from fatty acids.

**Keywords:** Poly(3-hydroxyalkanoate), Biodegradable polymer, *Escherichia coli*, fatty acid metabolism, arcA, Recombination

### Introduction

Poly(3-hydroxyalkanoates), or PHAs, are a group of biodegradable polymers produced by a variety of microorganisms as a form of carbon storage (Lu et al. 2009). These PHAs are typically classified as short chain-length (SCL) PHAs, which contain repeating units of 3–5 carbons; medium chain-length (MCL) PHAs containing 6–14 carbons. The physical properties of PHAs are dependent on monomer composition; MCL PHAs are generally stiff and brittle while MCL PHAs are more elastomeric, and co-polymerization of the two groups allows for great variability in material properties (Laycock et al. 2013). Previous studies have shown that MCL PHAs can be effectively produced in recombinant *E. coli* lacking the fatty acid degradation gene *fdhB* when utilizing a related carbon source such as fatty acids, although the monomer composition of the resulting polymers was heterogeneous and uncontrolled (Langerbach et al. 1999; Qi et al. 1997).

Recently, it was shown that the monomer identity can be precisely controlled in both PHA homo- and co-polymers synthesized by recombinant *Escherichia coli* strain LS07 (Tappel et al. 2012a, b). This was accomplished by deleting both the *fdhB* and *fdhD* genes in *arcA* mutant LS07, rerecombinantly co-expressing the *phaC2* gene from *Paenibacillus polymyxa* KT2440 with the highly active and broad substrate utilizing *phaC1(STQ)* genes, and feeding in specific ratios of fatty acids for conversion to PHAs.
Consolidated bioprocessing of poly(lactate-co-3-hydroxybutyrate) from xylan as a sole feedstock by genetically-engineered Escherichia coli

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Consolidated bioprocessing of lignocelluloses is an attractive strategy for the sustainable production of petroleum-based alternatives. One of the underutilized sources of carbon in lignocelluloses is the hemicellulose fraction which makes up about 30% of the polysaccharide xylan. In this study, Escherichia coli (E. coli) was engineered to express recombinant xylanase and poly(lactate-co-3-hydroxybutyrate) (PLA-co-3HB) from xylan as a consolidated bioprocess. The results show that E. coli T500985 was capable of producing PLA-co-3HB when xylan was the only feedstock. In addition, the growth and product parameters were examined in order to improve yields. The highest yields of PLA-co-3HB were obtained in this study using xylan added during mid-exponential growth after cells had been grown at high shaking speeds (200 rpm). The results showed an inverse relationship between total PLA production and Xa-consumer incorporation into the copolymer. Protein nuclear magnetic resonance ($\chi$ NMR) gel permeation chromotography (GPC), and differential scanning calorimetry (DSC) analyses confirmed that the polymers produced maintain physical properties characteristic of $\chi$ incorporating PLA-based copolymers. The present study achieves the first ever engineering of a consolidated bioprocessing bacterial system for the production of a bioplastic from a hemicellulosic feedstock.

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Keywords: Poly(lactic-co-3-hydroxybutyric) acid (PLA-co-3HB); Xylanase; Biotechnological engineering; Xylan; Biodegradable plastics; Novel systems; Consolidated bioprocessing;
Effect of acetate as a co-feedstock on the production of poly(lactate-co-3-hydroxyalkanoate) by pflA-deficient Escherichia coli RSC10

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Developing E. coli strains that are tolerant to acetate toxicity is important in light of an increased interest in the efficient utilization of lignocellulosic biomass feedstocks for the biosynthesis of value-added products. In this study, four strains known to produce polyhydroxyalkanoates (PHAs) from the typical hexadecanoic acid (HDA) were used for their tolerance to acetate. E. coli RSC10 was found to be tolerant to acetate, both in growth and fermentation studies. In the presence of acetate the strain showed a 2× fold increase in overall yields compared to using xylose alone as the feedstock. More importantly, the strain was found to be able to utilize acetate as a feedstock for biosynthesis of PHAs, with complete depletion of acetate (25 mM) at 9 h when acetate was the sole feedstock. Higher concentrations of acetate showed greater inhibition of fermentation than growth with a reduction of 30% in PHA yields at 100 mM. Additionally, the present work provides data to support the potential of acetate as a moderator for the control of composition of PHAs that incorporate lactate (LA) monomers into the copolymer from hemicellulose-derived sugars.

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Increased interest in utilization of lignocellulose as a feedstock for the biosynthesis of commodities (i.e., fuels, plastics) which are currently derived from petroleum requires focusing research efforts into engineering microbial factories that can not only synthesize the target product, but can also achieve high production yields. Pretreatment of lignocellulosic biomass can result in the accumulation of toxic byproducts that negatively affect yields either by slowing microbial growth or inhibiting fermentative processes (1–5). Acetate, furfural, and hydroxyacetone are among the most common byproducts derived from the hemicellulose portion of lignocellulosic biomass subjected to thermochemical treatments (6–8). Acetate is a common byproduct released from pretreatment of xylan, a component of hardwood lignocellulose, which contains up to 30% of its weight as acetyl groups (9,10). Detoxification of lignocellulosic hydrocarbons prior to fermentation is possible and many strategies have been developed (11–14); however, these processes significantly increase production costs (15,16). A potential strategy to bypass extensive detoxification processes is the development of bacterial strains that are more resistant to inhibitory effects of pretreatment byproducts.

In E. coli, acetate has been shown to negatively affect growth and some biosynthetic pathways (17,18). Additionally, acetate produced from overflow metabolism of sugars is excreted into the media as growth progresses, becoming an obstacle for production of high yields of recombinant proteins (19–21). Nevertheless, some E. coli strains have been found to be partially tolerant to acetate. For example, E. coli KO11 showed only a slight reduction in ethanol yields over long production periods or by adjustment of the culture pH, allied cellular growth was negatively affected (22). In the present study, the effect of acetate on the cell growth and fermentation of four E. coli strains (BW25113, JM103 ΔpflA, ΔpflAΔpoxB, and RSC10) known to produce polyhydroxyalkanoates (PHAs) from xylose (22–25) was examined using xylose and acetate as feedstocks. These strains were engineered to express the PHA synthase from Pseudomonas sp. BS-3 harboring a Ser225→Thr or Gln136→lys mutation (PscP136G136N), a pyruvyl-CoA transferase (PCT) from Mycobacterium celatulans, and a D-lactate:CoA synthase (FLaA) and NADP-dependent acetate-CoA reductase (PhaB) from Ralstonia eutropha under a N. europaeanus constitutive promoter in order to produce the PHA copolymer poly(lactate-co-3-hydroxybutyrate) (PLA-PHB) (26). E. coli strains BW25113, JM103 ΔpoxB, and LS218 showed reduced cell growth while utilizing xylose as a feedstock in the presence of acetate, leading to reduced overall yields of PHAs. Conversely, strain RSC10 showed tolerance to acetate both in terms of cellular growth and fermentation. Additionally, this strain was able to utilize acetate as a feedstock leading to a 2× fold increase in overall yields of PHA compared to using xylose as sole feedstock. Yields were further increased by 3× when addition of substrates was delayed...
Increased Production of the Value-Added Biopolymers Poly(R-3-Hydroxyalkanoate) and Poly(γ-Glutamic Acid) From Hydrolyzed Paper Recycling Waste Fines

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Reject fines, a waste stream of short lignocellulosic fibers produced from paper inboard recycling, are a cellulose-rich paper mill byproduct that can be hydrolyzed enzymatically into fermentable sugars. In this study, the use of hydrolyzed reject fines as a carbon source for bacterial biosynthesis of poly(R-3-hydroxyalkanoate) (PHA) and poly(γ-glutamic acid) (PGA) was investigated. Recombinant Escherichia coli harboring PHA biosynthesis genes were cultivated with purified sugars or crude hydrolysate to produce both poly(R-3-hydroxybutyrate) (PHB) homopolymer and medium chain length-containing copolymer (PHB-co-MCL). Wild-type Bacillus subtilis XW-02 were cultivated with crude hydrolysate to produce PGA. Both PHB and short chain length-co medium chain length (SCL-co MCL) PHA yields from crude hydrolysate were 2-fold improvement over purified sugars, and the MCL monomer fraction was decreased slightly in copolymers produced from crude hydrolysate. PGA yield from crude hydrolysate was similarly increased 2-fold. The results suggest that sugars from hydrolyzed reject fines are a viable carbon source for PHA and PGA biosynthesis. The use of crude hydrolysate is not only possible but beneficial for biopolymer production, eliminating the need for costly separation and purification techniques. This study demonstrates the potential to divert a lignocellulosic waste stream into valuable biomaterials, mitigating the environmental impacts of solid waste disposal.

Keywords: biopolymers, polyhydroxyalkanoates, polyglutamic acid, biosynthesis, lignocellulosic, waste stream, lignocellulosic recycling
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EDUCATION

2015-2020  SUNY ESF  Syracuse, NY
Advisor: Dr. Christopher T. Nomura
Ph.D. Department of Chemistry, concentration in Biochemistry. Research focused on bacterial gene regulation for (1) membrane transport in Pseudomonas aeruginosa and (2) biosynthesis of polyhydroxyalkanoates in Escherichia coli.

2011-2014  SUNY ESF  Syracuse, NY
Bachelor of Science in Environmental Biology with Honors, minoring in Chemistry and Biotechnology, Magna Cum Laude (GPA 3.58). Participated in honors research culminating in a thesis and presentation. Thesis: Deregulation of fatty acid transport in Escherichia coli for enhanced control of biodegradable plastic copolymer production.

2007-2011  SUNY Monroe Community College  Rochester, NY
Associates in Liberal Arts and Sciences (GPA 3.97)

PEER-REVIEWED PUBLICATIONS


RESEARCH EXPERIENCE

2015-Present  Doctoral Candidate, Department of Chemistry  SUNY ESF
Advisor: Dr. Christopher T. Nomura
Research focus: Metabolic engineering of Escherichia coli to enhance biosynthesis of biodegradable poly(R-3-hydroxyalkanoates) (PHA), and characterization of a gene regulatory network in Pseudomonas aeruginosa. Currently focused on optimizing high-density fermentation methods for medium chain length (MCL) PHA biosynthesis, synthesis of functionalized PHA nanoparticles for targeted drug delivery,
and engineering a novel metabolic pathway for poly(4-hydroxybutyrate)-co-MCL PHA for high-density fermentation.

Summer 2014 **Research Internship, Third Millennium Alliance**
Manabi, Ecuador
Advisor: Jordan Trujillo
Pursued an ethnobotanical research project on natural insect repellents in the coastal rainforest of Ecuador, worked closely with local community members, and presented research results in Spanish to the village of Camarones. Volunteered for the CEIBA foundation assisting Camarones women with piñón planting.

2012-2014 **Undergraduate Research, Nomura Research Group**
SUNY ESF
Advisors: Dr. Christopher T. Nomura, Dr. Benjamin R. Lundgren, Dr. Ryan Tappel
Research focus: Genetic engineering of *Escherichia coli* to enhance uptake and metabolism of related and un-related substrates for poly(R-3-hydroxyalkanoate) biosynthesis. Resulted in preliminary data for graduate thesis research.

**Fellowships and Scholarships**

2016-2018 **NIH Predoctoral Fellowship (F31)**
SUNY ESF
Awarded for research proposal investigating a putative uncharacterized enhancer binding protein and its regulon in *Pseudomonas aeruginosa*.

2011-2014 **Phi Theta Kappa Scholarship, International Honor Society for 2- and 4-Year Colleges**
Awarded for exemplary academic achievements.

**Honors and Awards**

May 2019 **Excellence in Academic Research Award, ESF Graduate Student Association**
SUNY ESF
May 2017 **2nd Place Research Presentation, 9th NYS Biotechnology Symposium**
Fall 2015 **1st Place Eastman Chemical Company Graduate Research Competition**
2013-2014 **Upper Division Honors Program**
2011-2014 **Dean's List (Fall semesters 2011-2013, Spring of 2014)**

**Teaching Experience**

Spring 2018 **Guest Lecturer, Biochemistry**
SUNY ESF
Prepared and presented a lecture on fatty acid biosynthesis and an introduction to polyketide biosynthesis for senior/graduate level course.

Fall 2017 **Lab Coordinator/Teaching Assistant, Biochemistry**
SUNY ESF
Maintained a lab inventory and ordered necessary chemicals for experiments, prepared buffers and reagents for students, set up research instruments and supervised their use, presented pre-lab lectures on enzyme characterization and kinetics, and instructed a 10-12 hour/week lab course.

Spring 2015 **Teaching Assistant, General Chemistry**
SUNY ESF
Supervised and instructed 2 lab sections in general chemistry techniques. Presented pre-lab lectures on principles of chemistry, assisted students in the lab, and mentored students during weekly office hours.

**Mentoring Experience**

2014-Present **Undergraduate Researcher Mentees**
SUNY ESF
Truong Ho (BS Biochemistry, 2021), Yuki Kageyama (MS Biological Chemistry and Engineering, International Research Experience Intern Fall 2019), Jennifer Callan (High School Mentorship Program,
LEADERSHIP EXPERIENCE

2017-2019  **President, Graduate Student Association (GSA)** SUNY ESF
Organized and chaired biweekly senate meetings in accordance with the GSA Bylaws and Robert’s Rules of Order. Prepared and circulated meeting agendas and was responsible for email communication to the graduate student body. Served as voting representative to the ESF Leadership Council and Academic Governance. Led the initiative to transform the organization from a club to an independent 501(3)(c) non-profit. Represented the GSA at biannual meetings of the SUNY Student Association, the university-wide student governance body of SUNY’s 64 campuses.

2016-2017  **Representative to Undergraduate Student Association, GSA** SUNY ESF
Acted as liaison between the Graduate and Undergraduate Student Associations. Served as one of eight voting members for GSA at Academic Governance and served on the Academic Governance committee on Instructional Quality and Academic Standards. Authored a resolution on Graduate Student Safety and planned social events on and off campus as a member of the GSA Social Activities Committee.

PROFESSIONAL EXPERIENCE

2007-2017  **Food Service Experience, 10 years** Rochester & Syracuse, NY
Worked in both front- and back-of-house positions at various restaurants while enrolled in college.

PRESENTATIONS

August 2018  **25th Bio-Environmental Polymer Society Meeting, Invited Speaker** Rensselaer Polytechnic
“Engineering *Escherichia coli* for enhanced biosynthesis of poly[(R)-3-hydroxyalkanoates]”

August 2017  **20th CBE Graduate Research Symposium, Poster presentation** University of Buffalo
“Enhancing poly(3-hydroxyalkanoate) production in *Escherichia coli* by the removal of the regulatory gene arcA”

May 2017  **9th NYS Biotechnology Symposium, 2nd Place, Poster presentation** SUNY ESF
“Enhancing poly(3-hydroxyalkanoate) production in *Escherichia coli* by the removal of the regulatory gene arcA”

May 2016  **8th NYS Biotechnology Symposium, Poster presentation** SUNY ESF
“Metabolic engineering of *Escherichia coli* LSBJ for improved production of poly (3-hydroxyalkanoates)”

March 2016  **Stevenson Biomaterials Lecture Series, Poster presentation** Syracuse University
“Metabolic engineering of *Escherichia coli* LSBJ for improved production of poly (3-hydroxyalkanoates)”

June 2015  **Boston Bacterial Meeting, Poster presentation** Harvard University
“Metabolic engineering of *Escherichia coli* LSBJ for improved production of poly (3-hydroxyalkanoates)”

April 2015  **Spotlight on Research, Graduate Poster presentation** SUNY ESF
“Metabolic engineering of *Escherichia coli* LSBJ for improved production of poly (3-hydroxyalkanoates)”

April 2014  **Spotlight on Research, Undergraduate Poster presentation** SUNY ESF
“Deregulation of fatty acid transport in *Escherichia coli* for enhanced control of biodegradable plastic copolymer production”

**LANGUAGES**
American English – Native/Bilingual (ILR Level 5)
Spanish – Limited working proficiency (ILR Level 2+)

**REFERENCES**

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