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Fermentative production of short-chain fatty acids and methyl ketones in Escherichia coli

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Fermentative production of short-chain fatty acids and methyl ketones in

*Escherichia coli*

by

Alexandra R. Volker

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Microbiology

Program of Study Committee:

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Iowa State University
Ames, Iowa
2015

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I would like to thank my major professor, Thomas Bobik, for all of the knowledge and help he has given me throughout my graduate career. His advice was invaluable to me and I appreciated his practical help at the bench when I was struggling with a difficult experiment.

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ABSTRACT

Dependence on petroleum for fuels is a well-known issue in the United States today. However, petroleum is also used to produce a broad range of chemicals that are used in applications such as plastics, fragrances, surfactants, detergents, food additives, and pharmaceuticals. As the world’s supply of petroleum dwindles, we must look to another method of procuring these chemicals. Biorenewable chemical production attempts to fill this void. Short-chain fatty acids and ketones are desirable precursors to many of these industrially relevant chemicals. Short-chain fatty acids are precursors to alpha-olefins, which are used as lubricants and surfactants in a variety of industries, including the automotive industry. They can also be used as precursors to fatty alcohols, which have potential applications as biodiesel. Methyl ketones are also a class of chemicals with many industrial applications. Butanone is a common industrial solvent, while 4-hydroxybutanone is used in pesticides, terpenoids, and most importantly, is an intermediate in the production of doxorubicin, an anticancer agent. Here we report the biorenewable production of short-chain fatty acids and methyl ketones from fermentation in *Escherichia coli*. A series of synthetic constructs were made to produce the desired metabolites utilizing glucose as the feedstock. Butyrate was produced at 9.670 g/L, hexanoate at 1.963 g/L, and octanoate at 0.216 g/L. In addition, 0.201 g/L of valerate was produced. Heptanoate production by fermentation in *E. coli* was reported for the first time, reaching a titer of 0.008 g/L. 4-hydroxy-2-butanone was produced by fermentation at a titer of 2.5 mM. To our knowledge this is the first report of production of 4-hydroxy-2-butanone by microbial fermentation.
CHAPTER ONE: GENERAL INTRODUCTION

I. Dependence on Petroleum

Petroleum is an essential source for fuels and chemicals in the world today. Petroleum products include transportation fuels, heating fuels, plastics, waxes, lubricants, and a variety of other chemicals. Much of the technology utilized today requires petroleum-based products in some way. However, petroleum is a finite resource. Some predictions indicate that in forty years, the majority of known oil reserves will be depleted (imeche.org). ExxonMobil has concluded that non-OPEC oil production peaked in 2010 and is no longer building new refineries (Cavallo, A. 2007). For this reason, it is essential that we look to renewable methods of producing chemicals that are currently derived from petroleum. In addition, current methods of producing the array of petroleum-derived products are not environmentally friendly, producing large amounts of emissions that contribute to greenhouse gases. Petroleum is the largest fossil fuel source for greenhouse gas emissions, contributing to 43% of total emissions in the United States (eia.gov). Current research is focusing on replacement of petroleum-derived chemicals and fuels with renewable options made from plant matter or microbial production methods (Choi et.al 2013, Dellomonaco et.al 2010, Fortman et.al 2008, Lu et.al 2008, Nikolau et.al 2008).

II. Biorenewable Fuels

Due to our reliance on petroleum for gasoline and other fuels, biorenewable fuels have been a topic of significant research. Ethanol is the most well-known renewable fuel in the United States, and is currently produced by utilizing corn. Ethanol can also be produced from cellulosic
feedstocks, although this is currently a less common production method. Biodiesel is another fairly common biorenewable fuel, made primarily from vegetable oils, although recent advances allow production from used cooking oils and animal fats as well. Biodiesel replaces regular diesel fuel and can be used in diesel engines without modification. However, biodiesel currently remains a small part of the market, with projections that it may account for 10% of the diesel market by 2022. In addition, current production cannot supplant the petroleum fuel demand. If all of the corn and soybean crops in the U.S. were dedicated to producing solely ethanol and biodiesel, only 12% of gasoline demand and 6% of diesel demand would be met (Hill et.al. 2006).

To address the gap in current biofuel production, research has turned toward utilizing microbes to produce fuels that could ultimately be made from lignocellulose. A variety of straight and branched-chain alkanes and alkenes are produced from fatty acid and isoprenoid biosynthetic pathways which can be used as substitutes to gasoline. In addition, biological alcohols such as butanol and isopentanol can also be produced, which increase the octane rating of gasoline while not decreasing its energy density (Fortman et.al. 2008). Butanol in particular has the possibility to be a viable alternative to ethanol, because it can be transported through existing oil pipelines, while ethanol cannot. Engineering of the fatty acid and isoprenoid biosynthetic pathways in microbes has made some modest gains in production of these fuels. Currently, the major challenges in microbial biofuel production is achievement of yields that will make these fuels cost-competitive with petroleum (Fortman et.al. 2008), and dealing with product toxicity to the host organisms.
In addition to producing biorenewable fuels, attention has turned to producing the array of chemicals currently made by petroleum in a biorenewable manner. Currently, the petroleum industry produces $400 billion worth of petrochemical products annually (Blue Ribbon Panel 2012). These chemicals are used as fuel additives, lubricants, surfactants, detergents, plastics, perfumes, food flavorings, and pharmaceuticals. Many of the same issues facing biofuel production are also challenges to biorenewable chemical production. A successful bio-based chemical industry would likely need to be structured as a platform chemical industry; several precursors and intermediates are produced, which can then be converted to a variety of end products (Nikolau et.al. 2008). Another possible approach is to functionally replace petrochemicals; rather than re-create the same chemical, a bio-based chemical with similar or improved functional properties could be produced. However, this could be difficult as introducing new chemicals to the market is quite expensive, with costs estimated of $1 billion (Nikolau et.al. 2008).

An important class of industrial chemicals are the carboxylic acids. The production of fatty acids of varying chain lengths ultimately can be used to produce alpha-olefins or fatty alcohols. Alpha-olefins of C4 to C8 chain length are often used as co-monomers in the production of polyethylene, while longer chain lengths are used as surfactants and lubricants (Linde Engineering). Fatty alcohols can be used in a variety of industrial applications, including agrochemicals, textile processing agents, soaps, polymer additives, and biocidal agents (Lennen et.al. 2012). Part of the work presented in this thesis focuses on the production of short-chain fatty acids from C4 to C8 in length.
Methyl ketones are another industrially relevant class of chemicals that could be produced in a biorenewable manner. Methyl ketones, including 2-butanone and derivatives, are used as flavor compounds in a variety of foodstuffs, especially those with a buttery flavor. They are also used as industrial solvents and are precursors to a variety of compounds. In addition to carboxylic acids, this work demonstrates some preliminary data in the production of butanone derivatives via microbial biorenewable methods. 4-hydroxy-2-butanone is a precursor to a variety of chemicals, including pesticides and terpenoids. Most importantly, it is an intermediate in the production of doxorubicin, a common anti-cancer drug. There is an ongoing shortage of doxorubicin in the United States, the causes of which are complex, but include a lack of manufacturers. Future shortages could be alleviated by having a biorenewable pathway to this drug.
CHAPTER TWO: LITERATURE REVIEW

I. Introduction

Fatty acids are the precursors to a variety of industrially relevant chemicals. Therefore, it makes sense to attempt to produce them in a biorenewable manner. Butyric acid production has been attempted in *Clostridium* by redesign of the fermentation pathway, with modest success, but is limited by the strict anaerobic requirements of the organism (Jang et.al 2013, Jiang et.al 2010). Some of these genes have been used for heterologous expression in organisms better suited to fermentative production, such as *Escherichia coli* and *Saccharomyces cerevisiae* with some success (Dekishima et.al 2011, Fischer et.al 2010). In addition, manipulation of the native fatty acid beta-oxidation pathway and fatty acid biosynthesis pathway in *E. coli* has been studied to produce fatty acids (Clomburg et.al 2012, Dellomonaco et.al 2011, McMahon et.al 2014, Seregina et.al 2010, Tseng et.al 2012).

Preliminary work has been done to explore the possibility of producing 2-butanone and other ketones in a biorenewable manner. 2-butanone is an important industrial solvent that is currently not produced biorenewably. 2-butanone can be produced by extending the 2, 3-butanediol pathway via heterologous expression of *Klebsiella pneumoniae* glycerol dehydratase *DhaB* and its reactivating factor (Yoneda et.al 2014). Another ketone, 4-hydroxy-2-butanone, is an essential intermediate for production of pesticides, steroids, and the anticancer drug doxorubicin. Currently there is no microbial production method for 4-hydroxy-2-butanone, although preliminary work has been done using immobilized enzymes to produce this chemical (Wang et.al 2012).
II. Microbial Production of Butyric Acid and Other Short-chain Fatty Acids

Butyric acid is an attractive target molecule for bio-based production because it is used in a variety of applications, including use in pharmaceuticals, plastics, and textiles (Zhang et.al. 2009). Biorenewable production of butyric acid also began by utilization of clostridial species as they naturally produce butyrate. Several Clostridium strains have been investigated and used in the past 20 years in industrial applications. They produce butyrate concomitantly with CO₂, H₂, acetate, and several other side products (Zhang et.al. 2009) from fermentation of glucose.

Figure 1: The metabolic pathway of the butyrate-producing clostridia (Zhang et.al. 2009). 1; EMP pathway and hexose phosphotransferase, 2; pyruvate-ferredoxin oxidoreductase, 3; hydrogenase, 4; acetyl-CoA acetyltransferase, 5; β-hydroxybutyryl-CoA dehydrogenase, 6; crotonase, 7; butyryl-CoA dehydrogenase, 8; phosphotransbutyrylase, 9; butyrate kinase, 10; phosphotransacetylase, 11; acetate kinase, 12; proposed enzyme butyryl-CoA acetate transferase, 13; NAD-independent lactate dehydrogenase.
While *Clostridium* can be used to produce butyrate, several issues arise. The first is that initial genetic engineering attempts were not necessarily helpful. Deletion of AK-PTA (acetate kinase-phosphotransacetylase) should have increased butyrate production, but these mutants suffered from less ATP production and overall less biomass production (Zhang et.al. 2009). Secondly, butyrate is toxic to the cells, resulting in eventual cell death. Lastly, the ideal way to produce butyrate would be from lignocellulosic materials, but the ability to degrade lignocellulose is not native to clostridia, and thus needs to be genetically engineered into production strains.

Immobilization of cells on a fibrous-bed bioreactor has been used to increase butyrate titers. This method increases the biomass in the reactor and facilitates continuous fermentation. Immobilized clostridial cells in a fibrous-bed reactor produced up to 0.44 g butyrate per g glucose compared to 0.28 g/g in traditional suspended cell fermentation (Jiang et.al. 2012). While immobilization increased butyrate production, it still suffered from toxicity issues.

Sugarcane bagasse was examined as a potential feedstock for butyrate production utilizing *Clostridium* in the immobilized fibrous-bed bioreactor. While production reached 0.48 g/g sugar, sugarcane bagasse requires extensive pretreatment (Wei et.al. 2013). Sugarcane bagasse is comprised of 38-43% cellulose, 26-28% hemicellulose, and 25% lignin. This composition requires acid pretreatment with 0.1N HCl (Wei et.al. 2013). The pretreatment solubilizes hemicelluloses and removes lignin. This is likely not ideal for commercial production, as it would require large amounts of HCl and a pretreatment of the sugarcane bagasse prior to microbial fermentation. However, at present this is the best attempt at utilizing lignocellulosic materials in clostridial fermentation.
Since *Clostridium* appears to be a less than ideal choice as a production microbe, attention has turned to engineering other organisms to produce short-chain fatty acids. *E. coli* is an obvious choice as it has been extensively studied, is stable and easy to grow, and has a wealth of tools with which to perform genetic manipulations. *E. coli* already possesses a fatty acid degradation pathway that converts fatty acids to acetyl-CoA, termed the beta-oxidation pathway. Thus efforts have been made to reverse this pathway and utilize it to create fatty acids of varying chain lengths (Dellomonaco et al. 2011; Seregina et al. 2010). Reversal of the beta-oxidation pathway will allow production of fatty acids from one carbon source, such as glucose.

![engineered reversal of the beta-oxidation cycle in *Escherichia coli* (Dellomonaco et al. 2011)](image-url)
The first step in reversing this pathway is to remove \textit{fadR}, which encodes a repressor of the major elements \textit{fadAB} (Seregina et al. 2010). This allows for constitutive expression of \textit{fad} regulon, which normally is repressed when not in the presence of fatty acids. Initiation of the reverse cycle begins by overexpression of the acetyl-CoA acetyltransferase \textit{atoB}; without this the reversal is not achieved (Dellomonaco et al. 2011). The appropriate termination enzymes must also be expressed to convert the CoA-thioester intermediates to the desired end products. Once the cycle has been reversed, intermediates can go through the pathway multiple times, resulting in longer chain end products such as C10 fatty acids (Dellomonaco et al. 2011). Reversal of the beta-oxidation cycle is an efficient method of producing fatty acids, but is somewhat constricted to which products can be made versus heterologous gene expression in \textit{E. coli}, which can create a variety of strains to produce many chemicals.

In addition to manipulating the beta-oxidation cycle, significant work has been done utilizing the native fatty acid biosynthetic pathway in \textit{E. coli}. Fatty acids are produced from acetyl-CoA, ATP, and NADPH. Acetyl-CoA carboxylase catalyzes the formation of malonyl-CoA; this is the first committed step of the fatty acid biosynthetic pathway. Fatty acid synthase (FAS) uses malonyl-CoA to create fatty acyl-ACPs, which can then be released by a thioesterase as a free fatty acid or incorporated into phospholipids (Lu et al. 2008) This is a highly regulated pathway and requires some genetic manipulation in order to produce free fatty acids at desired titers. First, the \textit{fadD} gene was deleted (Lu et al. 2008). \textit{FadD} encodes an acyl-CoA synthetase and deletion of this gene blocks activation of the beta-oxidation pathway to degrade fatty acids. Next, the chain length of the fatty acids can be manipulated by changing the thioesterase expressed. Thioesterases are a large class of enzymes that are found in a variety of organisms and each has its own chain length specificity. While some thioesterases are very promiscuous
and function on many fatty acids, others are more specific to a certain chain length or type of chain, such as straight or branched fatty acids. Third, the malonyl-CoA pool was increased by overexpression of ACC (Lu et al. 2008). This ensures that there is a large enough pool of malonyl-CoA to maintain continuous fatty acid synthesis. This system resulted in a production of 2.5 g/L of total fatty acids, with chain lengths of C12 to C18 primarily produced. 70% of the fatty acids produced were in free fatty acid form, rather than being incorporated into esters of polyols. However, less than 10% of the fatty acid pool was secreted into the fermentation medium (Lu et al. 2008). This has the potential to be an issue as extraction costs for products can be problematic in commercial production. As a prototypical process, this has potential for production of longer chain fatty acids.

Lastly, introduction of synthetic biosynthetic pathways for fatty acids into E. coli has been examined. One initial study constructed a butyrate production pathway using genes from several organisms. First, several genes were deleted in E. coli to minimize undesirable side product formation. The deleted genes were adhE, ato, frdABCD, ldhA, paaFGH, and pta. The synthetic construct consisted of crt and hbd from Clostridium acetobutylicum, ter from Treponema denticola, and tesB from E. coli. Under anaerobic conditions, this strain accumulated 4.35 g/L of butyrate with fairly high selectivity (Lim et al. 2013). This study provides the basis for further study that occurs in this thesis to improve titers and selectivity.

Butanol is a potential fuel substitute and chemical feedstock for bio-based chemical production. It is naturally synthesized by Clostridium species via a CoA-dependent pathway (Shen et al. 2011). This pathway produces butyryl-CoA, the precursor to both butyrate and butanol. By examining prior methods to increase butanol titers, we may be informed about how to produce butyrate in a similar fashion. However, clostridial species are difficult to work with
given their need for strict anaerobic conditions. In addition, there are fewer genetic tools available to manipulate these organisms, so they are not ideal for commercial production of bio-based chemicals. Several attempts have been made to move the butanol production pathway from *Clostridium* to organisms better suited for production such as *E. coli, Saccharomyces cerevisiae* and *Bacillus subtilis*. However, these have resulted in much lower titers of butanol production and much higher titers of related compounds such as ethanol, isobutanol, and isopropanol (Shen et al. 2011).

To improve butanol titers, a trans-enoyl-CoA reductase (Ter) from *Treponema denticola* was utilized to provide an irreversible reaction that would help drive butanol production. Ter catalyzes the reduction of crotonyl-CoA to butyryl-CoA, which then is converted to butanol (Shen et al. 2011). This addition created higher titers of butanol produced during anaerobic fermentation. These results inform later methods to produce high titers of butyric acid.

In addition to butyrate, there are other short chain fatty acids that are attractive biorenewable chemicals. One of these is hexanoate, which is both a chemical precursor and a biofuel precursor and additive. Hexanoate was reported as a major metabolic product of *Clostridium* sp. BS-1 when grown on D-galactitol, a derivative of D-galactose in seaweed (Jeon et al. 2010). This was a newly reported strain of *Clostridium* without a known hexanoate synthetic pathway. There are several hurdles to be overcome in this production scheme: further elucidation of the hexanoate synthesis pathway is needed, as well as optimization of culture conditions and removal of the end-product as it is toxic to the production organism.
Selection for longer chain fatty acids and their derivatives, linear alcohols, was improved in *E. coli* by modification of a previously made n-butanol pathway. Replacement of the *C. acetobutylicum* Hbd with the homologue PaaH1 from *Ralstonia eutropha* increased hexanol production ten-fold (Machado et.al. 2012).

![Figure 3: Higher alcohol production scheme in *Escherichia coli*. (Machado et.al. 2012)](image)

Directed evolution of the PaaH1 gene was used to improve the selectivity for longer chain alcohols. Several *paaH1* mutants showed improved titers of n-hexanol and n-octanol. In addition, a mammalian thioesterase was introduced which is more specific for longer-chain fatty acids rather than C4 products. This reduced C4 production and also provided recycling of the
CoA cofactor to allow the pathway to continue. These studies on production of longer-chain fatty acids provides some of the framework for the production of longer-chain fatty acids in this thesis and informs our thioesterase study to improve chain-length specificity.

Production of odd-chain fatty acids and alcohols is also an area of interest, particularly that of valerate and n-pentanol. Odd-chain fatty acids are used as plasticizers, herbicides, and in the fragrance industry (Torella et.al. 2013). The Prather group adapted the butanol biosynthetic pathway to produce odd-chain molecules. Expression of the bktB thiolase from Cupravidus necator allows propionyl-CoA to condense with an acetyl-CoA molecule, creating ketovaleryl-CoA, a five carbon precursor that can be processed to become valerate or n-pentanol. Further modifications of the pathway can be made to produce a variety of even and odd-chain fatty acids (Tseng et.al. 2012). Supplementation of the production medium with propionate is required, however, which is not ideal for commercial production. Comprehensive production of all fatty acids of chain lengths 4-14 was demonstrated in E. coli by varying the thioesterases expressed while also adding propionate to the medium (Torella et.al. 2013). Genetic engineering was used to add a propionyl-CoA production pathway to these strains, resulting in production of odd-chain acids without the need to supplement with propionate. In addition, the usually low concentration of medium-chain acyl ACPS in growing cells was enhanced by inhibition of the KAS enzymes FabB and FabF, which are responsible for creating long-chain acyl ACPS (Torella et.al. 2013). These studies showed that microbial production of odd-chain fatty acids is possible, but they fell short of reaching titers that are commercially viable. In addition, many of these studies required addition of propionate to the culture medium. Ideally, a production pathway would be created to make these fatty acids from one single feedstock such as glucose, which in the long term might be derived from lignocellulose.
III. Production of 2-butanone and Other Methyl Ketones

Certain ketones that are industrially relevant may also be produced by microbial fermentation. 2-butanone is utilized as a solvent in many industries and is currently made from butanol. While biorenewable butanol could be used to produce 2-butanone, it would be ideal to produce 2-butanone as is. However, this requires significant amounts of genetic engineering as 2-butanone is not naturally produced in any significant quantity. A 2,3-butanediol pathway has been expressed in *E. coli*, and this provided the basis for 2-butanone production. This pathway, which converts glucose to 2,3 butanediol, was extended by expression of the glycerol dehydratase from *Klebsiella pneumoniae* to produce 2-butanone (Yoneda et.al. 2014). Expression of the glycerol dehydratase reactivating factors improves the 2-butanone titer. These strains co-produce isobutyl alcohol as part of a side reaction catalyzed by endogenous alcohol dehydrogenases. This work proves that it is possible to engineer 2-butanone production into *E. coli*. However, there is little other research on this topic currently, and thus it is possible that other production schemes may be more efficient.

4-hydroxy-2-butanone is an important intermediate to several chemicals, including steroids, pesticides, terpenoids, and the anticancer agent doxorubicin (Wang et.al. 2012, Ichikawa et.al. 2005). Recent shortages of doxorubicin make this an attractive target molecule for biorenewable production. There are no current attempts to produce 4-hydroxy-2-butanone using microbes as a production organism. Current research utilizes an enzymatic production method that couples an NAD$^+$ dependent glycerol dehydrogenase and NAD$^+$ regenerating NADH oxidase that are immobilized on carbon nanotubes (Wang et.al. 2012). This system produced 6.5 mM of 4-hydroxy-2-butanone at its maximum efficiency. It is possible that microbial systems could be engineered to produce 4-hydroxy-2-butanone at higher titers and
without the need for complex immobilization systems, but further research is needed into this topic.
CHAPTER THREE: FERMENTATIVE PRODUCTION OF SHORT-CHAIN FATTY ACIDS IN ESCHERICHIA COLI

A paper published in the journal Microbiology

Alexandra R. Volker\textsuperscript{1, 2}, David S. Gogerty\textsuperscript{3}, Christian Bartholomay\textsuperscript{3}, Tracie Hennen-Bierwagen\textsuperscript{3}, Huilin Zhu\textsuperscript{3}, and Thomas A. Bobik\textsuperscript{2}

Abstract

\textit{Escherichia coli} was engineered for the production of even- and odd-chain fatty acids (FAs) by fermentation. Co-production of thiolase, hydroxybutyryl-CoA dehydrogenase, crotonase and trans-enoyl-CoA reductase from a synthetic operon allowed the production of butyrate, hexanoate and octanoate. Elimination of native fermentation pathways by genetic deletion ($\Delta ldhA$, $\Delta adhE$, $\Delta ackA$, $\Delta pta$, $\Delta frdC$) helped eliminate undesired by-products and increase product yields. Initial butyrate production rates were high (0.7 g l\textsuperscript{-1} h\textsuperscript{-1}) but quickly levelled off and further study suggested this was due to product toxicity and/or acidification of the growth medium. Results also showed that endogenous thioesterases significantly influenced product formation. In particular, deletion of the yciA thioesterase gene substantially increased hexanoate production while decreasing the production of butyrate. \textit{E. coli} was also engineered to co-produce enzymes for even-chain FA production (described above) together with a coenzyme B12-dependent pathway for the production of propionyl-CoA, which allowed the production of odd-chain FAs (pentanoate and heptanoate). The B12-dependent pathway used

\textsuperscript{1} Primary researcher
\textsuperscript{2} Author
\textsuperscript{3} Secondary researcher
here has the potential to allow the production of odd-chain FAs from a single growth substrate (glucose) in a more energy-efficient manner than the prior methods.

**Introduction**

Fatty acids (FAs) of various chain lengths are important industrial compounds. They are used by the food and beverage and pharmaceutical industries, and can be converted chemically or enzymatically into important biofuels including FA methyl esters, fatty alcohols, methyl ketones, alkenes and alkanes (Dellomonaco et al., 2010; Peralta-Yahya et al., 2012; Zhang et al., 2009). Current methods for industrial FA production often rely on petroleum, a finite resource, whose extraction and usage create energy security and environmental concerns. Hence, bio-based routes of FA production are of interest (Dellomonaco et al., 2010; Peralta-Yahya et al., 2012; Zhang et al., 2009). Research on the bio-based production of FAs has been mostly conducted in *Escherichia coli* and *Clostridium*. The three main approaches that have been used are diversion of phospholipid biosynthesis, reverse β-oxidation and fermentation (Clomburg et al., 2012; Dellomonaco et al., 2010, 2011; Lennen & Pfleger, 2012; Peralta-Yahya et al., 2012; Steen et al., 2010; Zhang et al., 2009). When specific thioesterases are produced at high levels in *E. coli* they hydrolyse the fatty acyl-carrier protein (ACP) intermediates of phospholipid biosynthesis and release free FAs (Lennen & Pfleger, 2012; Lu et al., 2008; Peralta-Yahya et al., 2012; Zhang et al., 2011). Diverting precursors of phospholipid biosynthesis in this manner has been used to produce FAs from C8 to C18 (Lennen & Pfleger, 2012; Peralta-Yahya et al., 2012). The chain lengths released depend on the specificity of the thioesterase, and mixtures of FA or in some cases single FAs are produced. Reverse β-oxidation has also been used to produce FAs in *E. coli* (Clomburg et al., 2012; Dellomonaco et al., 2011). Normally, β-oxidation is used for the degradation of FAs as carbon and energy sources, but *E. coli* has been engineered to reverse this
pathway for the production of FAs of various chain lengths (Clomburg et al., 2012; Dellomonaco et al., 2011). Reverse β-oxidation is potentially more efficient than redirection of phospholipid biosynthesis because FAs are produced by condensation of acetyl-CoA rather than malonyl-ACP whose synthesis requires additional ATP (Dellomonaco et al., 2011).

FAs have also been produced by microbial fermentation. Certain bacteria have native fermentation pathways that produce butyrate and hexanoate and have been used for the production of these FAs. *Clostridium tyrobutyricum* was used to produce high levels of butyric acid from various substrates in a fibrous bed reactor (Jiang et al., 2010; Wei et al., 2013). *Clostridium* sp. BS-1 and *Megasphaera elsdenii* were used to produce hexanoic acid from D-galactitol and sucrose, respectively (Choi et al., 2013; Jeon et al., 2010). In addition, *Clostridium acetobutylicum* has been engineered for high-level butyrate production (Jang et al., 2013). However, the development of clostridia for efficient production of diverse FAs is encumbered by slow growth, a requirement for strictly anaerobic culture conditions, sporulation and relatively limited genetic tools (Baek et al., 2013; Fischer et al., 2010; Lim et al., 2013; Zhang et al., 2009). Consequently, butyrate production was engineered into E. coli (Baek et al., 2013; Lim et al., 2013; Seregina et al., 2010). The reaction sequence used was similar to the butyrate fermentation of Clostridium to the point of butyryl-CoA formation. Two acetyl-CoA are condensed to acetoacetyl-CoA, and then converted in three steps to butyryl-CoA. However, the final step was the hydrolysis of butyryl-CoA to HS-CoA and butyrate by the TesB thioesterase, which differs from the clostridial pathway. The enzymes used for butyrate production in *E. coli* came from diverse sources and were mainly chosen based on studies aimed at optimizing 1-butanol production, which proceeds via a similar pathway to the point of butyryl-CoA (Baek et al., 2013; Bond-Watts et al., 2011; Lim et al., 2013; Shen et al., 2011). Of particular note is the use of
NADH-dependent trans-enoyl-CoA reductase (Ter) in place of a flavoprotein-dependent butyryl-CoA dehydrogenase (Bcd) for the reduction of crotonyl-CoA to butyryl-CoA (Bond-Watts et al., 2011; Shen et al., 2011). Ter enzymes were found to substantially increase the production of 1-butanol (Bond-Watts et al., 2011; Shen et al., 2011) and also work well for butyrate production (Lim et al., 2013). Besides the use of Ter, butyrate production by *E. coli* was substantially enhanced by genetically deleting competing native fermentation pathways (Lim et al., 2013) as was previously done for the fermentative production of ethanol (Jarboe et al., 2007) and other bio-based products (Lim et al., 2013; Shen et al., 2011; Zhu et al., 2011). In addition to butyrate production, reactions similar to those described above for the conversion of two molecules of acetyl-CoA to butyryl-CoA together with TesB have been used under aerobic conditions to produce functionalized short-chain carboxylic acids, including 3-hydroxyvalerate (3-hydroxy-pentanoate) (Tseng et al., 2010), dihydroxybutyrate (Martin et al., 2013), 3-hydroxy-4-methylvalerate (Martin et al., 2013) and a variety of alcohols (Tseng & Prather, 2012). Similar to reverse β-oxidation, fermentative pathways proceed by sequential condensation of acetyl-CoA and are more energy efficient than the interruption of phospholipid biosynthesis.

The three general approaches to FA production described above proceed by condensation reactions that add two carbon atoms per cycle, and hence result in the production of even-chain compounds. However, each of these systems was modified to produce odd-chain-length products. This was done by providing a source of propionyl-CoA, which reacts with acetyl-CoA (or malonyl-ACP) in the first condensation step to produce odd-chain-length compounds. Propionyl-CoA was provided by addition of propionate to the medium (Dellomonaco et al., 2011) or by diversion of threonine biosynthesis (Tseng & Prather, 2012), which is advantageous
because it provides a route to propionyl-CoA from glucose allowing the production of odd-chain products from a single relatively inexpensive growth substrate.

In this report, we describe the engineering of *E. coli* for the production of even- and odd-chain FAs by fermentation. Enzymes related to those previously used for the production of butyrate and 1-butanol are used for the production of butyrate, hexanoate and octanoate (Fig. 1). Studies also indicated that product toxicity and thioesterase specificity were critical to product yield and specificity. In addition, pentanoate and heptanoate were produced using a coenzyme B12-dependent pathway for propionyl-CoA production. This pathway has the potential to allow the production of odd-chain FAs from a single growth substrate (glucose) in a more energy-efficient manner than the prior methods used.

**Materials and Methods**

**Micro-organisms and media**

The bacterial strains used in this study are derivatives of *E. coli* K-12 MG1655 (Table 1). The media used for bacterial growth included lysogeny broth (LB; Difco) M9 minimal medium and terrific broth (TB). M9 minimal medium consisted of (per litre) 64 g Na$_2$HPO$_4$, 7H$_2$O, 15 g KH$_2$PO$_4$, 2.5 g NaCl, 5.0 g NH$_4$Cl, 0.493 g MgSO$_4$ . 7H$_2$O, 0.0147 g CaCl$_2$ . 2H$_2$O, 1 g yeast extract and 4 or 20 g glucose as indicated in the text. TB medium contained (per litre) 12 g tryptone, 24 g yeast extract, 100 ml 0.17 M KH$_2$PO$_4$, 100 ml 0.72 M K$_2$HPO$_4$ and 4 or 20 g l$^{-1}$ glucose as indicated. Kanamycin was used at 50 mg l$^{-1}$, ampicillin at 100 mg l$^{-1}$, chloramphenicol at 20 mg l$^{-1}$, coenzyme B12 at 200 nM and IPTG as indicated.
Chemicals and reagents

Antibiotics and coenzyme B12 were from Sigma Chemical Company. IPTG was from Diagnostic Chemicals Limited. Restriction enzymes and T4 DNA ligase were from New England Biolabs. Ethidium bromide, 2-mercaptoethanol and SDS were from Bio-Rad. Other chemicals were from Fisher Scientific.

Construction of E. coli strains for the fermentative production of FAs

Plasmids were constructed using standard methods for restriction digestion, ligation and electroporation (Sambrook & Russell, 2001) (Table 1). A λDE3 lysogenization kit from EMD4 Biosciences was used for site-specific integration of the T7 RNA polymerase gene under lacUV5 control into E. coli strains where indicated. The enzymes chosen for the fermentative production of even-chain FAs included the Clostridium kluyveri DSM 555 acetyl-CoA C-acetyltransferase (CkThl), accession number EDK35681, Clostridium beijerinckii NCIMB 8052 3-hydroxybutyryl-CoA dehydrogenase (CbHbd), accession number ABR32513, Clostridium kluyveri DSM 555 3-hydroxybutyryl-CoA dehydratase (CkCrt), accession number YP_001393856, and Euglena gracilis trans-2-enoyl-CoA reductase minus the first 134 aa (transit-peptide) (EgTer’), accession number AAW66853. A codon-optimized synthetic operon containing the genes for CkThl, CbHbd, CkCrt and EgTer’ was obtained commercially (Genscript), and subcloned into a modified pET41a vector (pBE522) that has both T7 and E. coli consensus promoters (Zhu et al., 2011). The enzymes used for production of propionyl-CoA were the PduCDE B12-dependent diol dehydratase (P37450, NP_460986, NP_460987) and the PduP aldehyde dehydrogenase (NP_460996), both from Salmonella enterica serovar Typhimurium LT2 (Bobik et al., 1997; Leal et al., 2003). These genes were also cloned as a
synthetic operon into pBE522. For co-expression of the enzymes for even-chain FA synthesis and propionyl-CoA synthesis, the genes for CkThl, CbHbd, CkCrt and EgTer’ were subcloned into a modified pACYC duet vector pBE1570. This vector has a p15a origin that is compatible with that of the colE1 origin of the pBE522 vector used for expression of propionyl-CoA synthesis genes. It also has both E. coli and T7 promoters under control of LacI\(^\text{q}\). To construct pBE1570, the following linker was cloned using EcoNI to XhoI into pACYCDuet (EMD4Biosciences):

```
AAATTAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTTTTGACA
GCTAGCTCAGTCCTAGGTATAATGACTAGAAATAATTTATGTTTACACTTTAATAAG
GAAGATCTCATATGAAGCCTTGTGCGACC.
```

![Diagram of fermentation-deficient E. coli](image)

Fig. 4. Pathway of fermentative FA production by acyl-CoA condensation and reduction. Two acetyl-CoA molecules are condensed, reduced and dehydrated to form butyryl-CoA, which is then hydrolysed to HS-CoA and butyrate by a thioesterase (Tes). Alternatively, butyryl-CoA can condense with acetyl-CoA and move through the same pathway to form hexanoyl-CoA, which is hydrolysed to hexanoate and HS-CoA. In principle, the pathway can function iteratively to form longer-chain FAs. In addition, provision of propionyl-CoA allows the formation of odd-chain products using the same enzyme reactions. The fermentation-deficient E. coli is E. coli K-12 MG1655 DE3, DldhA, DadhE, DackA, Dpta and DfrdC. Thl, thiolase; Hbd, hydroxybutyryl-CoA dehydrogenase; Crt, crotonase; Ter, trans-enoyl-CoA reductase.
For expression and purification of his-tagged enzymes, codon-optimized genes were subcloned into T7 vector pTA925 (Johnson et al., 2001) using BglII and HinDIII sites, and transformed into *E. coli* BL21 DE3 RIL (Stratagene). The resulting protein production strains were BE1126, BE1165, BE1572 and BE1575 (Table 1). All the clones described above were verified by DNA sequencing.

**Preparation of cell-free extracts for protein purification**

A 1 litre baffled Erlenmeyer flask containing 400 ml of LB medium was inoculated with 10 ml of an overnight culture grown on LB kanamycin medium. Cells were grown at 37 °C with shaking at 250 r.p.m. using an Innova I2400 incubator shaker (New Brunswick Scientific). When cells reached an OD<sub>600</sub> of 0.6–0.8 the temperature was reduced to 30 °C, and protein production was induced by addition of IPTG to 0.5 mM. Six hours after induction, cells were harvested by centrifugation at 5000 g for 10 min using a Beckman JA-25 centrifuge and JA-10 rotor. Culture pellets (~1 g of cells) were resuspended in 3 ml of lysis buffer (50 mM HEPES, pH 7.0, 300 mM NaCl, 20 mM imidizole, 5 mM AEBSF protease inhibitor, 1 mM DTT), and broken using a French pressure Cell (Thermo Scientific) at 20 000 p.s.i. (1.386105 kPa). Crude cell extracts were cleared by centrifugation using a Beckman J-25 centrifuge with a JA-17 rotor at 25 000 g for 20 min. Cleared cell extracts were filtered using a Nalgene 0.45 mm syringe filter and used directly for enzyme assays. Protein concentrations were determined using the Bio-Rad Protein Assay Reagent.

**His-tag protein purification**

Cell-free extracts (prepared as described above) were combined with 1 ml of Qiagen Ni-NTA Superflow and mixed on an orbital shaker for 30 min. The resin was placed in a 1 ml
polypropylene column (Qiagen) and washed once each with 5 ml of buffer that contained 50 mM HEPES, pH 7.0, 300 mM NaCl and 50, 80 or 150 mM imidazole. Lastly, the his-tagged protein was eluted with 5 ml of 300 mM imidazole in the same buffer.

### Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
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<td>BL21(DE3)</td>
<td>F′ ompT gal dcm len hsdSB (rB− mB−) λBL21(DE3)</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>DH5α</td>
<td>fhuA2 Δ(argF-lacZ)U169 phosphA glmV44 φ80A (lacZ)M15 gyrA96 recA1 relA1 thi-1 hsdR17</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>BL21DE3 RIL</td>
<td>E. coli B F′ ompT hsdS (rB− mB−) dcm+ Tet’ gal λBL21(DE3) endA Hte [argU ileY leuW Cam’]</td>
<td>Stratagene</td>
</tr>
<tr>
<td>BE400</td>
<td>E. coli K-12 MG1655 (wild-type)</td>
<td>Laboratory collection</td>
</tr>
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<td>BE1126</td>
<td>E. coli BL21DE3 RIL/pTA925-H6-CxThl, kanR</td>
<td>This study</td>
</tr>
<tr>
<td>BE1165</td>
<td>E. coli BL21DE3 RIL/pTA925-H6-CxHbd, kanR</td>
<td>This study</td>
</tr>
<tr>
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<td>E. coli BL21DE3 RIL/pTA925-H6-CxCrt, kanR</td>
<td>This study</td>
</tr>
<tr>
<td>BE1575</td>
<td>E. coli BL21DE3 RIL/pTA925-H6-EgTer', kanR</td>
<td>This study</td>
</tr>
<tr>
<td>BE1578</td>
<td>E. coli K-12 MG1655 DE3</td>
<td>This study</td>
</tr>
<tr>
<td>BE1576</td>
<td>E. coli MG1655 DE3, ΔadhE::FRT, ΔadhA::FRT, ΔackA-pta::FRT, ΔfrdC::FRT/pBE522-thl-hbd-crt-ter' (CxThl-CxHbd-CxCrt-EgTer')</td>
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<td>BE1576 ΔyecA::FRT</td>
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<td>BE1576 ΔyegC::FRT</td>
<td>This study</td>
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<tr>
<td>BE1908</td>
<td>E. coli MG1655 DE3 ΔadhE::FRT, ΔadhA::FRT, ΔackA-pta::FRT, ΔfrdC::FRT/pBE522-pduP-pduCDE-pduGH</td>
<td>This study</td>
</tr>
<tr>
<td>BE1952</td>
<td>E. coli MG1655 DE3 ΔadhE::FRT, ΔadhA::FRT, ΔackA-pta::FRT, ΔfrdC::FRT/pBE522-pduP-pduCDE-pduGH (aldehyde dehydrogenase, diol dehydratase and reactivase), pBE1570 thl-hbd-crt-ter' (CxThl-CxHbd-CxCrt-EgTer')</td>
<td>This study</td>
</tr>
</tbody>
</table>

*All strains are derivatives of E. coli K-12 MG1655.

### Enzyme assays

The Thl assay was based on previously established protocols (Hartmanis & Stadtman, 1982; Sliwkowski & Hartmanis, 1984; Sliwkowski & Stadtman, 1985). Briefly, activity was measured in a 1 ml assay that contained 100 mM Tris/HCl, pH 8.2, 25 mM MgCl₂, 33 mM acetoacetyl-CoA (Sigma), 90 mM CoA (Sigma) and 5 µl of purified enzyme. Assays were incubated at 30 °C and absorbance at 303 nm was monitored using a Varian Cary 50 Bio UV-
visible spectrophotometer. This assay measures the degradation of acetoacetyl-CoA to two molecules of acetyl-CoA. The initial rate of the Thl reaction was calculated using an extinction coefficient of 14 mM\(^{-1}\) cm\(^{-1}\) for acetoacetyl-CoA. The Hbd assay was based on a previously published protocol (Colby & Chen, 1992). The 1 ml assay contained 50 mM MOPS, pH 7.0, 1 mM DTT, 75 mM acetoacetyl-CoA (Sigma), 0.15 mM NADH (Sigma) and 5 µl of purified enzyme. Assays were carried out at 30 °C and absorbance at 340 nm was followed. The initial rate of Hbd was calculated using an extinction coefficient of 6.22 mM\(^{-1}\) cm\(^{-1}\) for NADH. The CRT assay was a modification of a prior protocol (Waterson & Hill, 1972). A 1 ml assay contained 45 mM Tris/ HCl, pH 7.6, 0.0045 % BSA, 10 mM KCl, 10 mM NaCl, 2 mM MgCl\(_2\) and 53.44 mM crotonyl-CoA (Sigma), with 5 µl of purified enzyme. Assays were incubated at 30 °C and the absorbance at 263 nm was measured. A decrease in absorbance at 263 nm is due to the hydration of the double bond in crotonyl-CoA. The initial rate of Crt was calculated using an extinction coefficient of 6.7 mM\(^{-1}\) cm\(^{-1}\). The crotonyl-CoA reductase (CCR) assay contained 45 mM Tris/HCl, pH 7.6, 0.0045 % BSA, 10 mM KCl, 10 mM NaCl, 2 mM MgCl\(_2\), 53.44 mM crotonyl-CoA (Sigma) and 0.15 mM NADH (Sigma), with 5 µl of purified enzyme in a total volume of 1 ml. Assays were incubated at 30 °C and the absorbance at 340 nm was followed. The initial rate of CCR was calculated using an extinction coefficient of 6.22 mM\(^{-1}\) cm\(^{-1}\) for NADH.

**Fermentation conditions**

Fermentation strains were grown overnight in 50 ml of LB medium with appropriate antibiotics at 37 °C with shaking at 250 r.p.m. Cells were pelleted by centrifugation at 5000 g (using a Beckman J-25 centrifuge and JA-17 rotor) and then resuspended in 10 ml TB or minimal medium supplemented with 0.4 or 2 % glucose and 200 nM coenzyme B12 (for
production of odd-chain FAs). The OD$_{600}$ was measured and cell suspensions were then placed in BellCo 18x150 mm anaerobic test tubes which were closed with serum stoppers and crimp seals. Oxygen was removed from the headspaces of the anaerobic tubes by flushing with 95% N$_2$/5% CO$_2$ and tubes were incubated for 18–24 h at 37 °C with shaking at 250 r.p.m.

**Measurement of glucose and fermentation products**

The fermentation products present in culture media were measured by HPLC and/or GC. Cells were removed from fermentation medium by centrifugation followed by filtration through a cellulose acetate membrane (0.2 µm pore size) (Thermo Scientific). Filtered medium was analysed using a Varian ProStar HPLC system, equipped with a 325 UV monitor (210 nm), a 355 RI detector and a Bio-Rad HPX-87H column. The column was developed with 5 mM H$_2$SO$_4$ at 0.4 ml min$^{-1}$, isocratic. Analytes were identified by retention time (and CG-MS as described below) and quantified by comparison of peak areas to a standard curve. The concentration of Analytes was normalized to a cell density of OD$_{600}$ of 20 (typical OD$_{600}$ values were 18–22). Glucose concentrations were determined using the Glucose (GO) Assay kit (Sigma) according the manufacturer’s instructions.

**Short-chain FA analysis by GC and GC-MS**

Short-chain FAs from anaerobic cell culture supernatants were detected and quantified using a Varian 450 gas chromatograph fitted with an Agilent HP-5MS column and flame-ionization detector. Supernatants were filtered using a Nalgene 0.2 µm syringe filter and 1 µl was injected into the chromatograph. The method used a 10:1 split ratio and a 30 ml min$^{-1}$ flow rate with helium as the carrier gas. The oven was held at 40 °C for 1 min followed by a ramp of 10 °C min$^{-1}$ up to 240 °C at which point the temperature was held at 240 °C for 15 min. For
analysis by GC-MS, 0.5 ml of culture supernatant was acidified with 50 µl 1 M HCl and extracted with 1 ml of chloroform/methanol (1:1) (Mayer & Shanklin, 2007) using heptanoic acid as an extraction control where indicated. The organic phase (1 µl) was injected onto an Agilent 6890 Series gas chromatograph equipped with an Agilent HP-5MS column and an Agilent single quadrupole 5973 mass spectrometer using electron ionization. The method used a splitless injection with a 50 ml min\(^{-1}\) flow rate and helium as the carrier gas, and a mean velocity of 36 cm/s. Oven temperature and run times were identical to GC-flame ionization detector described above.

**Results**

**Enzymes for the fermentative production of even-chain FAs by E. coli**

The enzymes used for fermentative production of even-chain FAs by E. coli were thiolase from C. kluyveri (CkThl), 3-hydroxybutyryl-CoA dehydrogenase from C. beijerinckii (CbHbd), crotonase from C. kluyveri (CkCrt) and trans-enoyl-CoA reductase from E. gracilis (EgTer\(^{+}\)) (see Methods). These enzymes are expected to catalyse production of FAs by the pathway shown in Fig. 4. The clostridial enzymes used were chosen from organisms that naturally produce butyrate and hexanoate by fermentation with the intention of producing longer-chain FAs (Seedorf et al., 2008). The C. beijerinckii Hbd was chosen because it is NAD-specific (Colby & Chen, 1992) and E. gracilis Ter was used because prior studies indicated that this enzyme improved production of butyrate and 1-butanol and that it is capable of reducing longer-chain enoyl-CoA compounds (Bond-Watts et al., 2011; Shen et al., 2011). All genes were obtained by gene synthesis and codon-optimized for protein production in E. coli.
Specific activities of enzymes for even-chain FA production in *E. coli*

To determine whether the enzymes chosen for even-chain FA production could be produced from codon-optimized genes with high activity in *E. coli*, we overproduced and purified his-tagged versions (Fig. 5). The specific activities of the N-terminally 6x his-tagged enzymes were 165.2, 438.5, 5224.7 and 76.5 µmol min\(^{-1}\) mg\(^{-1}\) for *Ck*Thl, *Cb*Hbd, *Ck*Crt and *Eg*Ter’, respectively. The specific activities for *Ck*Thl and *Cb*Hbd are somewhat higher than previously reported activities of 115 and 41.6 µmol min\(^{-1}\) mg\(^{-1}\), respectively (Sliwkowski & Hartmanis, 1984; von Hugo et al., 1972). The activity of *Ck*Crt is similar to that reported for the *C. acetobutylicum* enzyme (6155 µmol min\(^{-1}\) mg\(^{-1}\)) (Waterson & Hill, 1972). With respect to *Eg*Ter, the cited specific activity for the recombinant enzyme in crude extracts was 3.7 µmol min\(^{-1}\) mg\(^{-1}\) (Shen et al., 2011). The specific activity determined in this study for the purified enzyme is approximately 19 times higher (76.5 µmole\(^{-1}\) min\(^{-1}\) mg\(^{-1}\)); thus, these activities are likely to be roughly similar for purified enzyme.
Strain engineering for even-chain FA production

The optimized genes described above were used to construct a synthetic operon for the production of the CkThl, CbHbd, CkCrt and EgTer’ enzymes. This operon was moved into strain ZH84 (E. coli K-12 MG1655 DE3, ΔldhA, ΔadhE, ΔackA, Δpta, ΔfrdC) which has the native fermentation pathways deleted to reduce the formation of undesired by-products (Jarboe et al., 2007). The activities of the CkThl, CbHbd, CkCrt and EgTer’ enzymes within the context of the synthetic operon were then measured in crude cell extracts, and found to be 10.4, 39.3, 1259.2 and 1.6 µmol min⁻¹ mg⁻¹, respectively. These activities are sufficiently high to allow the production of substantial quantities of FAs. Thus, the resulting strain (BE1576) was expected to
produce even-chain FAs by the pathway shown in Fig. 4 with reduced by-product formation due to genetic deletion of competing native fermentation pathways.

**FA production via fermentation**

To test *E. coli* strain BE1576 for FA production by fermentation, cells were grown aerobically and then resuspended at an OD$_{600}$ of about 20 under anaerobic conditions. Under these conditions BE1576 did not grow appreciably and was functioning as a cell catalyst. When cells were incubated with 0.4 % glucose in M9 minimal medium for 20 h, HPLC analyses showed that the culture medium contained 0.815±0.005 g butyrate l$^{-1}$ and 0.395±0.002 g hexanoate l$^{-1}$. In addition, GC analyses were able to detect 0.051±0.007 g octanoate l$^{-1}$. During these fermentations, about 93 % of the glucose was utilized. Control strains having plasmid without insert did not produce detectable amounts of FAs. Thus, the results indicated that strain BE1576 produced significant quantities of butyrate, hexanoate and octanoate by fermentation of glucose.

**GC-MS to verify the FAs produced**

Initial identification of butyrate, hexanoate and octanoate in culture media was based on HPLC retention times. To verify the identity of these fermentation products, GC-MS was performed on FAs extracted from culture medium using chloroform/methanol (Mayer & Shanklin, 2007). The total ion chromatogram showed peaks at 10.3, 12.6 and 15.0 min, which matched the retention times of the butyrate, hexanoate and octanoate standards (Fig. 6). In addition, the mass spectrum from each peak was used to query the National Institute of Standards and Technology library and results showed near-perfect matches to spectra for butyrate, hexanoate and octanoate (Fig. 6). No FA peaks were detected in assays containing control cells.
lacking the synthetic operon for even-chain FA production. These results confirmed that butyrate, hexanoate and octanoate accumulated in the culture medium.

**Effect of excess glucose**

In the experiments described above (which used 0.4 % glucose) >90 % of the glucose was consumed. To test whether additional glucose would increase the production of C4, C6 and C8 FAs, fermentations were done using 2 % glucose in both M9 and TB media. Cells incubated in M9 media for 24 h accumulated 1.632±0.008 g butyrate l⁻¹, 0.586±0.011 g hexanoate l⁻¹ and 0.028±0.001 g octanoate l⁻¹. When incubated in TB medium, the short-chain FA production after 24 h incubation was 1.312±0.009 g butyrate l⁻¹, 0.686±0.015 g hexanoate l⁻¹ and 0.216±0.108 g octanoate l⁻¹. Somewhat higher hexanoate and substantially more octanoate was produced on TB medium compared with on M9. This suggests that the carbon sources present in TB provided additional reducing power (NADH) for the production of hexanoate and octanoate. For each of the experiments described above, control strains having plasmid without insert did not produce detectable amounts of butyrate, hexanoate or octanoate. Somewhat unexpectedly, under the conditions used, only about 25 % of the added glucose was consumed.
Fig. 6. Identification of butanoic, hexanoic and octanoic acids by GC-MS. Top: elution times were 10.3 (butanoic), 12.6 (hexanoic) and 15 min (octanoic). Middle row, left to right: spectra for butanoic, hexanoic and octanoic acids produced by fermentation. Bottom row, left to right: spectra for butanoic, hexanoic and octanoic acids obtained from the National Institute of Standards and Technology library.

**Time-course of butyrate production**

We also looked at the time-course of butyrate formation by strain BE1576 using TB medium with 2 % glucose. Results showed that butyrate accumulated rapidly for the first hour, reaching a level of 0.79±0.05 g l⁻¹ then levelled off abruptly increasing to a final yield of 1.77±0.05 g l⁻¹ after 24 h (Fig. 7). Again, less than 25 % of the added glucose was consumed. It seemed possible that the fall off in FA production and the incomplete glucose consumption may
have been due to FA toxicity inhibiting the metabolism of the production strain, BE1576 (Nunn et al., 1979). Given that low pH increases the toxicity of FAs, we looked at butyrate production on TB plus 2 % glucose while controlling the pH in a range between 7.0 and 7.1. This was done over a 24 h period, followed by anaerobic incubation without pH control for an additional 20 h. After the initial 24 h period, 68.18 % of the glucose had been consumed and butyrate, hexanoate and octanoate had been produced at 7.47, 0.37 and 0.043 g l\(^{-1}\), respectively. Following the additional 20 h incubation period, 82.7 % of the glucose had been consumed and the FA titres were at 9.67, 0.365 and 0.0425 g l\(^{-1}\), respectively. The reason that the relative production of butyrate, hexanoate and octanoate varied with and without pH control (see above) is uncertain. The medium pH might affect thioesterase expression but alternative explanations are also possible. More importantly, however, pH control substantially increased FA production.

![Fig. 7. Time-course of fermentative butyrate production. Butyrate production began rapidly but quickly levelled off, suggesting that productivity was limited by product toxicity or medium acidification. The strain used for butyrate production was BE1576 (Table 1). The medium was TB supplemented with 2 % glucose.](image-url)
Involvement of native thioesterases in FA production by strain BE1576

In the studies described above, no recombinant thioesterases were produced in the FA production strain (BE1576). Thus, although other possibilities were not ruled out, it seemed likely that hydrolysis of acyl-CoA compounds to FAs was mediated by endogenous thioesterases. To test for a possible role of native thioesterases in the production of FAs, we made single gene knockout mutations in four genes that are annotated as thioesterases in *E. coli*: *tesB, paaI, yciA* and *ybgC*. Each mutation was moved individually into FA production strain BE1576 and its effect on fermentative FA production was tested (Fig. 8). Deletion of the *tesB, paaI* or *ybgC* genes had little effect on the fermentative production of FA. However, deletion of the *yciA* gene substantially decreased butyrate formation while increasing hexanoate formation. This suggested that YciA plays a significant role in the hydrolysis of butyryl-CoA to butyrate.

![Fig. 8. Effects of thioesterase deletions on the fermentative production of butyrate (light grey) and hexanoate (dark grey). The strains used were BE1576, BE1869, BE1871, BE1872 and BE1873 (Table 1). The medium was TB supplemented with 2 % glucose.](image-url)
Production of odd-chain FAs

The enzymes used for the production of even-chain FAs described above (CkThl, CbHbd, CkCrt and EgTer’) are also expected to mediate the production of odd-chain FAs, if a source of propionyl-CoA is provided. In this case, propionyl-CoA will condense with acetyl-CoA in the first step of the pathway to produce 3-ketovalerate, which will proceed through the pathway similarly to its C4 analogue (Fig. 4) and produce pentanoate. To produce propionyl-CoA, we chose a coenzyme B12-dependent pathway. 1, 2-Propanediol can be converted to propionyl-CoA by the sequential action of coenzyme B12-dependent diol dehydratase (PduCDE) and HS-CoA-dependent propionaldehyde dehydrogenase (PduP) (Fig. 9). This pathway is potentially advantageous because 1, 2-propanediol can be efficiently produced from glucose (Clomburg & Gonzalez, 2011). Accordingly, we cloned the genes for diol dehydratase (pduCDE), diol dehydratase reactivase (pduGH) and propionaldehyde dehydrogenase (pduP) from Salmonella enterica into pBE522 as a synthetic operon. To determine whether this operon produced active enzymes in E. coli, we measured the activities of SePduCDE, SePduGH and SePduP in crude cell extracts of strain BE1908. The activities of the PduCDE diol dehydratase and the PduP aldehyde dehydrogenase were 51.0 and 9.2 µmol min⁻¹ mg⁻¹, respectively. There is no quantitative assay for reactivation of PduCDE by PduGH; however, we found that the expression plasmid was able to complement a pduGH deletion mutant of S. enterica for growth on 1, 2-propanediol, establishing the active expression of the SePduGH reactivase (data not shown). Given the measured activities of SePduCDE, SePduP and SePduGH, we expected this operon to effectively mediate the production of propionyl-CoA from 1, 2-propanediol.
Next, we combined plasmids for the production of even-chain FAs (pBE1570-thl-hbd-ccr-ter) and for propionyl-CoA production (pBE522-pduP-pduCDE-pduGH) into a background where the native fermentation pathways were deleted. The resulting strain (BE1952) was assayed for FA production by fermentation using TB medium supplemented with 2 % glucose, 1 % 1, 2-propanediol and 200 nM coenzyme B12. HPLC analyses showed the production of propionate (1.19±0.23 g l$^{-1}$) butyrate (0.23±0.03 g l$^{-1}$) and pentanoate (0.20±0.01 g l$^{-1}$). In addition, GC analyses found a small amount of heptanoate (0.008±0.004 g l$^{-1}$). The authenticity of the pentanoate and heptanoate were verified by GC-MS (as described above). Both synthetic operons (CkThl-CbHbd-CkCrt-EgTer and SePduP-SePduCDE-SePduGH) were required for the production of pentanoate, and none was detected when 1, 2-propanediol was omitted from the fermentation medium. Thus, we infer that pentanoate and heptanoate were produced using propionyl-CoA derived from 1, 2-propanediol.
Fig. 9. Pathway for production of propionyl-CoA as a precursor for the production of odd-chain FAs. Dihydroxyacetone phosphate (DHAP) is diverted from glycolysis to form 1,2-propanediol, and then 1,2-propanediol is converted to propionyl-CoA by a coenzyme B12-dependent diol dehydratase (PduCDE) and propionaldehyde dehydrogenase (PduP). The propionyl-CoA formed is condensed with acetyl-CoA to form 3-keto-pentanoate, which is converted to pentanoic acid by the enzymes shown in Fig. 4.

Discussion

Three general approaches for the microbial production of FAs have been studied most extensively: early termination of lipid biosynthesis, reverse β-oxidation and fermentation (Clomburg et al., 2012; Dellomonaco et al., 2010, 2011; Lennen & Pfleger, 2012; Peralta-Yahya et al., 2012; Steen et al., 2010; Zhang et al., 2009). Prior studies of the fermentative production of FAs used clostridia to produce high levels of butyrate or hexanoate from glucose or galactitol, respectively (Jiang et al., 2010; Wei et al., 2013). However, clostridia are disadvantageous due to slow growth, a requirement for strictly anaerobic culture conditions, sporulation and relatively limited genetic tools for use in strain improvement and product
diversification (Baek et al., 2013; Fischer et al., 2010; Lim et al., 2013; Zhang et al., 2009). To circumvent these problems, *E. coli* was engineered for fermentative butyrate production (Baek et al., 2013; Lim et al., 2013; Seregina et al., 2010). This was done by deleting *E. coli*’s native fermentation pathways and engineering it to produce enzymes for butyrate formation that catalyse the condensation and reduction of acyl-CoA compounds: AtoB and TesB from *E. coli*, Hbd and Crt from *C. acetobutylicum* and Ter from *Treponema denticola* (Baek et al., 2013; Fischer et al., 2010; Lim et al., 2013; Zhang et al., 2009). Here, we used an *E. coli* strain deleted for the native fermentation pathways (Δ*adhE:: FRT*, Δ*ldhA:: FRT*, Δ*ackA-pta:: FRT*, Δ*frdC:: FRT*) and an analogous set of enzymes for fermentative FA production by *E. coli* (*CkThl-CbHbd-CkCrt-EgTer*) (strain BE1576). However, in the studies reported here, not only was butyrate produced but also hexanoate and octanoate. This was probably due to the substrate specificities of the particular enzymes used as prior studies aimed at the production of fuel alcohols by an analogous pathway found that the enzymes used for acyl-CoA condensation and reduction affected the amounts of longer-chain alcohols produced (Dekishima et al., 2011). The yields of butyrate reported here are similar to the levels previously obtained in *E. coli*, 9.670 versus 7.2 g l\(^{-1}\) in prior studies (Baek et al., 2013). The highest levels of hexanoate produced here were about 10-fold higher than prior studies, 1.963 versus about 0.180 g l\(^{-1}\) (Machado et al., 2012). The highest amount of octanoate measured here was 0.216 g l\(^{-1}\) but in prior studies octanoate levels were not reported. Thus, the system reported here provides a reasonable basis for the production of even-chain FAs in *E. coli* including some longer-chain molecules.

In this report, we produced odd-chain FAs using a new pathway that has some potential advantages compared with prior methods (Fig. 9). The production of odd-chain FAs starts with the condensation of propionyl-CoA and acetyl-CoA followed by reduction and dehydration.
What is new here is that a coenzyme B12-dependent pathway was used to produce propionyl-CoA from 1, 2-propanediol. The production of propionyl-CoA from 1, 2-propanediol is of interest because prior studies engineered E. coli for the production of 1, 2-propanediol directly from glucose via dihydroxyacetone phosphate (DHAP); hence, there is the possibility of combining these pathways to produce propionyl-CoA from glucose in five steps with an energy cost of two ATP (the conversion of DHAP to propionyl-CoA would bypass two ATP-forming steps of glycolysis). Previously, odd-chain FAs were produced by addition of propionate to the medium for proof-of-concept, but this approach would generally be too expensive for commercial applications. In other work, propionyl-CoA was produced from phosphoenolpyruvate (derived from glucose or glycerol) via threonine in nine steps with an energy cost of three ATP per propionyl-CoA (Tseng & Prather, 2012). Lengthy pathways are more difficult to engineer and ATP consumption generally reduces product yield (particularly under anaerobic conditions) as some feedstock must be consumed to supply the needed energy. Thus, the B12-dependent pathway used here (in combination with a previously described pathway for 1, 2-propanediol production from glucose; Clomburg & Gonzalez, and 2011) is potentially advantageous due to fewer steps and a lower energy cost. The amounts of pentanoate and heptanoate produced here by combining enzymes for acyl-CoA condensation with the B12-dependent pathway of propionyl-CoA formation were 0.201±0.01 and 0.008 g l⁻¹, respectively. In prior studies that produced propionyl-CoA via threonine, the combined amounts of C5 compounds (pentanoate and pentanol) produced from glucose or glycerol were about 0.03 or 0.2 g l⁻¹, respectively. To our knowledge, heptanoate has not been previously produced in E. coli by fermentation. Thus, the approach reported here appears to be a promising basis for further
development of odd-chain FA production not only by fermentation, but also by premature termination of lipid biosynthesis or reverse β-oxidation.

A notable difference between the system for FA production described here and prior systems was that no thioesterase activity was engineered into the strains described here. Thus, the cleavage of acyl-CoA derivatives to the corresponding FAs was catalysed by endogenous enzyme systems (or occurred chemically), most likely by thioesterases. To test this idea, we individually deleted four thioesterase genes: \( \text{tesB}, \text{paaI}, \text{yciA} \) and \( \text{ybgC} \). Deletion of \( \text{tesB}, \text{paaI} \) or \( \text{ybgC} \) has little effect on the amount or types of FAs produced. It was somewhat surprising to us that deletion of \( \text{tesB} \) had little effect as production of recombinant TesB has been previously used for short-chain FA production in \( E. \ coli \) (Baek et al., 2013; Martin et al., 2013; Tseng et al., 2010; Tseng & Prather, 2012). By contrast, deletion of the \( \text{yciA} \) gene had a large effect, substantially increasing the amount of hexanoate at the expense of butyrate (Fig. 8). These results show the endogenous thioesterases play an important role in FA production in the system described here. They are also consistent with a recent survey study that used the control of thioesterase production to improve yields of pentanoate, trans-2-pentenoate and 4-methyl-pentanoate (McMahon & Prather, 2014). Thus, genetic deletion of native thioesterases that cleave shorter chain FAs is a potential approach for increasing yields of longer chain products or to control product specificity.

In the studies described here, we also looked at the time-course of even-chain FA production. Results showed a relatively high initial rate of butyrate production for the first hour: 0.79 g l\(^{-1}\) h\(^{-1}\) (0.2 g l\(^{-1}\) h\(^{-1}\) per gram of dry cells), which is about 10 % of a commercially meaningful rate. The rapid decline in the rate of FA production a later time points was probably due to the accumulation of FAs that are known to be toxic or to medium acidification (Desbois &
Smith, 2010; Lennen & Pfleger, 2012; Royce et al., 2013). We also found that pH control (FAs are more toxic at a lower pH) substantially increased total FA production (butyrate+hexanoate+octanoate) by about 450%. Thus, it is likely that the fermentative production of FAs by *E. coli* is mainly limited by product toxicity and/or medium acidification and that the system used here could support substantially higher levels of FA production if this difficulty could be reduced or eliminated by the use of resistant production strains or fermentation processes that continuously remove product.

**Acknowledgements**

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Edited by: S. Kengen
CHAPTER FOUR: FATTY ACID CHAIN LENGTH EXTENSION UTILIZING THE CANDIDA BOIDINII FORMATE DEHYDROGENASE

Introduction

The previously described butyrate production pathway relies on oxidation-reduction reactions that require nicotinamide adenine dinucleotide (NAD) as a cofactor. In order for the reactions to proceed, it is necessary for the NADH pool to be continually regenerated. If there is not enough NADH available, the final metabolite concentration is expected to be lower. We hypothesize that our even-chain production pathway is not redox balanced for production of fatty acids longer than C4, such as hexanoate and octanoate, which explains their lower titers in the fermentation medium. During anaerobic fermentation, glucose is converted to 2 acetyl-CoA and 2 formate and 2 NADH are produced. When 2 acetyl-CoA is condensed to form 1 butyryl-CoA, 2 NADH are used. These reactions are redox balanced. However, when hexanoate is made, 1 butyryl-CoA and 1 acetyl-CoA are condensed, but this uses another 2 NADH and the reactions are no longer balanced. This decreases the NADH pool faster than butyryl-CoA production alone. To remedy this issue, the NADH pool must be increased.
Fig 10: The butyrate pathway, composed of thl-hbd-crt-ter, utilizes two NADH molecules for every turn of the pathway. Two NADH are required to make one molecule of butyrate, four NADH are required to make one molecule of hexanoate, and so on.

One method to increase the NADH pool is to introduce an enzyme that will utilize formate, a by-product of our fermentations, and convert it to CO$_2$ while regenerating NADH from NAD$^+$. Fdh1 from *Candida boidinii*, which converts formate and NAD$^+$ to CO$_2$ and NADH, has been successfully used in other systems to increase NADH pools (Berrios-Rivera et.al. 2002, Balzer et.al. 2013, Ma et.al. 2013). It has been demonstrated that Fdh1 expression shifts the metabolic profile of *E. coli*, increasing ethanol and succinate formation while decreasing the amounts of acetate and lactate produced (Berrios-Rivera et.al. 2002). Expression of Fdh1 in a succinate production strain improved succinate titers and 1 mol NADH was produced per mol of formate (Balzer et.al. 2013). This study also shows that Fdh1 expression can efficiently remove
formate from the fermentation medium, solving the issue of how to remove it as an otherwise unnecessary byproduct. In *Klebsiella pneumoniae*, Fdh1 was expressed and the resulting higher availability of NADH improved glycerol metabolism and 1,3 propanediol production (Ma et.al. 2013). Based on the success of these prior studies, we chose to produce Fdh1 in conjunction with the butyrate production pathway to determine if increasing the available NADH pool will result in an increase in titers of hexanoate and octanoate.

**Materials and Methods**

**Microorganisms and media**

The bacterial strains used in this study are derivatives of *E. coli* K-12 MG1655 (Table 2). The media used for bacterial growth included lysogeny broth (LB; Difco) and Terrific broth (TB). TB medium contained (per litre) 12 g tryptone, 24 g yeast extract, 100 mL KH$_2$PO$_4$, and 4 g glucose. Chloramphenicol was used at 20 mg/L and kanamycin was used at 50 mg/L.

**Construction of *E. coli* strains for fermentative production of fatty acids**

Plasmids were constructed using standard methods for restriction digestion, ligation, and electroporation. *Candida boidinii* Fdh was synthesized by Genscript and codon-optimized for expression in *E. coli*. Fdh1 was subcloned into pBE1570, a modified pACYCDuet (EMD4Biosciences) vector as previously described. *Thl-Hbd-Crt-Ter*, which converts two acetyl-CoA molecules into butyryl-CoA (Fig.10), was subcloned into pBE522.

**Fermentations**

50 mL cultures were grown overnight at 37 °C and 275 rpm. The cells were pelleted and resuspended in 10 mL of TB media supplemented with 2% glucose. Cultures were transferred to
glass flasks and the headspace evacuated with 95% N\textsubscript{2} 5% CO\textsubscript{2}. Fermentations were allowed to proceed for 24 hours at 37 °C and 275 rpm.

**Fatty acid detection and quantification**

Fermentations were collected at 24 hours. Samples were centrifuged at 5000 \textit{x} g for 15 min and the supernatant was filtered with a 0.22 \textmu m syringe filter. Samples were analyzed on a Varian ProStar HPLC system, equipped with a 325 UV monitor (210nm), a 355 RI detector, and a Bio-Rad HPX 87H column. The column was developed with 5 mM H\textsubscript{2}SO\textsubscript{4} at 0.4 mL min\textsuperscript{-1}. Metabolites were identified and quantified by peak retention time and comparison to peak areas of standard curves.

Table 2: Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
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<tr>
<td>ZH84</td>
<td>\textit{E. coli} K12 MG1655 \textbackslash\textbackslash \Delta\textit{adhE::FRT}, \Delta\textit{ldhA::FRT}, \Delta\textit{ackA-pta::FRT}, \Delta\textit{frdC::FRT}</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>BE1576</td>
<td>ZH84/pBE522-\textit{thl-hbd-crt-ter}</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>ARV11442</td>
<td>BE1576/pBE1570-\textit{fdh1}</td>
<td>This study</td>
</tr>
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</table>

**Results**

Co-expression of \textit{fdh1} with \textit{thl-hbd-crt-ter} and anaerobic fermentation resulted in the production of a mixture of short-chain fatty acids (fig 11). As expected, the major constituents of this mixture were butyrate, hexanoate, and octanoate. Butyrate was still produced at the highest
titer, but with Fdh1 production lowered the concentration from 10.44 mM to 5.77 mM. Hexanoate production was variable in both the BE1576 and ARV11442, ultimately showing no discernable difference in titer. However, the titer of octanoate was more than doubled with Fdh1 expression, increasing from 0.3487 mM to 1.37 mM. Thus it is reasonable to conclude that \( fdh1 \) expression is increasing the available NADH in the cell and therefore allowing longer chain fatty acids to be produced at a higher titer.

![SCFA production with and without Fdh1](image)

Figure 11: Production of SCFA with and without Fdh expression. In blue, BE1576 (no \( fdh1 \)); in red, ARV11442 (\( fdh1 \)). Octanoate levels are increased in ARV11442.

**Discussion**

Expression of formate dehydrogenase has previously been shown to increase NADH pools (Berrios-Rivera et.al 2002, Balzer et.al 2013, Ma et.al 2012). Formate dehydrogenase from *C. boidinii* converts formate to CO\(_2\) and in the process regenerates NADH from NAD\(^+\). Expression of this enzyme creates a larger pool of available NADH which can be used by the synthetic fatty acid production pathway expressed in *E. coli*. With increased NADH available,
the pathway can go through more multiple iterations, resulting in the increase in titers of longer chain fatty acids, specifically octanoate. Future work could involve co-expression of \textit{fdh1} in strains designed for more specific production of one fatty acid, potentially leading to a production strain that is both specific and has high yield.
CHAPTER FIVE: FERMENTATIVE PRODUCTION OF 4-HYDROXY-2-BUTANONE

Introduction

Methyl ketones are another class of industrially relevant chemicals that could benefit from a biorenewable production method. This class of chemicals includes 2-butanone, 3-hydroxy-2-butanone, and 4-hydroxy-2-butanone. 2-butanone is a common industrial solvent as well as being used in food flavorings, particularly in foods with a buttery flavor (Yoneda et.al 2014). 4-hydroxy-2-butanone is an important intermediate to several chemicals, including pesticides, steroids, and terpenoids, as well as the chemotherapeutic agent doxorubicin (Wang et.al 2012, Ichikawa et.al 2005). 3-hydroxy-2-butanone, also known as acetoin, is used as a food flavoring in butter, yogurt, and foods containing apple, blackberry, and several other flavors. Acetoin is also used in pesticides and plant growth promoters. Currently 4-hydroxy-2-butanone is not produced from renewable feedstock. Here we show that part of the glycerol dehydratase pathway can be combined with a thiolase reaction to create a mixture of 4-hydroxy-2-butanone via fermentation.

Materials and Methods

Microorganisms and media

All bacterial strains are derived from E. coli K-12 MG1655 (Table 3). The media used for bacterial growth included lysogeny broth (LB; Difco) and M9 minimal medium. M9 minimal medium consisted of (per liter) 64 g Na₂HPO₄ · 7 H₂O, 15 g KH₂PO₄, 2.5 g NaCl, 5.0 g NH₄Cl,
0.493 g MgSO₄ · 7 H₂O, 0.0147 g CaCl₂ · 2 H₂O, 1 g yeast extract, and 20 g glucose or 20 g glycerol as indicated. Kanamycin was used at 50 mg/L and chloramphenicol at 20 mg/L.

**Construction of *E. coli* strains for production of 4-hydroxy-2-butanone**

*DhaB1-3* and *ddr* from *K. pneumoniae* were synthesized by Genscript and codon-optimized for expression in *E. coli*. *DhaB1-3* and *ddr* were subcloned into pBE522 using the *BglII*, *HindIII*, and *XhoI* restriction sites. *PduP* was amplified from *S. enterica* and subcloned into pBE522 using the *Ndel* and *BglII* restriction sites. *BktB* from *Megasphaera* spp. and *3HP-ACS* from *Metallosphaera sedula* were synthesized by Genscript and subcloned into pET41a. His-tagged versions of *BktB* and *3HP-ACS* were also synthesized for protein purification.

**Preparation of cell-free extracts for protein purification**

A 125 mL Erlenmeyer flask containing 50 mL LB medium was inoculated with 500 µL of an overnight culture grown on LB medium. Cells were grown at 37 °C with shaking at 275 rpm using an Innova I2400 incubator shaker. When cells reached an OD₆₀₀ of 0.6-0.8, the temperature was reduced to 30 °C, and protein expression was induced with 1 mM IPTG. After 6 hours of induction, cells were harvested by centrifugation at 5000 x g for 15 min using a Beckman JA-25 centrifuge and a JA-17 rotor. Cell pellets were resuspended in 20 mL of lysis buffer (potassium phosphate buffer, pH 8.0, 5 µM AEBSF protease inhibitor, 1 mM DTT, DNase) and lysed using a sonicator (amplitude 4, 2 min in 30 s bursts). Crude cell extracts were cleared by centrifugation using a Beckman J-25 centrifuge with a JA-17 rotor at 25000 x g for 20 min. The supernatant was separated from the pellet and the pellet was resuspended in potassium phosphate buffer. The supernatant and resuspended pellet were used directly in enzyme assays. Protein concentration was determined using the Bio-Rad Protein Assay Reagent.
His-tag protein purification

Cell-free extracts were combined with 1 mL of Qiagen Ni-NTA Superflow and mixed on an orbital shaker for 30 min. The resin was placed in a 1 mL polypropylene column (Qiagen) and washed once each with 1 mL of buffer that contained 50 mM HEPES, pH 7.0, 300 mM NaCl and 50, 80, or 150 mM imidazole. Samples of each fraction were run on SDS-PAGE to identify the his-tagged proteins and determine their location in either the supernatant or pelleted fraction of the extracts.

Enzyme assays

Activity of glycerol dehydratase was determined using the MBTH method (Tobimatsu et.al. 1996). Briefly, activity was measured in a 1 mL assay. It contained assay buffer composed of 35 mM potassium phosphate pH 8.0, 50 mM KCl, 200 mM 1,2-propanediol, and 300 nM Ado-\textsubscript{B12}. 10 µg of enzyme was added to the assay buffer and the reaction was initiated by addition of Ado-\textsubscript{B12}. After incubation for 1 min at 37 °C, 100 mM potassium citrate, pH 3.6 was added to stop the reaction. 0.1% MBTH solution was added and the reaction was incubated for 15 min at 37 °C. The absorbance was read at 305 nm and specific activity calculated using an extinction coefficient of 13,300 M\textsuperscript{-1}cm\textsuperscript{-1}.

Activity of BktB was determined using the previously established protocol of the Thl assay (Hartmanis & Stadtman, 1982; Sliwkowski & Hartmanis, 1984; Sliwkowski & Stadtman, 1985). Briefly, activity was measured in a 1 mL assay. It contained 100 mM Tris/HCl pH 8.2, 25 mM MgCl\textsubscript{2}, 33 µM acetoacetyl-CoA (Sigma), 90 µM CoA (Sigma), and 5 µL of enzyme. Assays were incubated at 30 °C for 5 min and absorbance at 303 nm was monitored using a Varian Cary 50 Bio UV-Visible spectrophotometer. The assay measures the degradation of acetoacetyl-CoA
into two molecules of acetyl-CoA. The rate of the Thl reaction was calculated using an extinction coefficient of 14 M$^{-1}$cm$^{-1}$ for acetoacetyl-CoA.

Activity of 3HP-ACS was determined using an assay that measures the formation of the thioester bond in 3-hydroxypropionyl-CoA (Kim et. al. 2001). Briefly, activity was measured in a quartz cuvette with a 1 cm optical path. The assay solution contained potassium phosphate buffer, pH 7.0, 0.4 mM Na acetate, 2 mM MgCl$_2$, 0.5 mM ATP, 0.2 mM CoA, and 5 µL enzyme, with a final volume of 1 mL. The creation of the thioester bond was monitored at 232 nm on a Varian Cary 50 Bio UV-Visible spectrophotometer.

**Fermentation conditions**

For anaerobic fermentations, a 50 mL overnight culture was grown in LB medium aerobically with shaking at 275 rpm. Cells were collected by centrifugation at 5000 $x$ g for 15 min. Cells were resuspended in 10 mL of fermentation medium, supplemented with 2% glucose or 2% glycerol, 1% propionate or 1% 3-hydroxypropionate, and 500 nM coenzyme B$_{12}$ as indicated. Resuspensions were moved to glass flasks, capped, and the headspace evacuated with 95% N$_2$ 5% CO$_2$. Fermentations were incubated at 37 °C with shaking at 275 rpm for 24 hours.

Microaerobic fermentations were carried out in 125 mL Erlenmeyer flasks. 50 mL of medium was supplemented with 2% glucose or 2% glycerol, 1% propionate or 3-hydroxypropionate, and 500 nM coenzyme B$_{12}$ as indicated. 1 mL of liquid cellular suspension was added and the fermentations were incubated at 30 °C and 200 rpm for 48 hours.
Metabolite analysis by HPLC and GC-MS

The fermentation products present in culture media were measured by HPLC and/or GC-MS. Cells were removed from fermentation medium by centrifugation followed by filtration through a cellulose acetate membrane (pore size 0.2 µm) (Thermo Scientific). Filtered medium was analyzed using a Varian ProStar HPLC system, equipped with a 325 UV monitor (210 nm), a 355 RI detector, and a Bio-Rad HPX-87H column. The column was developed with 5 mM H$_2$SO$_4$ at a flow rate of 0.4 mL min$^{-1}$, isocratic. Analytes were identified by retention time and quantified by comparison of peak area to a standard curve.

For analysis by GC-MS, 0.5 mL of filtered supernatant was acidified with 50 µL of 1M HCl and extracted with 1 mL of 1:1 chloroform:methanol. 1 µL of the organic phase was injected onto an Agilent 6890 series gas chromatograph equipped with an Agilent HP-5MS column and an Agilent single quadrupole 5973 mass spectrometer using electron ionization. The method used a splitless injection with a 50 mL min$^{-1}$ flow rate and helium as the carrier gas, and a mean velocity of 36 cm/s. The oven was held at 40 °C for 1 min followed by a ramp of 10 °C/min up to 240 °C, at which point the temperature was held for 15 min. Analytes were identified by comparison to known standards in the NIST database.
Table 3: Bacterial strains used in this study

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<th>Strain</th>
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<td>ZH84 pBE522-pduP-dhaB1-2-ddr, pBE1570-empty</td>
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<td>ZH84 pBE522-empty, pBE1570-thl-hbd-crt-ter</td>
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<tr>
<td>ARV2936</td>
<td>ZH84 pBE522-pduP-dhaB1-2-dd, pBE1570-bktB</td>
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<td>ZH84 pBE522-pduP-dhaB1-3-dd, pBE1570-thl-hbd-crt-ter-tesB</td>
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<tr>
<td>ARV21112</td>
<td>ZH84/pBE522-prpE, pBE1570-bktB</td>
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Results

Discovery of 4-hydroxy-2-butanone as a fermentation product

The butyrate operon and glycerol dehydratase construct were co-expressed and the resulting strain used in an attempt to produce 5-hydroxyvalerate. However, when HPLC was performed, no 5-hydroxyvalerate was detected. The specific activity of DhaB1-3 was measured at 2.4 µmol min\(^{-1}\) mg\(^{-1}\), which is lower than previously reported activities but reasonable for
product formation. We hypothesize there is a blockage in the pathway from glycerol to 5-hydroxyvalerate. Previous studies have shown that all the required enzymes have been produced with high activity. Therefore, the problem is likely that the terminal hydroxyl group on 3-hydroxypropionyl-CoA is not accepted on substrates for one of the enzymes involved (Fig 12).

Interestingly, small amounts of 4-hydroxy-2-butanone were detected from these fermentations. This metabolite is made by a diversion in the 5-hydroxyvalerate pathway (Fig 12). Acetyl-CoA and 3-hydroxypropionyl-CoA condense to form 5-hydroxy-3-ketovaleryl-CoA. Rather than progress down the 5-hydroxyvalerate pathway, this molecule is acted upon by a thioesterase, releasing the CoA group. The resulting molecule undergoes non-enzymatic decarboxylation to produce 4-hydroxy-2-butanone. This suggests that the Hbd enzyme cannot accept a hydroxyl group. After the discovery of small amounts of this product, we chose to focus our efforts on 4-hydroxy-2-butanone production.
Figure 12: Production pathway of 4-hydroxy-2-butanone. Acetyl-CoA and 3-hydroxypropionate condense to form 5-hydroxy-3-ketovaleryl-CoA. An endogenous thioesterase cleaves the CoA group. The resulting molecular undergoes non-enzymatic decarboxylation to produce 4-hydroxy-2-butanone.

**Engineering strains for 4-hydroxy-2-butanone production**

We next chose to engineer several strains with the potential to produce 4-hydroxy-2-butanone. BktB was chosen as a replacement thiolase for the $Ck$Thl in the butyrate operon. BktB has been reported to function more promiscuously than $Ck$Thl and thus may have better activity towards 3-hydroxypropionate as a starter molecule. 3HP-ACS from *Metallosphaera sedula* was chosen to replace $DhaB-DDR$. 3HP-ACS converts 3-hydroxypropionate and ATP to 3-hydroxypropionyl-CoA. Using this ACS will require the addition of 3-hydroxypropionate to the fermentation medium. TesB from *E. coli* was also expressed on the end of the $Thl-Hbd-Crt-Ter$
construct. Overexpression of a thioesterase may direct 5-hydroxy-3-ketovalerate towards the 4-hydroxy-2-butanone pathway. Control strains were also made that expressed one gene or operon on one plasmid while the other remained empty to ensure that any metabolite production was due solely to the expressed genes and not endogenous processes.

ARV2936 (bktB-pduP-dhaB1-3-ddr) performed the best of all the production strains, with the titer of 4-hydroxy-2-butanone reaching 2.5 mM. This strain was also the most consistent in its production. While ARV2681 (pduP-dhaB1-3-ddr) also reached titers of 2.5 mM, it was less consistent and would sometimes produce much less. Interestingly, none of the strains utilizing the 3HP-ACS produced the desired metabolite. To determine why this was occurring, the his-tagged BktB and 3HP-ACS were purified by nickel chromatography and all of the extracts were run on SDS-PAGE and subject to enzyme assays. BktB enzyme activity was measured at 8.4 µmol min⁻¹ mg⁻¹. However, no ACS activity was measured, and the SDS-PAGE showed that the
ACS was segregating into the pellet fraction of the cell extracts, indicating that is likely nonfunctional in vivo. To address this issue, 3HP-ACS was replaced by prpE from E. coli, which is known to express in an active form.

4-hydroxy-2-butanone production utilizing prpE

PrpE was amplified from the E. coli chromosome and subcloned into pBE522. It was co-expressed in the production strain with bktB. Fermentations were performed with the addition of 3-hydroxypropionate at 100 mM. This strain, ARV21112, produces 4-hydroxy-2-butanone at a titer of approximately 1 mM.

Discussion

Methyl ketones are a class of industrial chemicals that have the potential to be produced biorenewably. 4-hydroxy-2-butanone is an important intermediate for a variety of chemicals, including the anticancer agent doxorubicin. We focused on producing 4-hydroxy-2-butanone by diverting the 5-hydroxyvalerate pathway using glycerol dehydratase and the butyrate operon. After initial success, we expanded our potential production strains by adding bktB and 3HP-ACS to replace portions of the original strain. BktB worked well but the 3HP-ACS did not function in vivo. It was replaced with prpE which functioned properly in the cells and produced 4-hydroxy-2-butanone. To our knowledge, this is the first reported production of 4-hydroxy-2-butanone via microbial fermentation.

The success in utilizing these constructs to produce 4-hydroxy-2-butanone could be leveraged to produce other useful methyl ketones, such as 2-butanone. In this scheme, propionate would be used instead of 3-hydroxypropionate as a starter molecule. The same pathway should
then produce 2-butanone. Future work could focus on the creation and optimization of strains to produce 2-butanone as well as optimizing the 4-hydroxy-2-butanone pathway.
CHAPTER SIX: CONCLUSIONS

Biorenewable production of fuels and chemicals is an emerging industry of great importance. As petroleum stocks decline, it is essential to be able to produce the variety of chemicals we currently rely on in a different manner. Biorenewable production has the potential to replace petroleum production of these chemicals. Fatty acids and methyl ketones are two important classes of molecules that are precursors to a variety of industrially relevant chemicals. Previous methods of producing these chemicals have included utilizing genes from the clostridial butanol pathway, reversal of the \( E. \ coli \) beta-oxidation pathway, and manipulation of the \( E. \ coli \) phospholipid biosynthesis pathway (Clomburg et al. 2012; Dellomonaco et al. 2010, 2011; Lennen & Pfleger 2012; Peralta-Yahya et al. 2012; Steen et al. 2010; Zhang et al. 2009). We created a synthetic construct composed of a thiolase from \( C. \ kluyveri \), hydroxybutyryl-CoA dehydrogenase from \( C. \ acetobutylicum \), crotonase from \( C. \ kluyveri \), and Ter from \( E. \ gracilis \) to synthesize butyrate from glucose (Fig. 4). During anaerobic fermentation, this strain produces a mixture of butyrate, hexanoate, and octanoate. We investigated the possibility of further engineering strains to produce one of these fatty acids by knocking out several thioesterases to determine their specificity. Of the thioesterases tested, only \( yciA \) was particularly specific. Knockout of \( yciA \) dramatically increased the titers of hexanoate while decreasing butyrate yield, indicating that it works preferentially on butyryl-CoA. We also investigated the possibility of increasing the NADH pool and increasing titers of higher chain length fatty acids by addition of the \( fdh1 \) formate dehydrogenase from \( Candida boidinii \). Expression of \( fdh1 \) significantly increased the titers of octanoate, indicating that higher NADH pools were necessary for increased production of longer chain length fatty acids in this system.
To produce methyl ketones, we initially used a combination of the butyrate synthetic construct and a construct composed of pduP from S. enterica and the glycerol dehydratase dhaB1-3 and its reactivating factor from K. pneumoniae (Fig 12). The initial condensation reaction creates 5-hydroxy-3-ketovaleryl-CoA. This is then acted upon by an endogenous thioesterase, and the resulting intermediate undergoes non-enzymatic decarboxylation to create 4-hydroxy-2-butanone. After these initial results, we further engineered several strains to attempt to produce 4-hydroxy-2-butanone in higher titers and consistently, as the initial strain was inconsistent in its production. Strains utilizing bktB were much more consistent in their production of 4-hydroxy-2-butanone, indicating that it is better than the CkThl at functioning on substrates with a hydroxyl group, such as 3-hydroxypropionyl-CoA. We also attempted to replace the glycerol dehydratase-pduP construct with a 3-hydroxypropionyl-CoA synthetase, however our initial choice of 3HP-ACS was nonfunctional in vivo. We replaced it with prpE from E. coli which was successful in replacing the glycerol dehydratase-pduP.

Future work in these areas will focus on increasing titers of the desired products and creation of strains with high specificity to produce one product. Ideally, there would be one strain that would produce only butyrate, another that would produce only hexanoate, and so on. This is preferable for commercial production, as separation and purification of the product increases costs. Further research into the specificity of thioesterases could determine the ideal thioesterase to express for each desired fatty acid. Overall titers could be increased by strain engineering to increase the tolerance to low pH and the accumulation of the desired products, as they have been shown to be toxic to the production organisms. In addition, the 4-hydroxy-2-butanone production strains can be further engineered to produce 2-butanone, a chemical that is
fairly ubiquitous in a variety of industries. Creation of a 2-butanone production strain would be an essential step towards biorenewable production of this chemical.
References


