MEGASPHAERA ELSDENII ATCC25940 AS A PLATFORM FOR ENGINEERING LONGER CHAIN-LENGTH ALCOHOL PRODUCTION

by

NEELY WOOD

(Under the Direction of Christine Szymanski)

ABSTRACT

Megasphaera elsdenii has the native ability to condense acetyl-CoA groups to efficiently generate C4 to C8 compounds from lactate and plant carbohydrates, including butyric (C4), valeric (C5), hexanoic (C6), and in some cases octanoic (C8) acids. Though its metabolism is not well understood, its genome is annotated to have a chain elongation pathway using acetyl-CoAs. *M. elsdenii*'s major fermentation products, including butyric and hexanoic acids, are potential precursors to the medium chain length alcohols butanol and hexanol. As the carbon chain length increases for the corresponding alcohols, fuel properties improve, making hexanol an appealing target as a next-generation drop-in biofuel, and *M. elsdenii* serves as a potential platform organism for engineering its production. In this work, a deletion of a putative propionyl Coenzyme A transferase was constructed in the *M. elsdenii* chromosome, which resulted in the elimination of propionate production as well as an overall increase of longer chain-length products.

INDEX WORDS: Non-model organism, VFA production, hexanoate, metabolic engineering, lactate fermentation, chain elongation

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NEELY WOOD

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NEELY WOOD

Major Professor:	Christine Szymanski
Committee:	Diana Downs
	Adam Guss
	Breeanna Urbanowicz

Electronic Version Approved:

Ron Walcott
Vice Provost for Graduate Education and Dean of the Graduate School
The University of Georgia
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CHAPTER 1

INTRODUCTION AND BACKGROUND

1.1 Biofuels

The impending climate crisis this planet is in as a result of greenhouse gas emissions and the enormous carbon footprint of transportation results in the need for the continued development of the biofuel industry to produce drop-in and replacement fuels for petroleum-based gasoline. The advancement of these carbon-neutral processes is critical to resolving the transportation industry's contribution to the climate crisis (Nelson, Choi, Lamsen). The current model for drop-in fuels is largely based on ethanol produced industrially from starches in corn feedstocks. Though this ethanol produced from starch has a positive energy balance and already contributes to lowered emissions when added to gasoline, land use is a concern for feedstock-based corn bioethanol production and have the potential to be greatly lowered (URL: https://afdc.energy.gov/fuels/ethanol_fuel_basics.html) (Tracy). The development of next-generation biofuels such as longer chain-length alcohols like butanol and hexanol offers the opportunity for increasing the efficiency of the biofuels produced and reducing carbon-based fuel emissions (Choi, Lamsen). Longer-chain alcohols and fatty acid production has been seen in a variety of organisms, including but not limited to many Clostridia (Choi, Tracy, Weimer). Chain-elongation pathways have been heterologously expressed in *Escherichia coli,* but organisms with native flux condensing acetyl-CoA groups are more robust in their ability to form the end-products, and, ultimately, engineering these pathways in *E. coli* was not seen to produce these alcohols in industrially relevant fluxes (Dekishima, Clomburg, Kataoaka, Dollomonaco, Kim).

Additionally, engineering efforts in chain elongation pathways have also been undertaken to domesticate a number of other non-model organisms and develop them into platform strains for alcohol and other bioproduct formation, most notably, *Clostridium thermocellum* (Tian).

1.2 Megasphaera elsdenii

Megasphaera elsdenii is a ruminal mesophilic obligate anaerobe that is a member of the Negativicutes that natively possesses high-flux energy metabolism from a variety of carbon sources to fatty acids including butyric acid, valeric acid, hexanoic acid, and even octanoic acid (Nelson, Prabhu). These native metabolic abilities make *M. elsdenii* a uniquely promising organism for metabolic engineering and optimization of drop-in biofuel production of the longer-chain alcohol biofuels of the future. Additionally, this work will take full advantage of this novel organism that has previously been used in fermentation studies (Nelson, Prabhu), but has not been genetically tractable to genetic engineering until recently (Riley et al., unpublished). With advanced sequencing technologies, transcriptomics, and cross-phylogeny implementation of other rapid genetic tools now available, we are positioned to exploit the abilities of *M. elsdenii* to serve as a platform for the production of long chain alcohols. The genome of *M. elsdenii* is fully sequenced and its genome annotations by KEGG and GenBank were used to elucidate a predicted metabolic model (Hatmaker, unpublished), and this was used to construct a predicted chain elongation pathway to its main fermentation products (**Figure 1**). These tools include the development of a strategy for the use of a counter-selectable marker in this work for targeted enzyme deletions to increase flux towards targeted products, utilizing the gene *upp* (*uracil phosphoribosyltransferase*), which allows for the use of counter-selection against an integrated vector using 5-fluorouracil.

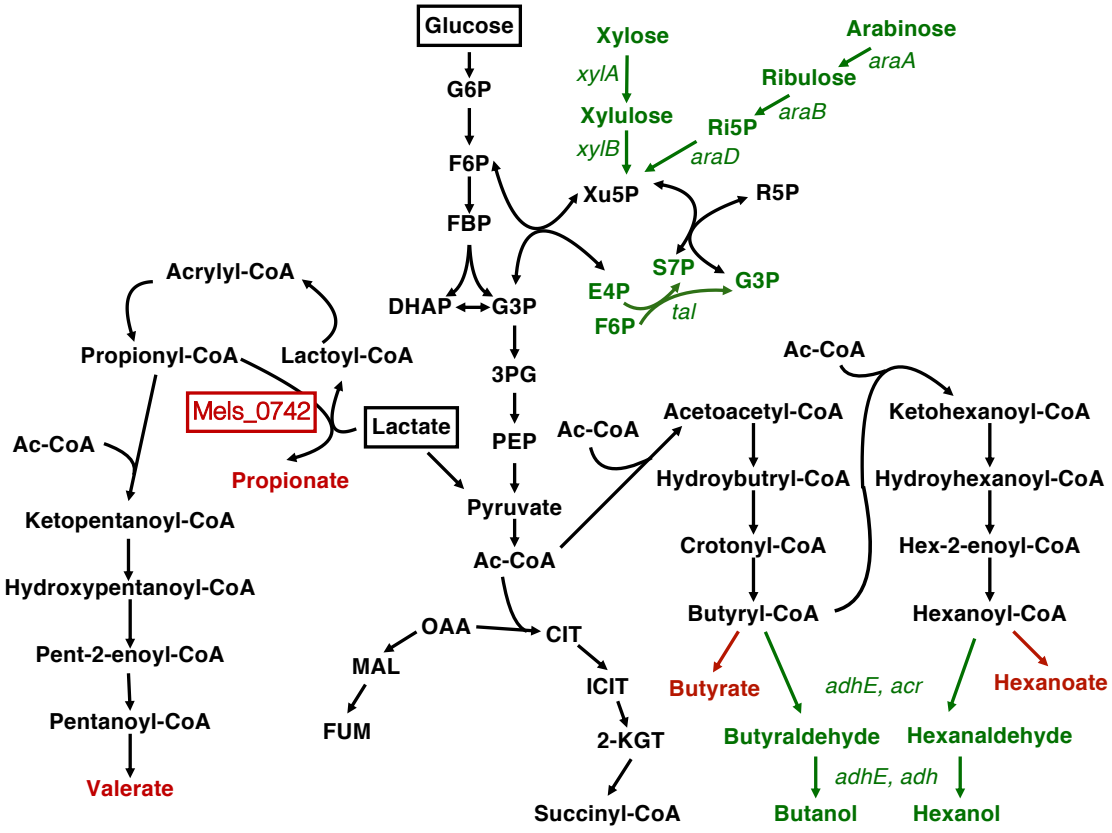

Figure 1. Metabolism and genetic targets in *M. elsdenii.* Black: Native metabolism in *M. elsdenii* ATCC 25940. Red: deletion of the CoA-transferase that leads to valerate production; alternative acyl-CoA transferases to investigate their roles in *M. elsdenii* metabolism and to lead to metabolic engineering with the goal of funneling carbon to longer chain fatty acids and alcohols. Green: Potential overexpression targets of heterologous genes for pathway expansion.

1.3 MELS_0742, putative coenzymeA transferase

Relatively little is known about *M. elsdenii* metabolism and the biochemical activities of its chain elongation and organic acid production enzymes. Metabolic engineering efforts in this organism will be greatly bolstered by a better understanding of the roles of these enzymes in metabolism and its flux. As a result of the ambiguous nature of genome annotation, the functions of its putative organic acid production enzymes are unknown, including its various acyl-CoA transferases. MELS_0742 is a putative propionyl-CoA transferase (*pct*) annotated in the genome

of *M. elsdenii* ATCC 25940 (GenBank). This enzyme has been biochemically characterized and though it was not tested for propionyl-CoA activity, it was predicted to be an enzyme responsible for lactate conversion to lactoyl-CoA and thought to be an acetyl-CoA: lactate CoA-transferase. It was found by (Zhang) via phylogenetic analysis to be fairly unique compared to 14 other CoA transferases, only closely linked to other *Megasphaera* sp. CoA transferases (Zhang, Prabhu). In this work, I begin to explore the genetic links between putative chain elongation enzymes and organic acid fermentation products in *M. elsdenii* utilizing the newly developed genetic tools to construct a chromosomal marker replacement of the putative *pct* gene, locus tag: MELS_0742. I then elucidated the resulting fermentation profile and growth phenotypes of this single mutant. This represents the first published gene knockout involved in the *M. elsdenii* ATCC 25940 putative chain elongation pathway to its various volatile fatty acid (VFA) products.

1.4 Approach to metabolic engineering

Our approach to genetic engineering in *M. elsdenii*, as a non-model organism with few available genetic tools and little known about its metabolism, is dual-pronged. The first part of the approach is based on understanding basic biology in this organism. That is, using the ability to make targeted gene knockouts in its genome and then observing any changes in phenotype to help elucidate the way its metabolism functions beyond the scope of what putative genome annotations can provide. The second part of the approach involves using genetic engineering to work towards altering and optimizing the organism's native metabolism to produce products of interest through a series of gene knockouts and pathway additions. The gene knockout described in this thesis, MELS_0742, was chosen as a candidate because of its putative role in a likely non-essential portion of *M. elsdenii* metabolism, the acrylate pathway, and because of relatively high expression of this gene on lactate as a carbon source (Riley, Ashok, ORNL, unpublished),

suggesting that it actively plays a role in metabolism and is not just a genetic artifact. As so, this deletion was constructed to both assign a suggested metabolic role to the enzyme it encodes, as well as to work towards a metabolic engineering effort that aims to increase flux towards medium chain length products butanoate and hexanoate.

The *M. elsdenii* ATCC 25940 genome is annotated to predict many putative acyl-CoA transferases, including propionate-, 4-hydroxybutyrate-, acetate-, and acetyl-CoA transferases, as well as an apparent gene cluster putatively involved in the cycle including a putative lactoyl-CoA dehydratase, and a putative glyoxylase. Aside from the deletion of the MELS_0742 putative propionyl-CoA transferase deletion constructed, a series of eight additional plasmids were constructed for targeted deletions in each putative acyl-CoA transferase genes, (Locus tags: MELS_0033, MELS_0034, MELS_0341, MELS_0430, MELS_0437, MELS_0464, MELS_0742, MELS_1130, MELS_1631). These targeted deletions were each attempted in the AG5855 background, but for reasons not currently understood, were not successfully constructed. The specific aims of this paper are as follows: (1) Construct a deletion in the MELS_0742 putative propionyl-CoA transferase gene in *M. elsdenii;* (2) Characterize growth and fermentation products of the deletion mutant; and (3) Make predictions in the function of the MELS_0742-encoded enzyme in its metabolism which may inform basic biological knowledge of the non-model organism. Included in Chapter 4.2 of this paper is a description of further genetic engineering possible in the strain yielded from the above described study, and in Chapter 4.3, a description of all unsuccessful constructions of putative CoA transferase genes in the organism. Chapter 5 of this paper also contains a comprehensive description of the continued development of genetic tools in *M. elsdenii*, including an additional counter-selectable marker,

pyrF, and its complementation, enabling use of two secondary antibiotics, and the design of plasmids to test and characterize a known phage-integrase integration system.

CHAPTER 2

METHODS

2.1 Plasmid construction

MELS_2191 (*upp*) deletion plasmid pLAR151 and MELS_0742 (*pct*) deletion plasmid pLAR179[1] were constructed via Gibson assembly as described below. All other plasmids in this study were designed via Geneious Prime and sequences were submitted to Genscript for synthesis into a pUC-mini vector to construct pMTV208. From there, pMTV208 was used as the backbone for synthesis of gene replacement homology arms constructs via Genscript. Each plasmid contains the *bla* gene, an origin of replication for *E. coli*, the *upp* gene controlled by the promotor controlling *cat* in the base plasmid, pMTL85151, P_{cat}, and the gene replacement construct for each targeted deletion, which contains 700-1000 bp upstream and downstream homology arms surrounding the chloramphenicol/thiamphenicol resistance gene, *cat*, controlled by a native promotor region, P_{MELS_0747}. The *cat* gene in these constructs is flanked by ϕC31 serine integrase attachment sites.

2.2 Chromosomal deletion of MELS_2191, upp

To construct a deletion of MELS_2191 in the *M. elsdenii* ATCC 25940 chromosome, plasmid pLAR151 was constructed by Gibson assembly via an intermediate plasmid, pLAR147. The pBC1 origin of replication was amplified from the pBC1 plasmid (DeRossi) cloned in place of pIM13 in the shuttle plasmid pMTL82151 (Heap), resulting in plasmid pLAR147. Then, 490

[1] pLAR151 and pLAR179 were constructed by Lauren Riley (ORNL, unpublished). All other deletion plasmids from this study were designed in Geneious Prime by myself and ordered by Melissa Tumen-Velasquez for DNA synthesis by GenScript.

bp up and downstream of the *upp* gene (MELS_2191) were amplified and cloned into the MCS of pLAR147, resulting in the plasmid pLAR151. Additionally, a point mutation was inadvertently obtained in the pBC1 origin of replication, rendering it non-functional. All PCR amplifications were performed using Phusion Master Mix (Thermo Fisher). The plasmid pLAR151 was transformed into *E. coli* strain AG4157 (**Table 1**) which expresses two methyltransferases and their corresponding specificity subunits (MELS_0051-0052, MELS_1615-1616) from *M. elsdenii* cloned into the chromosome. Electrocompetent cells of *M. elsdenii* ATCC 25940 were prepared via three subsequent washes with a 10% glycerol 250 mM sucrose solution at room temperature after growth to stationary phase, and methylated plasmid DNA was subsequently isolated and used to transform *M. elsdenii* ATCC 25940 (Riley, ORNL, unpublished). Transformants were selected on RCM (HIMEDIA) agar plates with 5 µg/mL thiamphenicol (TM) and were incubated for 72 hours. Colonies were picked into RCM medium (BD Difco) with 5 µg/mL TM and incubated overnight. The liquid cultures were subcultured into RCM (HIMEDIA) liquid medium and subsequently plated in RCM (BD Difco) with 20 µg/mL 5-fluorouracil. The plates were incubated overnight, and colonies were streaked on RCM plates. Single colonies were picked into RCM medium (HIMEDIA) and PCR screened for the chromosomal deletion of *upp*.[2]

2.3 Chromosomal deletion of MELS_0742, pct

Plasmid pLAR179 was constructed via Gibson assembly. The plasmid's backbone was PCR amplified from plasmid pMTL85141 (Heap, 2009) to linearize the plasmid, excluding the *cat* gene. MELS_2191, the uracil phosphoribosyltransferase*,* was amplified from the *M. elsdenii* chromosome and was cloned in place of the *cat* gene for the purposes of counter-selection.

[2] The chromosomal deletion of MELS_2191, *upp*, was constructed by Lauren Riley (ORNL, unpublished)

Additionally, a cassette containing ~800 bp upstream and downstream of MELS_0742 flanking a *cat* gene driven by the promoter region of MELS_0747 (P_{MELS_0747}) and phiC31 *att*B/P sites, was synthesized by Twist Bioscience. Subsequently, the cassette was amplified and cloned into the final construct downstream of the *upp* gene. The plasmid was transformed into *E. coli* strain AG4157 (**Table 1**), and isolated. Electrocompetent cells of *M. elsdenii* ATCC 25940 were prepared (Riley, ORNL, unpublished), and methylated plasmid pLAR179 was subsequently isolated and used to transform AG5855 (*M. elsdenii* ATCC25940 Δ*upp*). Colonies were selected on RCM agar plates containing 5 µg/mL thiamphenicol and incubated at 37 degrees C for 72 hours. Colonies were picked into liquid RCM medium (Difco) with 5 µg/mL TM, incubated overnight, and then plated on RCM (Difco) with 5 µg/mL TM and 50 µg/mL 5-fluorouracil for counter-selection against the plasmid. Plates were incubated for 48 hours at 37 degrees C and single colonies were picked into RCM (Difco) liquid medium with 5 µg/mL TM and screened for the marker-replacement. Three single colony purification steps were needed to isolate a pure culture of the deletion from the initial merodiploid.[3]

2.4 Fermentations and product quantification

M. elsdenii ATCC 25940 wild type, *M. elsdenii* ATCC 25940 Δ*upp* (strain AG5855), and *M. elsdenii* ATCC 25940 MELS_0742::phiC31-*cat*-phiC31 (strain JWME04) were grown in 5 mL RCM medium (HiMedia) + 5 µg/mL thiamphenicol, if necessary, overnight. Then, 50 µL of each strain was added to Balch tubes containing 10 mL of modified RCM with 1) 5 g/L Na-lactate instead of glucose, and separately 2) with glucose. Each strain was cultured in duplicate for 72 hours at 37 degrees C. Samples were taken at 24-hour intervals, optical density measurements taken, and fermentation products were quantified using HPLC. Lactate, glucose,

[3] Plasmid pLAR179 was designed and constructed by Lauren Riley (ORNL, unpublished). The protocol for MELS_0742 deletion was also designed by Lauren Riley.

acetic acid, propionic acid, butyric acid, valeric acid, and hexanoic acid were quantified on an Agilent 1260 infinity series HPLC with an Aminex-HPX-87H column (Bio-Rad). The mobile phase was 5 mM sulfuric acid. The column was heated at 65 degrees C, the flow rate was 0.6 mL/min, and the chromatograph was visualized using an RI detector.

CHAPTER 3

RESULTS

3.1 Deletion of M. elsdenii uracil phosphoribosyltransferase (Δ*upp*)

Uracil phosphoribosyltransferase (*upp*) converts the uracil analogue, 5-fluorouracil (5-FU), to a toxic product, fluorodeoxyuridylate (FdUMP) (Singh). *M. elsdenii* was found to be sensitive to 25 µg/ml 5-FU. Growth of the wild type strain on 5-FU also resulted in spontaneous mutations in this gene indicating that it is responsible for conversion of 5-FU to FdUMP. A deletion of the uracil phosphoribosyltransferase (*upp*, MELS_2191) in the *M. elsdenii* chromosome resulted in a strain, AG5855 (**Table 1**), that is resistant to 50 µg/ml 5-FU. This chromosomal deletion allowed for the counter-selection of plasmids containing a copy of the wild type allele. This is the first counter-selection strategy developed in *M. elsdenii*.[4]

3.2 Deletion of a M. elsdenii *putative propionyl-CoA transferase* (Δ*pct*)

There are four annotated putative propionate-CoA transferases, (*pct*) in *M. elsdenii* (Kyoto Encyclopedia of Genes and Genomes, KEGG), MELS_0742, MELS_0464, MELS_1631 and MELS_1130. RNAseq and proteomic analysis revealed that expression of MELS_0742 was highest, and MELS_1130 was not detectable (Riley and Ashok, unpublished data). To investigate the role of the most highly expressed of these, MELS_0742, in organic acid production from lactate, a deletion of this gene was constructed in the AG5855 (Δ*upp*) background strain (**Figure 2**), generating strain JWME04. The plasmid designed to generate this deletion contained a *cat* gene encoding chloramphenicol and thiamphenicol resistance with

[4] The deletion of this gene (*upp*) was constructed by collaborator Lauren Riley (ORNL).

upstream and downstream homology to MELS_0742 with phiC31 attachment sites (*attB* and *attP*) flanking the *cat* gene (**Figure 3**). The *cat* gene allowed selection of marker replacement events and the phiC31 attachment sites should allow removal of the *cat* gene for subsequent engineering (**Figure 2**).

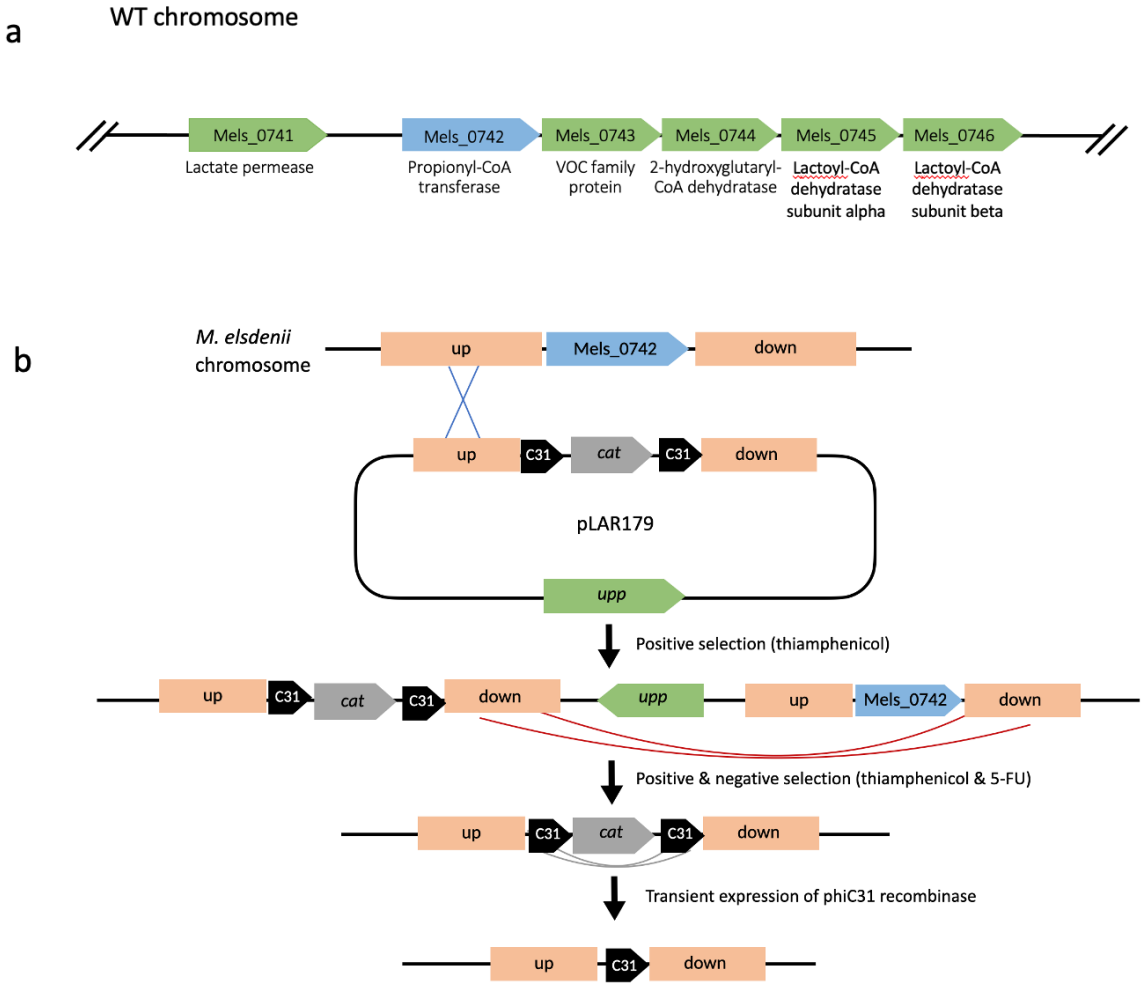

Figure 2. Deletion of putative propionyl-CoA transferase MELS_0742. (a) Chromosomal region surrounding MELS_0742, the gene for targeted marker replacement. (b) Plasmid pLAR179 containing a native copy of *upp,* and the *cat* gene flanked by sites for homologous recombination for marker replacement of MELS_0742 on the *M. elsdenii* chromosome. Transformants were selected on reinforced Clostridial medium (RCM) + thiamphenicol, and then counter-selected with RCM+5-FU while maintaining positive selection for the *cat* gene. Plasmid was resolved via single-colony isolation in the presence of thiamphenicol.

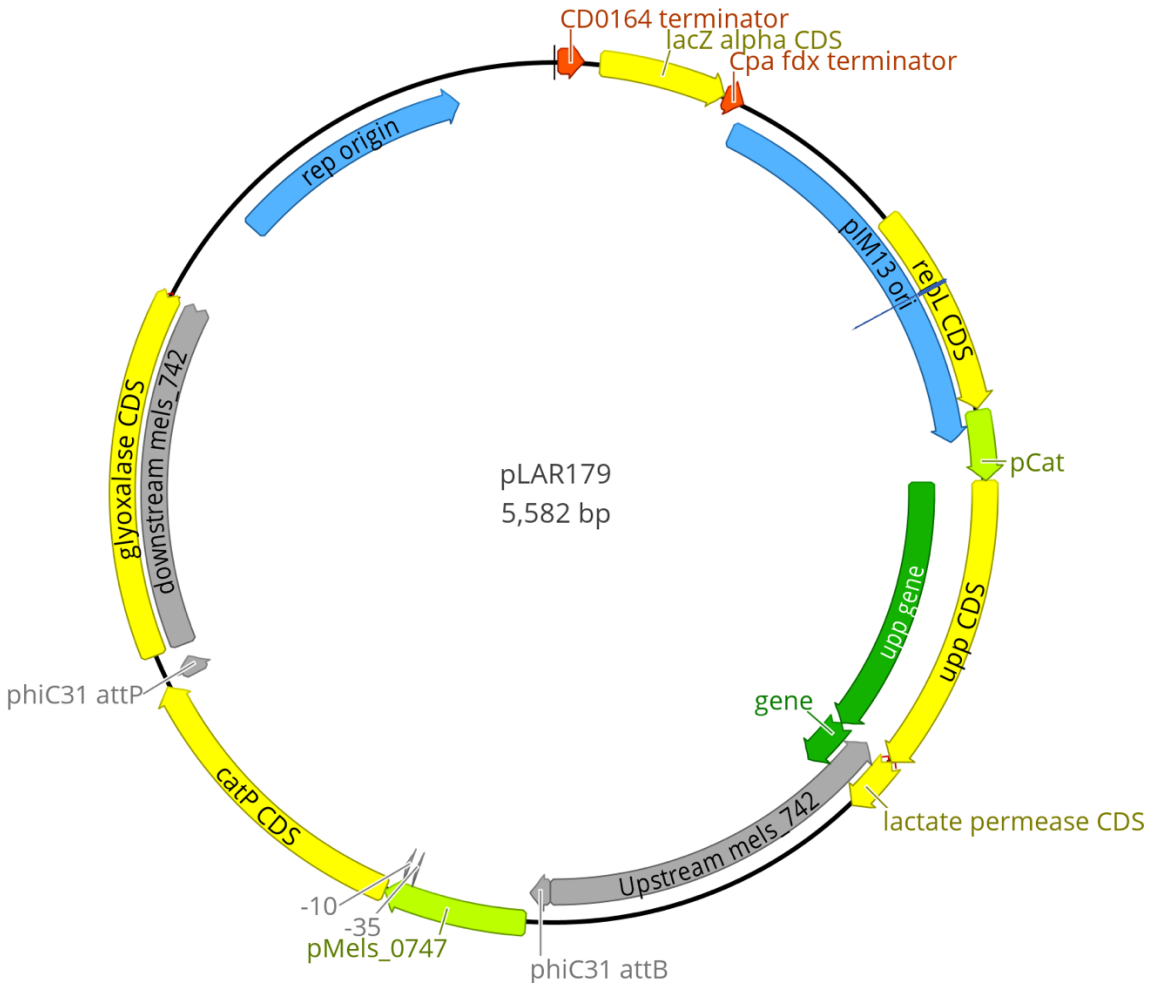

Figure 3. Plasmid map of deletion vector, pLAR179. The plasmid's backbone was amplified from plasmid pMTL85141 (Heap, 2009) to linearize the plasmid, excluding the *cat* gene. MELS_2191, the uracil phosphoribosyltransferase, was amplified from the *M. elsdenii* chromosome and was cloned in place of the *cat* gene for the purposes of counter-selection. Additionally, a cassette containing ~800 bp upstream and downstream of MELS_0742 flanking a *cat* gene driven by the promoter region of MELS_0747 (p_{MELS_0747}) and phiC31 *att*B/P sites, was synthesized by Twist Bioscience. Subsequently, the cassette was amplified and cloned into the final construct downstream of the *upp* gene.

3.3 Comparison of growth of Δupp Δpct, Δupp, and wildtype strains

Growth of AG5855 (Δ*upp*) and JWME04 (Δ*upp* Δ*pct*::phiC31-*cat*-phiC31) was compared to the wild type on both glucose and lactose (**Figure 4**). While both strains grew better on glucose compared to lactate, growth of the deletion strains was indistinguishable from wild type suggesting that these deletions, in general, had no effect on growth on either glucose or

lactate. A two-way ANOVA was performed to determine there was no significant difference in growth between strain JWME04 and others on glucose, at timepoints t=0 and t=72. At t=24, strain JWME04 showed statistically different growth from strain AG5855 ($p<0.05$). At t=48, strain JWME04 showed statistically different growth from the WT ($p=0.017$). On lactate, strain JWME04 showed statistically different growth from the other strains at t=24, where JWME04 grew significantly lower than AG5855 ($p=0.004$) and at t=72 ($p=0.008$) grew significantly lower than WT. With a confidence interval of 0.005, there is only one statistical difference between JWME04 growth and other strains and that is on glucose at t=48 compared to AG5855.

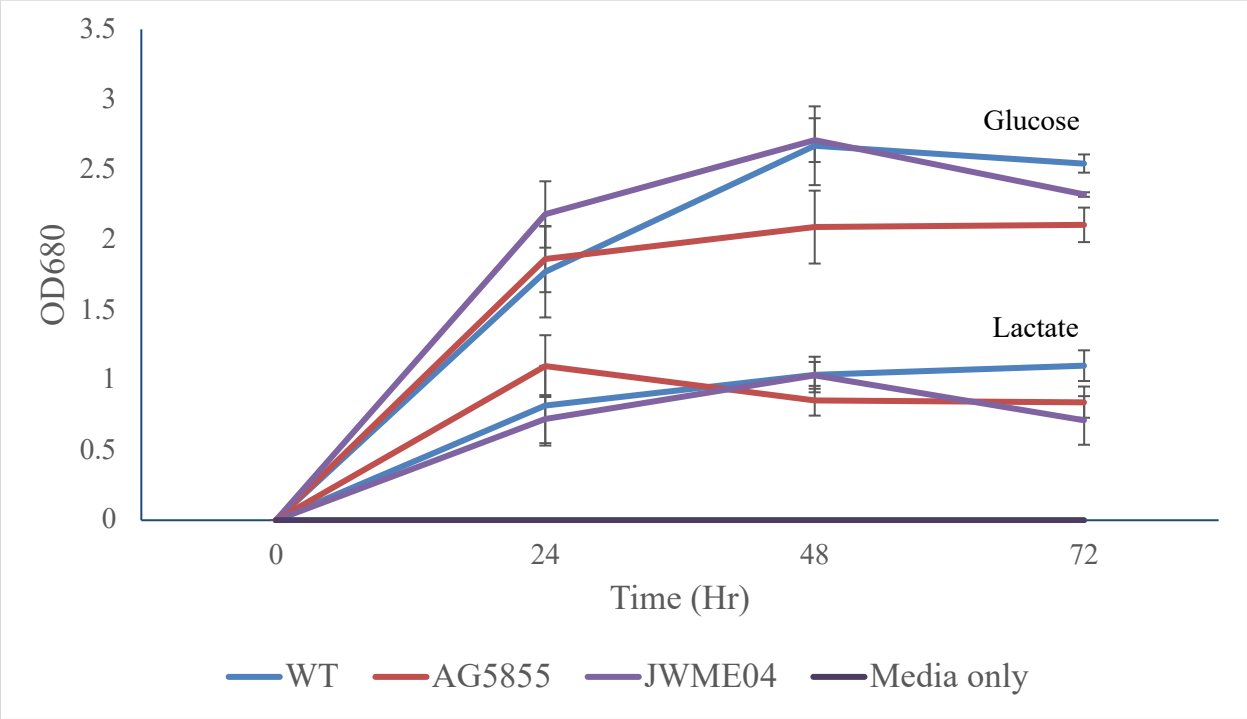

Figure 4. Growth of ΔMELS_0742 mutant on lactate and on glucose. *M. elsdenii* ATCC 25940 wild type, *M. elsdenii* Δ*upp* (strain AG5855), *M. elsdenii* MELS_0742::phiC31-*cat*-phiC31 (JWME04) were grown in 5 mL RCM + 5 µg/mL thiamphenicol, if necessary, overnight. Then, 50 µL of each strain was added to Balch tubes containing 10 mL of modified RCM with lactate and separately with glucose for 72 hours at 37 degrees C. The OD was measured at 680 nm wavelength. Error bars represent standard deviation from the mean of triplicates at each data point. A two-way ANOVA was performed and statistical significance was determined if $p<0.05$.

3.4 Observed changes in fermentation profile, strain JWME04

To investigate the effect of MELS_0742 on organic acid production, high performance liquid chromatography (HPLC) analysis was performed on cells grown on lactate. As shown in **Figure 5** and confirmed by a two-way ANOVA, there was a complete loss of propionate production ($p<0.0001$) and a reduced level of valerate production (although not statistically significant) in strain JWME04. There was about a 2-fold increase in butyrate (C4) production ($p<0.0001$) and a 10-fold increase in hexanoate (C6) production ($p<0.0001$) in the mutant strain JWME04 when compared to its parent strain and the wild type strain. Total C4 and C6 products in the mutant strain increased from about 24 mM to about 39 mM. This strain also consumed significantly more acetate than the WT or AG5855 parent strain ($p<0.0001$).

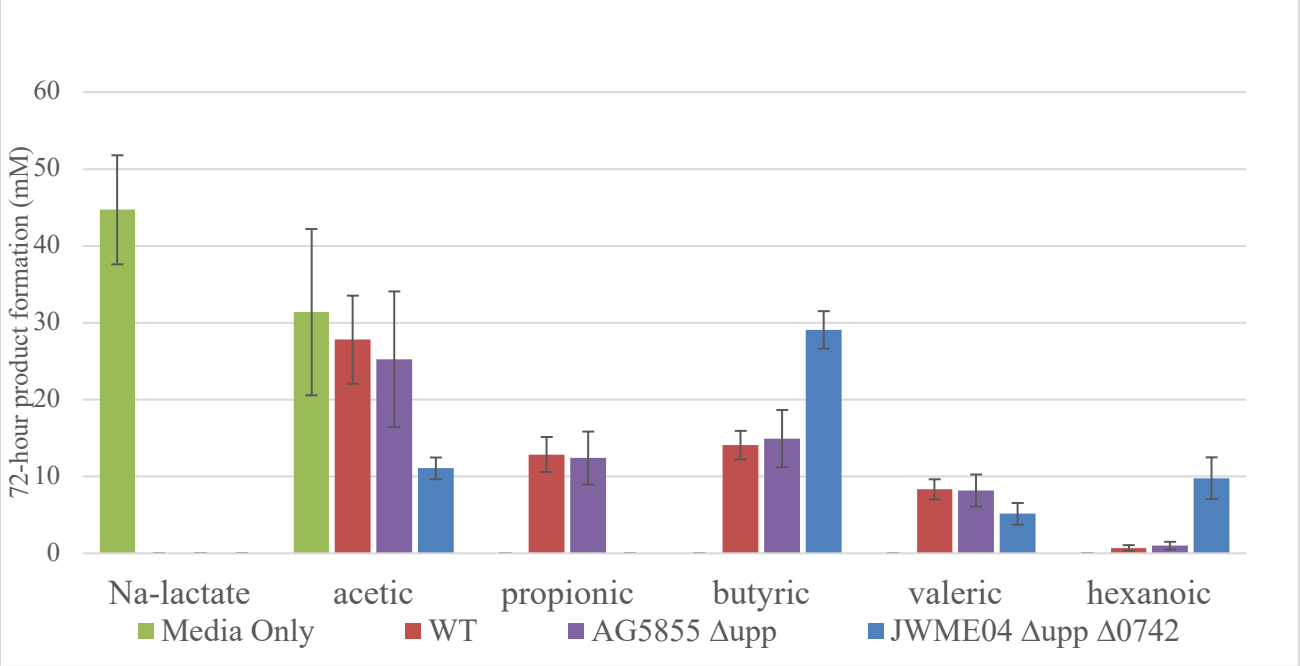

Figure 5. Effect of MELS_0742 deletion on fermentation products. *M. elsdenii* ATCC 25940 wild type, *M. elsdenii* Δ*upp* (strain AG5855), *M. elsdenii* ΔMELS_0742::phiC31-*cat*-phiC31 (strain JWME04) were grown in 5 mL RCM + 5 µg/mL thiamphenicol, if necessary, overnight. Then, 50 µL of each strain was added to Balch tubes containing 10 mL of modified RCM with lactate and separately with glucose for 72 hours at 37 degrees C. Sodium lactate (Na-lactate), acetic, propionic, butyric, valeric, and hexanoic acids in the supernatants following culture were quantified via HPLC. Error bars represent standard deviation from the mean of five replicates. A two-way ANOVA and results were determined to be significant when $p<0.05$.

CHAPTER 4

DISCUSSION AND FUTURE DIRECTIONS

4.1 Discussion of results

The chromosomal knockouts of both genes, *upp* and *pct*, did not seem to have an effect on growth when compared to wild type *M. elsdenii* ATCC25940 (**Figure 4**). This is essential when making gene knockouts in an organism, especially gene knock-outs that could potentially have an effect on growth as a whole. Though the MELS_0742 deletion had no significant effect on growth, it did have a significant effect on metabolism. Not only did the mutant Δ*pct* strain provide evidence of the link between the function of the putative propionyl-CoA transferase enzyme to the gene locus in the chromosome, but also allowed us to elucidate that lactoyl-CoA production, as it enters the putative acrylate cycle, can be provided by some other enzyme that does not produce propionate (**Figure 1**). This is because biochemical characterization and gene annotations predict *pct* is an enzyme that performs a coupled reaction. That is, it is predicted to be responsible for the lactate to lactoyl-CoA conversion as well as the propionyl-CoA to propionate conversion steps via a CoA transfer, the lactate to lactoyl CoA step predicted to be the sole entry into the acrylate pathway producing valerate (Hatmaker).

An overall increase in longer chain length products was seen when propionate, a side product in *M. elsdenii* metabolism was eliminated via the deletion of MELS_0742 (*pct*), increasing total C4 and C6 products in the mutant strain from about 24 mM to about 39 mM combined, including a 10-fold rise in the C6 product hexanoate. As a result, it appears the *M. elsdenii* strain JWME04 consumes more acetate to balance redox without the production of

propionate (**Figure 5**). This is an indicator of *M. elsdenii*'s high potential for becoming a platform organism for engineering fermentations of biomass sugars, byproduct waste, and acetate to medium chain-length products, and eventually to C4 and C6 alcohols. While C4 products have been shown to be produced at decent titers in a variety of Clostridia (Choi, Tracy, Weimer), and even engineered into model organisms such as *E. coli* (Clomburg), C6 products were rarely seen, where as in strain JMWE04, 9.7 mM (1.13 g/L) hexanoate (caproate) was seen even in small volume sealed Balch tube fermentations. The drastic improvement of hexanoate production compared to the increase in butyrate production was surprising. Since hexanol is the ideal goal biofuel in the future of this work, this deletion may serve as a key contributor to the necessary increased flux towards that product in a future engineered strain.

4.2 Further genetic engineering in strain JWME04

In *M. elsdenii* strain JWME04 (Δ*upp* Δ*pct*::ϕC31*att*B-*cat*-ϕC31*att*P), further engineering is possible using the *cat* gene that was used for marker replacement selection if it is removed from the ΔMELS_0742 locus via the ϕC31 *att* sites present flanking the *cat* gene. For this purpose, I designed an expression vector containing the ϕC31 integrase gene and a second selection marker, apramycin, as well as the *upp* gene. At the time our studies concluded, we had been troubleshooting the expression of the ϕC31 integrase gene on plasmid pMTV554. One challenge associated with this was that the integrase was driven in the plasmid by what could be a lactate-inducible promoter, which originated from the regulatory region of the MELS_0747 gene. A second challenge associated with expression of this plasmid is the need to both amplify and methylate the plasmid in *E. coli* strain AG4157, and the possibility for mutations to occur in the integrase gene, as a phage protein, rendering it nonfunctional. However, with proper ϕC31 integrase expression in this strain, the *cat* selectable marker should become available once again

for future plasmid selection and subsequent gene deletions, and the plasmid should be curable from the strain with 5-FU exposure.

4.3 Attempted constructions of acyl-CoA transferase deletions

The final step in *M. eldenii*'s metabolism producing VFAs, including propionate, butyrate, valerate, and hexanoate – where the acyl-CoA is converted to the VFA product – is catalyzed by an acyl-CoA transferase. In the *M. elsdenii* genome, there are nine predicted putative CoA transferases, some of which are predicted to have acyl-CoA transferase activity. In order to both explore the basic biology of *M. elsdenii* metabolism and assign putative CoA transferase enzymes to specific functions, as well as to work towards increasing flux through the pathways to C6 products, deletions were attempted in all nine putative CoA transferases in the Δ*upp* background strain AG5855. These gene annotations included propionate-, 4-hydroxybutyrate-, acetate-, and acetyl-CoA transferases. As the nature of the gene annotation is predicted merely by gene sequence and protein sequence homology to known genes in other organisms, genetic or biochemical characterization of proteins is necessary to increase accuracy of gene annotation, of which acyl-CoA transferases, in general, are lacking. In order to construct these targeted deletions of the eight additional CoA transferases in the *M. elsdenii* ATCC25940 genome, I designed plasmids that were synthesized to replace each gene with a chloramphenicol/thiamphenicol marker and counterselection against the plasmid backbone using the same *upp* expression cassette as used in the successful MELS_0742 deletion, and using the same strategy and steps; however, in the additional eight attempted CoA transferase deletions, including MELS_0464, the final deletion was never observed. Additionally, a construction of the

predicted acrylate pathway deletion (MELS_0742-0746) was attempted in the same manner, and it was also not successfully constructed.[5]

4.4 Limitations and challenges

There are a significant number of challenges in working to engineer bioproduction in a non-model organism that has few developed genetic tools. One of these major challenges is that in *M. elsdenii*, specifically, there is a very small number of known functional promoters, merely including two promoters, one of which is a native Clostridial promoter that drives expression of the *cat* resistance marker in base plasmid pMTL85141 (Heap). The second is a native *M. eldsdenii* promoter that drives MELS_0747, reported by collaborators to be relatively strong based on transcriptomics data. Other promoters tested by collaborators in *M. elsdenii* to date, including P_{tac}, and P_{tac} with an upstream (UP) element associated with a native *M. elsdenii* promoter, have not been seen to function on a replicating plasmid (unpublished). Without a functional promoter library, rational tuning of gene expression in *M. elsdenii* is impossible, which is key in metabolic engineering for bioproducts. Having very few functional promoters has also added potential challenges to counter-selection in constructing chromosomal deletions, where it is difficult to determine if a plasmid backbone containing a counter-selectable marker is being counter-selected in the presence of the agent, i.e. *upp* counter-selection in the presence of 5-fluorouracil. This same concept also applies to the ability to develop and test expression cassettes for additional antibiotic resistance genes on plasmids in strains where an initial resistance gene, i.e. *cat*, is integrated into the genome.

More specific limitations in metabolic engineering in this organism are a result of limited genetic tools. Taking the deletion of a putative acyl-CoA transferase as an example, the first

[5]Gene deletions of MELS_0464 and MELS_0034 were attempted entirely by Zachary Obenhoff (UGA, unpublished) using plasmids deigned by me.

challenge associated with constructing a deletion is having a reliable and consistent counter-selectable marker system involved, such as *upp*. Immediately, the counter-selectable gene must be implemented into the deletion plasmid under control of a promoter that efficiently promotes its expression, allowing counter-selection against the backbone of the plasmid. Whether or not a native copy of the gene placed under a new promoter in a plasmid will produce the desired effect in consistent counter-selection is not always known. A layer of complexity is added when attempting to construct a deletion of a gene that may be a key contributor to metabolism. That is, it is difficult to predict the efficiency of counter-selection in deleting or replacing a gene with a selectable marker, and the efficiency of the many other factors at play including rates of mutation in a counter-selectable gene and rates of homologous recombination between homology arms in a deletion vector and the chromosome. Some other factors that can make this difficult include efficiency of transformation of a deletion vector, as well as whether or not it is best to have a functional origin of replication on the deletion vector. In many ways, early genetic tool development in a non-model organism requires reliance on estimation and expectations based on related organisms, as well as development of genetic tools by trial-and-error.

A last limitation associated with metabolic engineering in a non-model organism like *M. elsdenii* deals with the nature of genome and metabolic exploration in a microorganism that has not been heavily studied. When choosing candidate genes to delete – which serves as a tool to elicit a predicted phenotype – there are significant limitations in basing strain construction on genome annotations alone. With little to no biochemical characterization of many of the enzymes produced from our many putative chain-elongation genes, the importance of the gene in metabolism, and even potential lethality, is difficult to predict. Additionally, with a lack of knowledge of flux and energy balance in the organism, the effect of pathway interruptions is

unknown until their deletions are constructed or they are determined to be lethal mutations. For that reason, it is important to consider the possibility of the deletion of acyl-CoA transferases with respect to lethality in the organism, bearing in mind the adjacency to energy flux and central carbon metabolism and our lack of understanding of the organism's metabolism as it stands. Any of these putative CoA transferase targets could interrupt flux and result in lethality, which complicates enzyme function assignments.

4.5 Implications of M. elsdenii *as a platform for biofuel production*

In this study, I have demonstrated a shift in the fermentation profile of *M. elsdenii* ATCC25940 to increased longer chain-length products via a single gene deletion in a propionyl-CoA transferase, MELS_0742 (**Figure 5**). This provides evidence that it may be possible to interrupt all acrylate cycle-dependent production of VFAs (propionate and valerate) in this organism and shift all carbon flux towards C4 and C6 products. It is important to consider the potential significant effects on energy and cofactor balance in *M. elsdenii* metabolism by deletion of entire pathways and products; however, more about flux in this organism must be understood through further studies. Additionally, I identified a cluster of genes that could be co-regulated and represent the acrylate pathway, presumably responsible for valerate and propionate production (**Figure 6**). Genes MELS_0742-0747, beginning with the *pct* gene, are annotated as a propionyl-CoA transferase, glyoxylase family protein, 2-hydroxyglutaryl-CoA dehydratase, two 2-hydroxyacyl-CoA dehydratases, and appear to be coregulated in a cluster in the genome. These genes are potential candidates for eliminating all propionate and valerate production, as MELS_0742 deletion has already been seen to have a dramatic effect on this pathway.

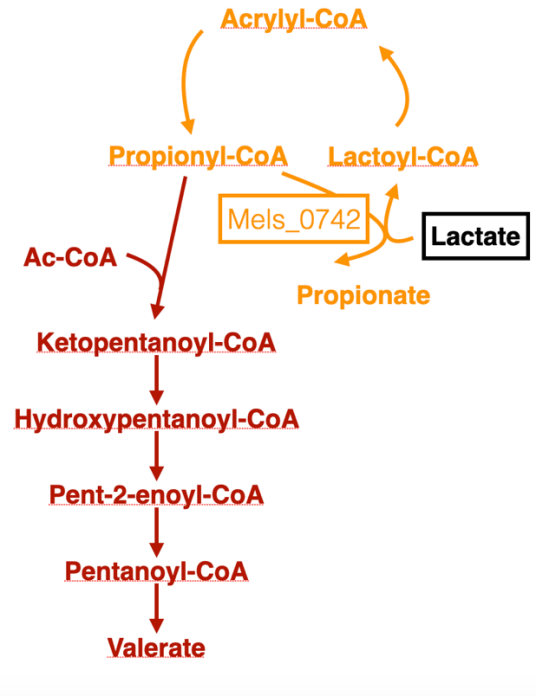

Figure 6. Putative lactate-dependent pathways in *Megasphaera elsdenii* ATCC25940. From genome sequencing and annotation, a metabolic map was created to represent *M. elsdenii* metabolism from glucose and lactate substrates. Pathways predicted to only be active during lactate metabolism are pictured, including propionate production, the acrylate cycle, and valerate production.

Further evidence that *M. elsdenii* could serve as a potential platform for C4-C6 alcohol production through metabolic engineering is demonstrated through work by collaborators at ORNL, where overexpression of a heterologous *adhE*2 produced butanol at 10% of theoretical yield (Riley, unpublished data). Hexanol production has not yet been seen, but with the deletions of the CoA transferases to butyryl and hexanoyl-CoAs, there is much potential for conversion of these precursors to butanol and hexanol when expressed with heterologous alcohol production pathways. Combined with increased flux towards the medium chain length products via side product elimination, there is a clear path toward producing and optimizing butanol and hexanol production in *M. elsdenii* ATCC25940.

CHAPTER 5

ADDITIONAL WORK

Working towards metabolic engineering in a non-model organism takes a multi-faceted approach to lead to the working genetic tools that enable further work in a strain. In *M. elsdenii*, many metabolic tools were underdeveloped or not yet developed for our use in future strain engineering. To this end, I designed experiments for and tested additional genetic tools for their use in *M. elsdenii* including enabling the use of a secondary counter-selectable marker via complementation analyses, testing secondary antibiotics for their use in thiamphenicol^R strains, and designed plasmids and an experiment to test efficacy and efficiency of a known integrase-based gene integration system.

5.1 Complementation of pyrF in ΔpyrF strain JWME03[6]

In a Δ*pyrF* strain (*pyrF* - orotidine 5'-monophosphate decarboxylase), I tested complementation of the *pyrF* gene (strain JWME03) using vectors I designed and that were synthesized at Genscript. Each construct contained *cat*, a thiamphenicol resistance gene, the pIM13 origin of replication, an *E. coli* origin of replication, and a copy of an orotidine 5'-monophosphate decarboxylase gene driven by the P_{cat} promoter. Vector pMTV207 contained a native copy of the *pyrF* gene, and vector pMTV246 contained a copy of the *pyrF* gene from *Clostridium thermocellum* DSM1313 (**Figure 7**). Each vector was transformed into JWME03, selected on thiamphenicol, and single colony isolates containing the replicating vectors were

[6] The pyrF deletion was constructed by Matthew Russo (Westphelng Lab UGA, unpublished) in a WT background to yield strain JWME03. Subsequently, the deletion plasmid was cured by myself from the strain via serial passaging.

screened for sensitivity to 2.5 mg/mL 5-fluoroorotic acid (5-FOA) in plates. Isolates containing pMTV207, which hosts the native copy of *pyrF* (MELS_0842), were not sensitive to 5-FOA, meaning this construct did not complement the deletion. However, isolates containing pMTV246, which hosts the *C. thermocellum* DSM1313 copy of *pyrF*, restored sensitivity to 2.5 mg/mL 5-FOA indicating that it complements the deletion.

The complementation of *pyrF* allows the complementation construct (*C. thermocellum pyrF*) to be cloned into a vector that can serve as a counter-selectable marker in a Δ*pyrF* background strain. Much like the use of *upp*/5-FU for counter-selection in *M. elsdenii*, this provides a system by which additional chromosomal deletions may be constructed, extending the genetic toolbox of the organism for future metabolic engineering work.

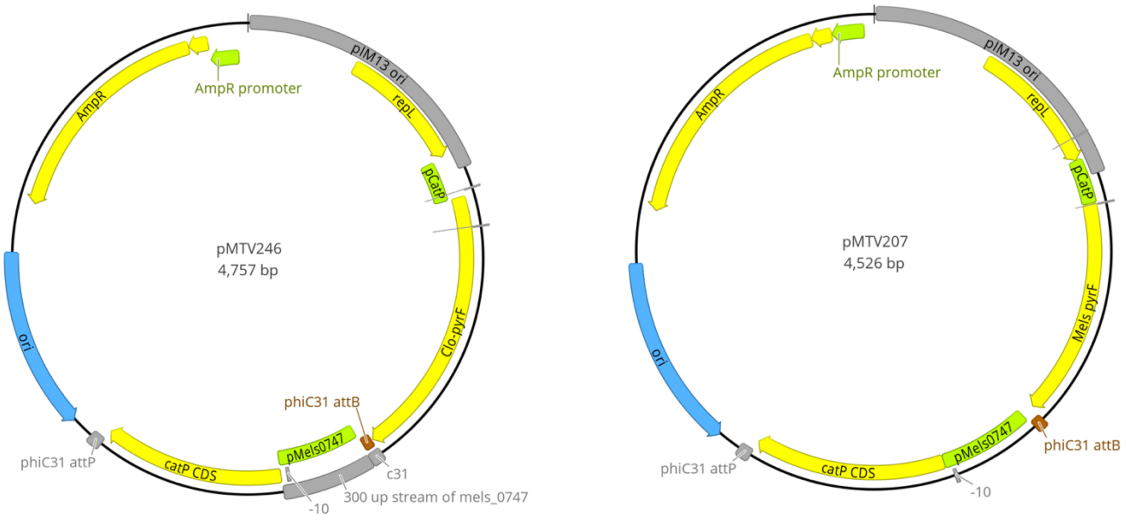

Figure 7. *PyrF* complementation vectors pMTV207 and pMTV246. Plasmids were designed via Geneious Prime and synthesized into a pUC-mini vector at Genscript. The *cat* gene is driven by P_{MELS_0747} and each *pyrF* copy is driven by the P_{Cat} promoter.

5.2 Testing secondary antibiotics

Historically, thiamphenicol selection utilizing the *cat* marker was the only reliable resistance marker in *M. elsdenii* ATCC25940. Though a range of antibiotics were tested and the microbe was found to be sensitive to them, specific antibiotic expression and selection for transformants using those markers needed to be tested. To this end, I designed vectors expressing four different antibiotic resistance genes which *M. elsdenii* previously was shown to have some level of native sensitivity to (Riley and O'Dell, ORNL, unpublished). Codon-optimized erythromycin, tetracyline, apramycin, and kanamycin resistance genes were synthesized in an operon with the *cat* marker into a pMTL85141 backbone vector by Genscript. Each vector was identical aside from the secondary marker that was synthesized into the test vector (**Figure 8**, pMTV405 Apramycin vector pictured).

Vectors were methylated by passage through strain AG4157. Then, each vector was transformed into *M. elsdenii* strain JWME04 via electroporation and selected on plates with thiamphenicol. pMTV403, pMTV404, and pMTV405 transformed successfully onto thiamphenicol. Then secondary transformations were performed and transformants were selected on their respective antibiotics to understand functionality of each antibiotic resistance construct (**Table 3**). Transformation efficiencies were calculated on each secondary antibiotic compared to the parent plasmid containing *cat* only, pMTL85141, on thiamphenicol. Kanamycin (350 µg/mL) and apramycin (150 µg/mL) were found to be functionally selective, and transformed at efficiencies within an order of magnitude of the parental plasmid pMTL85141 on thiamphenicol (**Table 4**).

The use of multiple antibiotics for plasmid selection in an organism is a key genetic tool. In the case of *M. elsdenii* ATCC25940, a functional secondary antibiotic was necessary for loop-out of the *cat* gene in constructs where marker-replacements were made, i.e. in the MELS_0742 deletion strain (JWME04), making them available for continued metabolic engineering. To this end, a ϕC31 expression plasmid was constructed containing the apramycin resistance gene found in pMTV405 for selection in a thiamphenicol resistant strain (Chapter 4.2).

Table 3. Antibiotic test plasmids and antibiotic test concentrations.

Plasmid	Antibiotic Resistance	Antibiotic Concentration (µg/mL)
pMTV402	TetR	2, 10
pMTV403	ErmR	50, 100
pMTV404	KanR	350
pMTV405	AmpR	150

Table 4. Efficiency of transformation of apramycin resistance gene expression vector, pMTV405.

Plasmid	CFU/µg	
	Thiamphenicol 5 µg/mL	Apramycin 150 µg/mL
pMTV405	0	420 +/- 20
pMTL85141	800	0

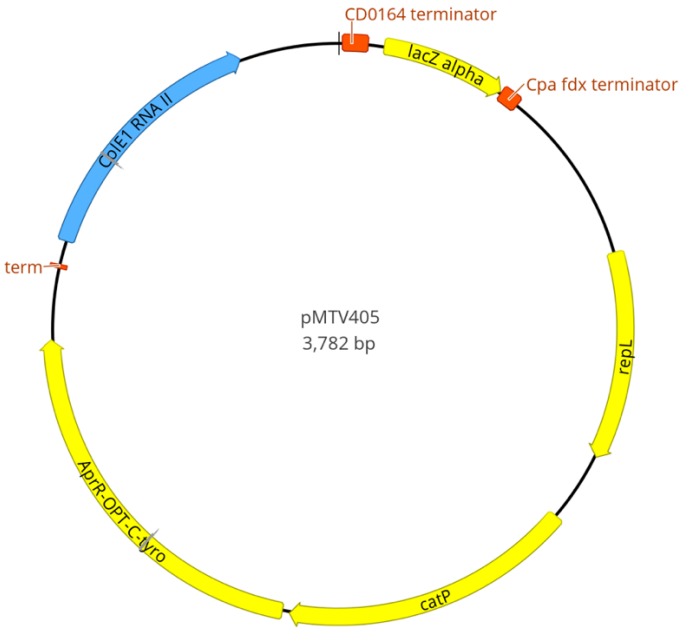

Figure 8. Secondary antibiotic test plasmid, pMTV405. Plasmids were designed via Geneious Prime and synthesized into pMTL85141 vector at Genscript. The *cat* gene is driven by p_{cat} promoter and the secondary antibiotic resistance gene, apramycinR, was constructed in-operon with *cat*.

5.3 Plasmid design for integrase system characterization

An important genetic tool to be characterized in *M. elsdenii* is a phage-integrase based gene integration system which has been developed in a range of other organisms, including *Pseudomonas putida* (Elmore et al., 2017), and shown to allow rapid, site-specific, multi-gene integrations into the organism's chromosome. In this system, there are multiple attachment sites (*att* sites) where corresponding integrases mediate recombination between an *att* site put on a plasmid (*att*P) and an *att* site on the chromosome (*att*B), allowing integration of specific metabolic genes of interest. In *M. elsdenii*, an *att*B cassette containing ten unique attachment sites was integrated into the *upp* locus (Riley, ORNL, unpublished). In order to characterize and optimize this integration system, collaborator Melissa Tumen-Velasquez and I together designed a library of integrase plasmids designed to each express one integrase to be co-expressed in

strains containing an *att*P cassette plasmid with *cat* in the Δ*upp*::*att*B-cassette strain . Each plasmid contained one of the following serine phage integrases: ϕ370, BL3, TG1, A118, Bxb1, ϕC1, MR11, R4, ϕBT1 (Brown), the pIM13 origin of replication, the apramycin resistance gene (*aprR*), and *upp* in an operon with *aprR* (**Figure 9**). This system is now ready to be tested, where integration efficiencies at each *att* site in the chromosome can be calculated by looking at the integration events of the *att*P plasmid containing a *cat* marker.

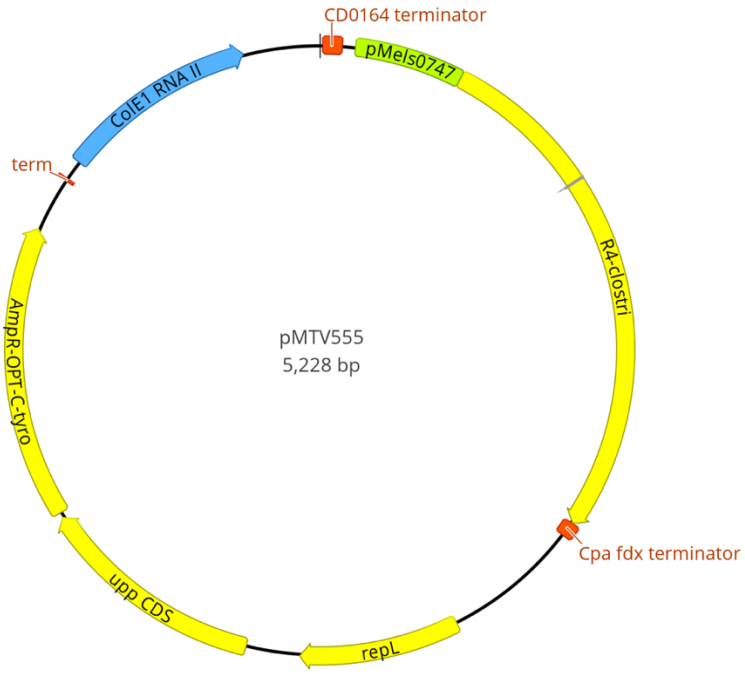

Figure 9. R4 integrase expression plasmid, pMTV555. Plasmids were designed via Geneious Prime and synthesized at Genscript. The *aprR* gene and *upp* gene are driven by p_{cat} promoter. The R4 integrase gene is driven by p_{MELS_0747}.

This system has been shown in other organisms to be highly efficient (Brown, Elmore), thereby greatly increasing the number of alternative heterologous genes and gene constructs that can be tested for pathway expansion in *M. elsdenii* simply by the nature of this strategy. While traditional means of gene integration via homologous recombination and counter-selection using

singular plasmids has been demonstrated to function in *M. elsdenii*, this strategy can be more time consuming and laborious (Riley LA, Guss AM, unpublished). Traditional methods are not excluded from our genetic toolbox, but a newly developed integrase system has the potential to rapidly speed up engineering. For example, using this strategy with twenty plasmids containing twenty different alcohol dehydrogenases to test hexanol production, one could perform 20 individual transformations into the same strain containing a replicating integrase vector and with the poly-*att*B cassette on the chromosome and quickly screen integrated genes for alcohol production. Similarly, it can be used to test different substrate utilization genes to eventually expand metabolism to lignocellulosic substrates like arabinose and xylose. Ultimately, this integration system, once optimized, has the potential to more quickly allow robust engineering of *M. elsdenii* for production of alcohols or other products from renewable feedstocks.

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Table 1. Strains and plasmids

Strains & plasmids	Genotype/phenotype	Source
E. coli		
AG4157	top10 dcm- HK::poly*att*-cassette R4::MELS_0051-52 λ::MELS_1616-17	Riley (upublished)
M. elsdenii		
AG5855	ATCC 29540 Δ*upp*	This study
JWME04	ATCC 29540 Δ*upp* ΔMELS_0742::phiC31-cat-phiC31	This study
AG6465	ATCC 29540 Δ*upp::adhE2* ΔMELS_0742::phiC31-cat-phiC31	Riley (upublished)
Plasmids		
pMTL85141	Shuttle vector containing pIM13 ori	Heap 2009
pMTL82151	Shuttle vector containing pIM13 ori	Heap 2009
pLAR147	Intermediate plasmid containing pBC1 ori	This study
pLAR151	*upp* deletion vector containing nonfunctional pBC1 ori	This study
pLAR179	MELS_0742 deletion vector	This study
pMTV208	backbone vector containing *upp*	This study
pMTV241	MELS_0033 deletion construct	This study
pMTV242	MELS_0034 deletion construct	This study
pMTV252	MELS_0430 deletion construct	This study
pMTV253	MELS_0437 deletion construct	This study
pMTV254	MELS_0464 deletion construct	This study
pMTV255	MELS_1130 deletion construct	This study
pMTV257	MELS_1631 deletion construct	This study
pMTV246	Clo_1313 pyrF complementation vector	This study
pMTV207	MELS *pyr*F complementation vector	This study
pMTV402	cat-tetracyclineR construct	This study
pMTV403	cat-ErythromycinR construct	This study
pMTV404	cat-KanamycinR construct	This study
pMTV405	cat-ApramycinR construct	This study
pMTV554	phiC31 integrase expression vector	This study
pMTV555	R4 integrase expression vector	This study

Table 2. Primers list

Primer	Sequence	Description
NW062	TACGTCTATCGGCTTTGTCAGCAGCG	Screening primers located inside MELS_0742 gene
NW063	TTTTCAAGCCGCCAGCCGTGAA	
NW066	TCAAAGGCCTCCGCAATACGATCAT	Screening primers located outside of flanking regions of MELS_0742 gene
NW067	ACATCGTCCACTTCGAAGGCGATGT	
NW065	GAACTTGAATTGCCAAAGGAAGT	Screening primer located inside the cat gene used in marker replacement of Mels_0742
147_backbone_F	gtaagctagcTCAGATCCTTCCGTATT	Primers to amplify backbone of pMTL85121, excluding pIM13 ori
147_backbone_R	aggtgtccatGCAGGTAAACCTCCTT	
147_insert_F	gtttacctgcATGGACACCTACGCG	Primers to amplify pBC1 ori from pBC1 plasmid for construction of pLAR147
147_insert_R	aaggatctgaGCTAGCTTACGCCG	
upp_up_F	CGCCTTTGAGTGAGCTGATACCGCACATCCAAGTCTATAATACGCCGCAGC	Primers to amplify 490 bp upstream of MELS_2191, *upp,* for construction of pLAR147
upp_down_R	CATGCTCCAACAGCAAAAAGGAAAATTAGTACTCGGCGCCGATCA	
151_backbone_F	TCCTTTTTGCTGTTGGAGCATG	Primers to amplify and linearize the backbone of pLAR151 from pLAR149 for construction of pLAR151
151_backbone_R	GCGGTATCAGCTCACTCAAAGGC	
upp_up_R	GATTTCACACTAATCGTTCATCGACGTA	Primers to amplify 490 bp downstream of MELS_2191, *upp,* for construction of pLAR151
upp_down_F	TGAACGATTAGTGTGAAATCTCCTTTCCTA	
179_backbone_F	aagcctggttgcatgCTTCAGGTTTGTCTGTAACT	Primers to amplify the backbone of pMTL85141, excluding the *cat* gene and its promoter, for construction of pLAR179
179_backbone_R	tacttgtacgtgcatCTAAGTTCCCTCTCAAATT	
179_upp_F	tgagagggaacttagATGCACGTACAAGTAATG	Primers to amplify MELS_2191, *upp*, for construction of pLAR179
179_upp_R	tcagggagatggcccTTATTTCGTGCCGA	
179_homology_F	ttcggcacgaaataaGGGCCATCTCCC	Primers to amplify homology arm cassette containing 800 bp upstream and downstream of MELS_0742, cat gene, and phiC31 sites, for construction of pLAR179
179_homology_R	cagacaaacctgaagCATGCAACCAGGCT	

LIST OF ABBREVIATIONS

*att*P/*att*B	Attachment sites B/P
C	Celsius
CoA	Coenzyme A
C4	4-Carbon length chain
C5	5-Carbon length chain
C6	6-Carbon length chain
FdUMP	Fluorodeoxyuridylate
g	Grams
HPLC	High performance liquid chromatography
KEGG	Kyoto Encyclopedia of Genes and Genomes
M	Molar
mL	Milliliters
mM	Millimolar
OD	Optical density
Ori	Origin of replication
PCR	Polymerase chain reaction
PCT/*pct*	Propionyl-CoA transferase
RCM	Reinforced clostridial medium
TM	Thiamphenicol
UP element	Upstream promotion regulation element
upp	Uracil phosphoribosyltransferase
VFA	Volatile fatty acids
5-FOA	5-fluoroorotic acid
5-FU	5-fluorouracil