METABOLIC ENGINEERING OF *MEGASPHAERA ELSDENII* FOR ENHANCED PRODUCTION OF MEDIUM-CHAIN AND LONG-CHAIN ORGANIC ACIDS

by

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(Under the Direction of Diana Downs)

ABSTRACT

Microbiology and metabolic engineering are emerging fields with significant potential for developing petroleum-free energy alternatives and advancing environmentally sustainable bioremediation methods. The 21st century has seen a renewed focus on biofuels as a critical alternative to fossil fuels, driven by the need to mitigate greenhouse gas emissions and combat climate change. Biofuels offer environmental benefits such as reduced reliance on non-renewable resources and lower carbon emissions. In this context, microorganisms, particularly *Megasphaera elsdenii*, are being explored as promising platforms for biofuel production due to their natural ability to generate medium-chain organic acids, such as butyric, hexanoic, and octanoic acids. Genetic modifications, including the deletion of the uracil phosphoribosyltransferase gene (*upp*) and the propionyl transferase gene (Mels_0742), have been made to optimize the production of longer-chain fermentation products, positioning *M. elsdenii* as a potential producer of valuable chemicals and fuels, including aviation fuel, biopolymers, and next-generation gasoline blend stocks. Further developments in genetic tools for *M. elsdenii* aim to enhance the conversion of lignocellulosic biomass into longer-chain alcohols and other chemicals, supporting the goal of creating sustainable, bio-based fuel alternatives.

INDEX WORDS: Bacteria, Anaerobe, Mesophile, Propionyl transferase, Biofuel

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B.S., University of Georgia, 2019

A Dissertation Submitted to the Graduate Faculty of the University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2025

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May 2025

ACKNOWLEDGEMENTS

I would like to dedicate a special part of this thesis to the memory of Janet Westpheling. It is with deep respect and gratitude that I acknowledge her profound impact on my academic journey. Jan's unwavering commitment to excellence, paired with a generosity of spirit, shaped my growth both as a scholar and as an individual.

Her generosity, not only with her time and expertise but also with her encouragement, gave me the confidence to push forward when I doubted myself. I would not have reached this point without her. Her belief in me, her mentorship, and her wisdom have left an indelible mark on my academic career, and I will carry those lessons with me for the rest of my life.

I am deeply grateful to all the members and contributors of the Westpheling Lab whose support and collaboration were instrumental in the success of this project. In particular, I want to thank Adam Guss and Mark Eiteman for their invaluable expertise and guidance, which have been crucial to my research.

A special thanks goes to my fellow lab members—Jordan Russell, Sun-ki Kim, Sangmin Shin, Neely Palacio, Zach Obenhoff, Saba Pasupathi, Jin Cho, and Meredith Mock. Not only did you assist me in navigating the complexities of science, but you also provided unwavering support as I navigated the challenges of life. Your camaraderie, encouragement, and shared insights made this journey not only possible but enjoyable.

I would like to express my deepest gratitude to Diana Downs for stepping in to guide me through the final months of writing my thesis. Her unwavering support, insightful feedback, and

encouragement were indispensable during this critical phase. Her willingness to invest time and energy into helping me refine my work has made all the difference, and I am incredibly thankful for her guidance. I will be forever grateful for her generosity, patience, and expertise.

Last but certainly not least, I want to thank my daughter, Zumi. Although she is still too young to fully grasp it, her presence in my life keeps me grounded in ways I can't fully express. She is my constant inspiration, and every step I take toward the future is motivated by the love and hope I have for her. Her joy, laughter, and pure heart remind me daily of what truly matters.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

I. History of microbes in applied processes

Microbes have been used in applied processes for thousands of years, long before any scientific understanding of their existence. Fermentation, the most widespread of these applied processes, was used by ancient cultures to make wine, beer and bread dating back to 7000 BCE (1,2). Yeasts, such as *Saccharomyces cerevisiae*, were used by the people of China and Mesopotamia to brew alcoholic beverages (3). Ancient Egyptians used this acquired knowledge to not only brew alcohol and make leavened bread, but to produce yogurt and cheese using bacteria we now know belong to species such as *Lactobacillus* and *Streptococcus* (4,5). Without understanding the role of microbes, these ancient societies laid the groundwork for biotechnology.

The 17th century advancements in microscopy by Antonie van Leeuwenhoek led to a greater understanding of microbes. Leeuwenhoek became the first person to view microorganisms with the power of light microscopy. In his work, Leeuwenhoek described the microbes as "animalcules" (6). It was not until the 19th century when the work of Louis Pasteur established the basis for the role of microbes in fermentation (7). However, Louis Pasteur is best known for developing pasteurization, the process that bears his name (8). Pasteurization is the process by which food and drink is heat-treated to prevent spoilage. Louis Pasteur, and separately Robert Koch, paved the way for medical microbiology by correlating specific diseases to specific microbes (9).

Applied processes in microbiology advanced many fields of research and extended well beyond food and medicine in the latter half of the 19th century. Agriculture benefitted from the improvement of soil fertility through nitrogen-fixing bacteria (10). The use of enzymes, and not just whole organisms, advanced many industrial processes including the production of antibiotics and pharmaceutical compounds (11). In an effort to use less petroleum-based energy, research has led to the successful supplementation of our current fossil-based fuels with ethanol from corn (12,13).

Today, microbiology and metabolic engineering are proving to be promising areas of research that demonstrate potential for developing petroleum-free energy alternatives and implementing environmentally safe bioremediation (14,15). While microbes have been used for thousands of years, we are still learning of different ways to improve upon older traditional practices. Using the scientific method through systematic and controlled processes, we are embarking upon an exciting age of large-scale industrial biotechnology.

II. Biofuel history, composition and requirements

Biofuels are defined as liquid fuels that are derived from renewable biological sources, often plants. The idea of biofuel use can be traced back to when humans first burned wood for heat and energy. Up until the 1850s, ethanol was the primary fuel used in lamps. In 1895 Rudolf Diesel developed an engine that could run on peanut oil (16). This potential of vegetable oils as fuel is still being explored today. In 1908, Henry Ford designed the Model T that ran on ethanol, suggesting that he envisioned a future where biofuels played a role in transportation (17). Thus, for centuries of human history, biofuels were the predominant source of heat and energy. The popularity of ethanol, and other biofuels waned after the development of petroleum fuels.

The widespread availability of inexpensive fossil fuels extinguished the growing interest in biofuels in the early 20th century. At the time, global consumption was relatively low and there was little to suggest that the supply of fossil fuels had limits. Petroleum-based fuel became scarce as a consequence of the two World Wars in the 1900s (18). Additionally, the 1970s saw a multinational oil crisis that led to higher prices at the pump (18). Each of these events contributed to a revived interest in development and use of biofuels.

The 21st century has ushered in an era of renewed interest in establishing an economically viable alternative to petroleum-based fuels. Efforts to develop renewable energy sources are driven by the desire to reduce the dependence of fossil fuels and address the rising and increasingly critical impact of greenhouse gas emissions on climate change (19). Today, biofuel research is an integral part of the renewable energy landscape with an eye to improving fuel efficiency, economic viability, and lower carbon emissions (20–22). Biofuels are renewable energy sources that offer significant environmental benefits. These benefits include decreased reliance on non-renewable fossil fuels and a reduction in greenhouse gas emissions, a major contributor to climate change. There is also a national security benefit inherent in a renewable energy structure that is contained within our national borders (23).

Biofuels can be classified into several categories based on their source, method of production, and intended use. Each biofuel is tailored to satisfy a specific energy need. By virtue of the biofuel feedstocks using atmospheric CO_2 for biomass production, these biofuels offer a reduction in net CO_2 released into the atmosphere when compared to their fossil-based alternative (24). Bioethanol is an alcohol produced from fermenting sugars derived from crops like corn and sugarcane. It is commonly blended with gasoline to reduce emissions and enhance fuel performance. In theory, the use of bioethanol helps in lowering the carbon footprint of

transportation fuels; however, the reduction in locally grown crops and the need to transport the crops for ethanol production and refining can offset the reduced emissions (24).

In contrast to bioethanol, biodiesel is a fuel that is produced through the transesterification of vegetable oils or animal fats. Biodiesel has the advantage that it can be used as a direct substitute for petroleum-based diesel in diesel engines (25). Biodiesel also offers advantages over conventional diesel fuels in that its burning results in lower emissions of particulate matter and hydrocarbons (26). However, the high cost of feedstocks and the competition with food crops makes the replacement of fossil-based fuels with biodiesel an economic challenge.

Biogas consists mainly of methane and carbon dioxide and is primarily generated from the anaerobic digestion of organic waste. Ultimately, biogas can be utilized for electricity generation and heating, thus it provides the potential for a renewable alternative to natural gas (27,28). An attractive aspect of biogas generation is that its production can help in managing waste by effectively turning landfills into methane production plants (29).

Another area of great promise are algae-based biofuels. These biofuels are emerging as a promising option due to the high oil yield of algae and the ability to grow algae in non-arable land (30). The algae used to produce algae-based biofuels can be grown where no crops of any kind can grow - food crops or feedstocks. To make algae-based biofuels economically viable will require infrastructure to be built in addition to a product yield great enough to offset the costs. Research in this area focuses on improving the efficiency and scalability of algal biofuel production (31).

Finally, bio-jet fuel is produced from organic feedstocks and can serve as a sustainable alternative to conventional aviation fuel. It has the potential to significantly reduce the carbon footprint of the aviation industry (32). However, the composition of standard jet fuel and aviation

oil is quite varied. Jet engines rely on fuel and aviation oil for much more than the energy that propels them. Jet fuel and aviation oil also act as a lubricant, an antifreeze, and to help manipulate the moving hydraulic parts of an airplane (33).

Efforts to integrate biofuels into our energy ecosystem faces multiple challenges. For instance, production of biofuels currently requires competition with food crops and at present the high production costs limit the ability to efficiently generate the biofuels at a scale needed (34). Advancements in biofuel technology, including the development of second-generation (derived from lignocellulosic biomass) and third-generation (derived from algae and other aquatic biomass) biofuels, seek to address some of these challenges. For instance, many of these newer biofuel processes utilize non-food feedstocks or waste products as precursors (35). This strategy reduces competition with food supply and enhances sustainability. Critically, the non-food feedstocks proposed or used in these processes, such as switchgrass, can grow on marginal lands that are unusable for traditional food crops (36). Use of switchgrass is particularly attractive since this feedstock can be harvested multiple times a year. Creative research and continuing innovation are expected to play a crucial role in overcoming the existing economic limitations and expanding the role of biofuels in the global energy landscape.

To be usable, biofuels must meet several key requirements that ensure they are effective, efficient, and compatible with existing energy systems. These requirements include physical and chemical properties, appropriate environmental impact, economic feasibility, and regulatory compliance (37). Perhaps most critical, biofuels must have an energy density sufficient to effectively replace fossil fuels (38).

The energy density affects the ability of a fuel to provide power and efficiency in engines and other energy systems. For example, biodiesel and bioethanol should have energy densities

comparable to or higher than conventional diesel and gasoline, respectively, to be effective (39). Biofuels need to burn cleanly and efficiently. To meet these criteria, the fuels must have suitable ignition properties, flame stability, and combustion temperatures (40). The fuels must also minimize emissions of particulates, carbon monoxide, and unburned hydrocarbons. For instance, bioethanol has a high oxygen content, which can lead to more complete combustion and lower emissions compared to gasoline (41). Biofuels must be chemically stable and must have a long shelf life so they can be stored and used effectively over time (42). Biofuels should resist oxidation and degradation, which can lead to fuel gumming or corrosion in storage tanks and engines. For example, biodiesel must be treated to reduce its susceptibility to oxidation and microbial contamination (25).

To be effectively incorporated into the energy landscape, several criteria must be considered and met. Biofuels need to be compatible with existing infrastructure, including engines, pipelines, and storage systems. Infrastructure compatibility requires ensuring that the biofuel does not cause adverse effects that would include corrosion, clogging, or degradation of materials used in these systems. For instance, bioethanol is currently often blended with gasoline at a low percentage (10% at most) (43). However, if higher concentrations of bioethanol are to be used, it may require modifications to engine components and fuel systems (44). Ideally biofuel production and use should result in a net reduction of greenhouse gas emissions compared to fossil fuels. To meet this criterion, the entire lifecycle emissions, including those from feedstock production, fuel processing, and end-use, need to be considered. Many biofuels, like bioethanol and biodiesel, are promoted for their potential to lower overall carbon emissions (41). To be sustainable as an energy source, production of biofuels cannot lead to significant adverse impacts on land use or food supply. For instance, biofuel production must use feedstocks that do not compete with food crops

or lead to deforestation or generate other ecological harm (45). Advanced biofuels from non-food crops or waste materials are preferred, since they allow the process to meet this standard (35). The production of biofuels needs to be efficient in terms of water and energy usage. High water and energy consumption in biofuel production can offset some of the environmental benefits (46). Sustainable practices and technological advancements focus on reducing these resource demands. To be considered realistic, biofuels will need to be economically competitive with fossil fuels. Such cost needs to incorporate production costs, market prices, and subsidies. At present, the cost of feedstocks, processing, and distribution impacts the overall cost-effectiveness of biofuels (47). The feasibility of widespread biofuel adoption depends on the availability and cost of the infrastructure for production, distribution, and consumption. Investments in new infrastructure or modifications to existing systems may be required and these requirements can influence the overall economic viability of embracing biofuels (48). Finally, biofuels must meet various regulatory standards and certifications to ensure quality and safety. This includes the need to comply with national and international standards for fuel quality, emissions, and environmental impact. For example, biodiesel must meet standards such as ASTM D6751 in the United States (49). Supportive policies and incentives can influence the adoption of biofuels. Government regulations, subsidies, and mandates can impact the production and use of biofuels by providing financial support or setting usage targets.

Most modes of human travel could be altered to run on electricity. For instance, automobiles, trains, and boats are, or will be in the near future, capable of using electricity as an energy source. In considering the two obvious exceptions of jet and space travel, biofuels can complement electricity as a productive energy source. Jet engines are a major contributor to carbon emissions but offer the most efficient travel from a time perspective. These features make the

prospect of devising a renewable jet fuel attractive, but bio-jet fuel production poses some unique challenges.

Bio-jet fuel, also known as aviation biofuel, provides a renewable alternative to conventional jet fuels that are derived from petroleum. Bio-jet fuel is designed to meet stringent standards that ensure safety, performance, and compatibility with existing aircraft engines and infrastructure (50). The physical properties of bio-jet fuel are critical for its effectiveness and include several key requirements. Bio-jet fuel must have a high specific energy (measured in MJ/kg), an essential feature to provide the necessary thrust of an aircraft (50). The specific energy of bio-jet fuel should be comparable to or exceed that of conventional jet fuels to ensure efficient performance (40,51,52). The energy density (measured in MJ/L) of bio-jet fuel is crucial for optimizing fuel storage and reducing weight. It should be comparable to that of conventional jet fuels to ensure that aircraft can carry sufficient fuel without compromising performance (53). The density of bio-jet fuel is typically required to be in the range of 0.775-0.840 g/cm³ at 15°C (54). This requirement is essential for ensuring the fuel's compatibility with aircraft fuel systems and for accurate fuel measurements and calculations. The density affects the flow characteristics and pressure in fuel systems. It must be consistent with that of conventional jet fuels to avoid operational issues in aircraft fuel systems. Furthermore, bio-jet fuel must have a maximum kinematic viscosity of 12 mm²/s at -40°C (55). This property is critical for proper atomization and combustion in jet engines. Viscosity affects the fuel's flow characteristics and its ability to atomize properly in the engine (53). The viscosity of bio-jet fuel must be consistent with that of conventional jet fuels to ensure reliable engine performance and avoid issues such as clogging or poor combustion (53).

Bio-jet fuel should have a freezing point lower than the operational temperatures encountered at high altitudes. The maximum freeze point for aviation fuels is typically -47°C (53,56). This ensures that the fuel remains in a liquid state during flight and does not clog fuel lines or filters. Low freezing points are essential to avoid fuel solidification, which can impair fuel flow and engine performance in cold environments. The flash point of bio-jet fuel, which is the temperature at which it can ignite in the air, must be above 38°C (57). This requirement ensures safe handling and reduces the risk of accidental ignition during storage and transportation. A high flash point is critical for maintaining safety standards in the aviation industry and ensuring that the fuel is stable and non-volatile.

Bio-jet fuel must have low sulfur content, typically less than 0.3% by weight (57). Low sulfur content is essential to minimize the formation of sulfur oxides during combustion, which can contribute to air pollution and engine corrosion (53). Reducing sulfur content is important for meeting environmental regulations and reducing the environmental impact of aircraft emissions (58). Bio-jet fuel should have a similar carbon chain length distribution to that of conventional jet fuels, which typically range from C8 to C16 (59). This ensures that the fuel has similar combustion characteristics and energy content (53). Maintaining similar carbon composition is crucial for ensuring compatibility with existing jet engines and infrastructure.

Bio-jet fuel must be thermally stable to withstand the high temperatures encountered in aircraft engines without degrading. This includes resistance to oxidation and thermal degradation (60). The fuel must also be stable during storage to avoid sediment formation and degradation over time. Bio-jet fuel may require certain additives to meet performance requirements, such as anti-icing agents or corrosion inhibitors. The use of additives must be compatible with the bio-jet fuel

and should not adversely affect its properties (53). Additives used must comply with aviation fuel standards and regulations to ensure safety and performance (53).

Microbes have been central to recent efforts to generate biofuels that meet the criteria described above (61). Carbon chain length is the barrier to most of the energetic requirements for biofuel, and particularly biojet fuel (62). This requirement of longer carbon chains also creates a cost-effective barrier. Few organisms have the synthetic ability to generate long chain alcohols, and the bioengineering has often sought to bring pathways in and/or create hybrid pathways.

The unique chemistry of *Megasphaera elsdenii* suggests it can be used to alleviate some cost and energy issues that have plagued other microbial systems. The native ability to condense acetyl-CoA groups to efficiently generate C4 to C8 compounds makes *Megasphaera elsdenii* a compelling platform to produce fuels and chemicals from lactate and plant carbohydrates (63).

III. Overview of the genus *Megasphaera*

Megasphaera is a genus of bacteria classified within the family *Veillonellaceae (64)*. These Gram-negative, non-motile, anaerobic cocci are commonly found in the gastrointestinal tract of humans and animals, and they play a role in the microbiome of various organisms (65). *Megasphaera* can utilize a range of substrates for growth. The ability to ferment lactate is particularly notable, as it contributes to the production of short-chain fatty acids (SCFAs), which can influence the pH of the gut and impact the growth of other microbial species (66).

M. elsdenii generates organic acids as fermentation products when growing on lactate and glucose. The organic acids butyric (four carbon), hexanoic (six carbon), and in some cases octanoic (eight carbon) acids are formed as major fermentation products (66). These acids are mostly likely formed via chain elongation using acetyl-CoA in a pathway that is conceptually similar to the one used by multiple *Clostridium* species to produce butyrate and butanol (67).

Importantly, as the carbon chain length increases, desirable biofuel properties improve (68). Specifically, the energy density increases and hygroscopicity decreases, such that hexanol > butanol > ethanol. Thus, hexanol is an appealing target as a next-generation gasoline blend stock beyond ethanol and potentially a component of biojet fuel. We hypothesize that *M. elsdenii* has the promise to efficiently produce next-generation, drop-in lignocellulosic fuels such as hexanol at high yield and titer. For this to be realized, *M. elsdenii* must be developed as a bioengineering platform using both native and introduced biochemical capacity. The initial steps in this development are the subject of this dissertation. Many of the pathways available for manipulation are shown in Figure 1.1.

IV. Metabolic pathways of interest and relevance

When considering the challenges in engineering organisms to produce valuable chemicals, particularly the long chain alcohols relevant for biofuels, there are some key pathways and enzymes that have been focused on.

The acrylate pathway is a biochemical route utilized by various microorganisms to convert substrates into acrylate, which is a key industrial chemical. Acrylate, or acrylic acid, is an important compound used in the production of polymers, superabsorbents, coatings, and various other materials (69). Its commercial significance has spurred interest in understanding and optimizing its biosynthetic pathways, particularly in the context of sustainable and bio-based production methods.

In microorganisms, the acrylate pathway involves the conversion of simple carbon sources, such as sugars or organic acids, into acrylate through a series of enzymatic reactions. The pathway typically includes the following key steps …

Conversion of Substrates to Propionyl-CoA: The process begins with the transformation of substrates into propionyl-CoA, a crucial intermediate. This step is often catalyzed by enzymes such as propionyl-CoA carboxylase or propionyl-CoA synthetase.

Formation of Propionate: Propionyl-CoA is then converted into propionate, which is a central intermediate in the pathway. This conversion involves the action of enzymes like propionate CoA-transferase or propionate dehydrogenase.

Oxidation to Acrylate: The final step involves the oxidation of propionate to acrylate. This reaction is typically catalyzed by the enzyme propionate dehydrogenase, which facilitates the conversion of propionate into acrylate and carbon dioxide.

The acrylate pathway has garnered significant attention due to the potential for sustainable production of acrylate. Traditional methods of acrylate production rely heavily on petrochemical feedstocks, leading to environmental concerns and resource depletion (70). The biosynthetic approach offers a greener alternative by utilizing renewable biomass as a starting material. Various microorganisms, including bacteria and yeast, have been engineered to enhance acrylate production via the acrylate pathway (71). For instance, strains of *Clostridium* and *E. coli* have been genetically modified to optimize the flux through the acrylate pathway, improving yields and making the process economically viable (71,72). Despite its potential, the acrylate pathway faces several challenges, including the need for efficient substrate utilization, high product yields, and the management of by-products and metabolic flux. Advances in metabolic engineering, synthetic biology, and enzyme optimization are critical for overcoming these challenges and making microbial acrylate production a commercially viable process (73).

Research into the acrylate pathway is focused on optimizing enzyme performance, improving microbial strains, and scaling up production processes. Continued advancements in genetic engineering and fermentation technologies are expected to enhance the feasibility of bio-based acrylate production, contributing to a more sustainable chemical industry.

Our focused interest is in the disruption or elimination of the acrylate pathway. It is our theory that with *Megasphaera*'s inability to produce propionate and valerate, the organism will depend on the putative chain elongation pathways using acyl-CoA transferases and as a result produce longer chain fatty acids. It is our goal to have the organism produce these longer chain fatty acids with predicted specificity.

Propionate-CoA transferases are key enzymes in the biochemical processing of propionate, a three-carbon fatty acid derivative, within various metabolic pathways. These enzymes play a crucial role in the transfer of Coenzyme A (CoA) from one molecule to another, facilitating the conversion of propionate into propionyl-CoA and vice versa. This reaction is central to numerous metabolic processes, including the production of important industrial chemicals, the catabolism of fatty acids, and the synthesis of biofuels and other bioproducts (74).

Propionate -CoA transferases catalyze the transfer of CoA between propionate and other coenzyme A derivatives, a reaction essential for the formation of propionyl-CoA from propionate. This enzymatic activity involves the exchange of CoA groups, which is crucial for maintaining cellular energy balance and metabolic flux (75,76). The general reaction is represented in Figure 1.2.

In microorganisms, propionate-CoA transferases are involved in the metabolism of fatty acids and other organic compounds. They play a significant role in the fermentation processes of

various microbes, such as those in the genus *Clostridium*, where they help convert propionate into propionyl-CoA for further utilization in energy production or biosynthesis (77). Additionally, in the context of biofuel production, these enzymes are instrumental in the biosynthesis of valuable chemicals like acrylate from renewable sources (78).

The ability to engineer propionate-CoA transferases for enhanced activity and specificity has important applications in biotechnology (78). By modifying these enzymes, researchers can optimize metabolic pathways in microorganisms to improve the efficiency of bioproduction processes. For example, propionate-CoA transferases are being investigated for their potential in optimizing the production of biofuels and biochemicals from lignocellulosic biomass, which is a promising approach for sustainable energy solutions (78,79).

One of the main challenges in working with propionate-CoA transferases involves achieving high enzyme activity and stability under industrial conditions. Advances in enzyme engineering, such as directed evolution and protein design, are being employed to enhance the performance of these enzymes (80). Additionally, the integration of propionate-CoA transferases into synthetic metabolic pathways requires careful consideration of cofactor availability and substrate specificity to maximize yield and efficiency (81).

Research on propionate-CoA transferases is likely to continue focusing on improving enzyme characteristics and understanding their roles in various metabolic contexts. Innovations in enzyme engineering and synthetic biology are expected to facilitate the development of more efficient and versatile biocatalysts, which will have significant implications for industrial biotechnology and the production of sustainable chemicals and fuels.

We hypothesize that a deletion of Mels_0742 (propionyl-CoA transferase) will require the organism to utilize other acetyl-CoA transferases to keep redox potential balanced and this will result in longer chain fatty acid production.

V. Genetic tools

a. The limitations of model organisms

Molecular genetic tools are essential to engineering an organism to incorporate desired metabolic capabilities. Strain modification is the dominant means to generate production of relevant products, such as those alluded to above. This capability exists in many model organisms, which is often the benefit of using them as engineering chassis. Throughout decades of metabolic research, model organisms have served as representative species that can help scientists understand biological processes that are conserved across a wide range of species and/or organisms. Model organisms are chosen, among other things, for their rapid reproduction rates, ease of manipulation in laboratory settings and the vast array of technical tools for which they are amenable (82). Using model organisms, researchers can investigate fundamental aspects of metabolism and engineer whole metabolic pathways to produce a number of desired products. However, with an end goal defined, not all model organisms are created equal, and each organism has definitive limitations.

There are often organisms that possess native physiological traits that make them attractive for biotechnological applications. Often these are metabolic, or physiological characteristics that offer a means to overcome limitations present in model organisms. Unfortunately, the lack of genetic tools in many of these potentially useful organisms presents a major barrier to their use in industrial applications. The native ability to condense acetyl-CoA groups to efficiently generate C4 to C8 compounds makes *Megasphaera elsdenii* a compelling platform to produce fuels and chemicals from lactate and plant carbohydrates (66). As such, efforts like those described in this

dissertation are being made to generate the genetic tools needed to allow the efficient use of *Megasphaera elsdenii* in engineering applications. Molecular biological tools are broadly available, but taking advantage of them requires protocols that depend on the physiology and metabolism of the target organism.

b. Defined media

Manipulating bacteria to take advantage of different metabolisms requires a defined medium on which specific traits can be assessed. Defined media, or chemically defined media, are specifically formulated nutrient solutions used in biological research to cultivate microorganisms, cells, or tissues. Unlike complex media, which contain natural extracts or undefined ingredients, defined media consist of precisely known quantities of pure chemicals. Consistent media composition helps researchers obtain reliable and repeatable experimental results. The use of defined media in research is crucial for several reasons. Defined media ensure that researchers control the composition of the growth environment. Such control is essential for reproducibility, since it provides a defined recipe that can be generated by each investigator. The use of defined medium allows researchers to standardize experimental conditions across different studies and laboratories. This standardization facilitates comparisons between experiments and across different research groups, ensuring that results are comparable and that findings can be validated. This consistency allows science to move forward by incorporating data from multiple studies.

A defined medium allows investigators to study the metabolic needs and pathways of organisms in detail. By providing a specific set of nutrients and observing how organisms respond, researchers can gain insights into their metabolic processes, nutrient utilization, and growth requirements. With defined media, researchers can precisely manipulate the concentrations of individual nutrients and additives. This precision enables detailed investigations into how specific

compounds and/or their concentrations influence growth, gene expression, and other biological processes. These data not only facilitate the understanding of different limitations on organismal growth and metabolism but allows the implementation of genetic tools, such as nutritional markers, which depend on this information. This physiologically relevant approach is essential for understanding how organisms adapt to nutrient constraints and allowing the identification of key factors that influence growth and fitness of an organism.

In genetic and molecular biology research, defined media allow researchers to study the effects of specific genes or genetic modifications under controlled conditions. By using media with known compositions, researchers can better understand how genetic changes impact growth, gene expression, and cellular function.

In industrial biotechnology and production processes, having a defined medium for the organism of interest is crucial. Defined medium contributes to better quality control in biotechnological applications by providing a stable and predictable growth environment. This stability is critical for producing high-quality and consistent products, such as pharmaceuticals, biofuels, and specialty chemicals. Furthermore, consistency is essential for the production of recombinant proteins, where precise control overgrowth conditions are necessary to maximize yield and facilitate protein purification. The use of defined media helps ensure that protein production systems are optimized for efficiency and purity. By controlling the nutrient composition, researchers and manufacturers can enhance the efficiency of microbial fermentation or cell culturing processes, which often improves productivity and reduces costs by excluding any unnecessary components (83).

Complex media often contain undefined components such as yeast extract or animal serum, which can introduce variability due to batch-to-batch differences. While defined medium

eliminates these unknown factors allowing researchers to isolate the effects of specific nutrients or conditions on their experimental subjects, it is not always possible to generate a defined medium. In the case of *M. elsdenii*, a defined medium has been generated (84).

c. *Genetic selection and counterselection*

Any manipulation of genetic material, particularly the introduction of new genetic material, is facilitated by a selection that distinguished the new genotype from the parent. It is most useful if this is a simple growth difference on a defined medium. In a best-case scenario, there will also be a counterselection, which allows one to refine the final genotype by eliminating the transfer vehicle - usually a plasmid. In many microorganisms, the biosynthetic pathway for uracil is an attractive genetic tool since it can provide both the selection and counterselection described above. Uracil is a crucial component of nucleic acids. It is one of the four nucleobases found in RNA, where it pairs with adenine. Uracil is involved in various metabolic processes, including the synthesis of nucleotides and nucleic acids (85).

In organisms capable of synthesizing uracil, the pathway typically involves the conversion of precursors like carbamoyl phosphate and aspartate into orotate, which is then converted to uridine monophosphate (UMP) and subsequently to uracil (86). Strains lacking one or more of the enzymes required for this biosynthetic pathway require that uracil be added to the medium. In yeast, for instance, null mutations in the URA3 gene, which encodes orotidine-5'-phosphate decarboxylase, can cause uracil auxotrophy (87). This gene is essential for the conversion of orotate to UMP, a precursor of uracil. Similarly, in bacteria, a mutant lacking a uracil biosynthetic gene provides a positive selection for plasmids (or other constructs) that express the wild-type gene. By complementing the auxotrophy with plasmids or other vectors carrying the wild-type gene, researchers can investigate gene function and regulate gene expression in a controlled

manner (88). In molecular cloning and recombinant DNA technology, uracil auxotrophic strains are often used as selectable markers. The presence or absence of uracil in the growth medium can be used to select for transformed cells that carry a plasmid with a *ura* gene (or *ura* homolog) or other desired genetic modifications (88).

Some chemical analogs are converted to toxic products by cellular enzymes. There are two gene products in uracil metabolism that have been used in counterselection protocols in multiple bacteria and yeast model systems. In each case, a toxic antimetabolite is used to determine if a strain has a functional enzyme. Specifically, 5-fluorouracil (5-FU) and 5-fluoroorotic acid (5-FOA) are converted to toxic compounds by uracil phosphoribosyltransferase (Upp), and orotidine-5'-phosphate decarboxylase (PyrF) respectively (88,89). Each of the resulting toxic molecules have toxic effects on cell growth and thus indicate the strains that have functional Upp and/or PyrF enzymes. Therefore, a defined medium coupled with a *upp* or *pyrF* mutation can offer means for both selection and counterselection.

VI. Dissertation outline

The dissertation that follows describes my efforts to develop foundational genetic tools to facilitate bioengineering studies in the bacterium *Megasphaera elsdenii.* As described above, this bacterium has features that suggest it will be a valuable addition to the systems available for producing precursors to biofuels.

In Chapter 2, I describe the enhancement of long-chain fatty acid production in *Megasphaera elsdenii* as a result of a deletion in the acrylate pathway, specifically propionyl-CoA transferase. A *pct* deletion has resulted in the elimination of propionate but displayed increased production of longer-chain organic acids, primarily butyrate and hexanoate.

In Chapter 3, I describe the development of further genetic tools to be used in *Megasphaera elsdenii*, specifically the engineering of a uracil auxotroph via a deletion in the *pyrF* gene. This deletion has provided reliable counterselection for plasmids containing the *pyrF* gene. Additionally, I describe the complementation of *pyrF* and the return to uracil prototrophy. This along with the development of a defined media is a promising method for the positive selection of plasmid DNA.

In Chapter 4, I summarize the results of my work and suggest directions and methods for continuing research and describe additional studies to further unlock the potential of this promising organism.

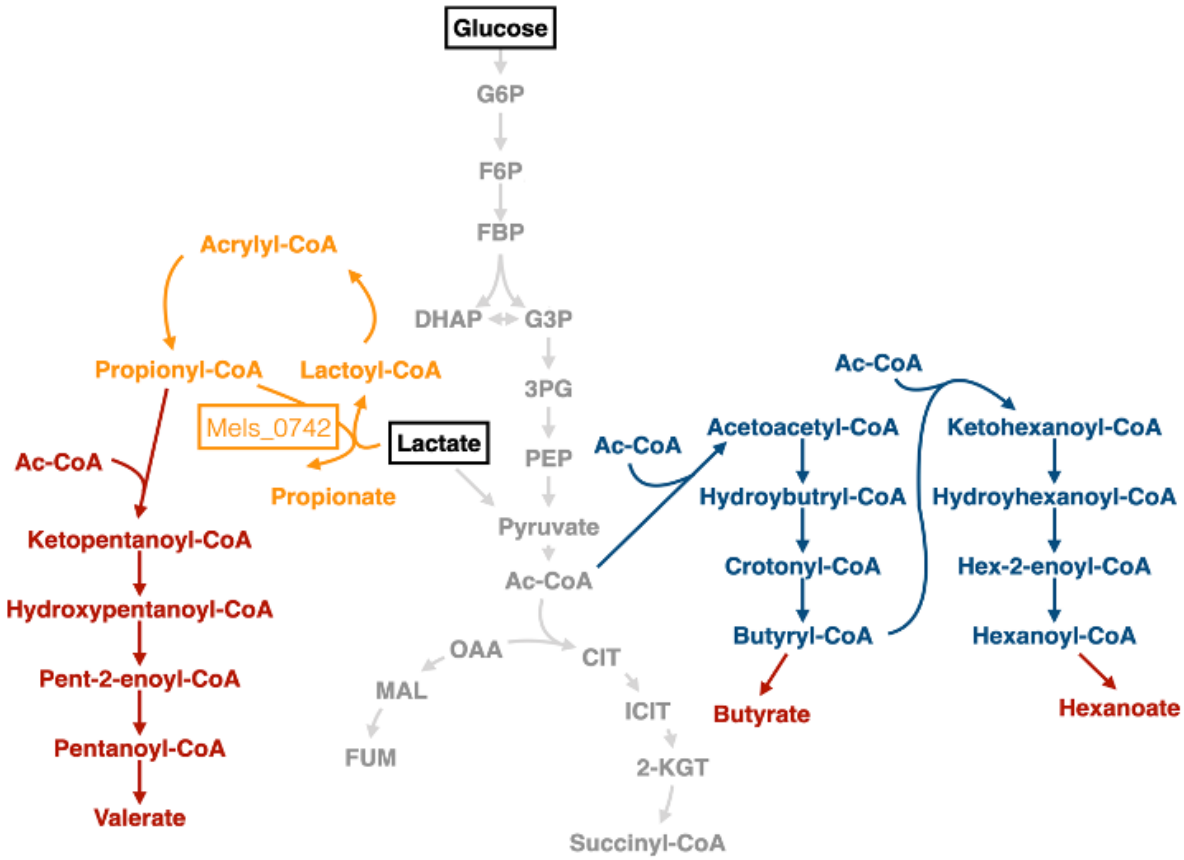

Figure 1.1: Predicted metabolic pathways in *Megasphaera elsdenii*. Gray: glycolysis and branched TCA cycle; Orange: acrylate cycle with the assignment of the Mels_0742 propionyl-CoA transferase; Red: putative chain elongation pathway to delete; Blue: putative chain elongation pathway to retain; Black boxes: growth substrates.

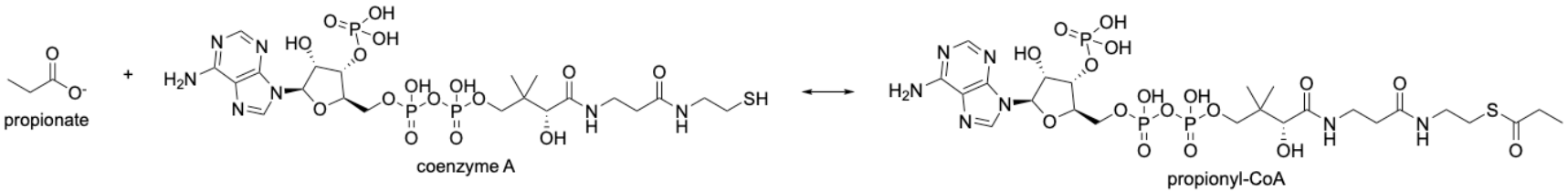

Figure 1.2: Proposed enzymatic reaction of Mels_0742. Propionyl-CoA transferase is an enzyme that catalyzes the reversible reaction converting propionyl-CoA into propionate and coenzyme A. Image prepared with ChemDraw® by Revvity Signals.

CHAPTER 2

DELETION OF A PUTATIVE ACYL-COA TRANSFERASE IN *MEGASPHAERA ELSDENII* ELIMINATES PROPIONATE PRODUCTION AND INCREASES MEDIUM CHAIN ORGANIC ACID PRODUCTION.[1]

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[1]Adapted from Russo M, Riley L, Wood N, Eschedor G, Blum D, Guss A, Westpheling J. 2025 To be submitted to Applied and Environmental Microbiology, 2025.

The work in this chapter was done with collaborators. I contributed to the design of experiments and writing of the work herein described. I contributed to and/or performed experiments depicted in Figures 2.5 and 2.6.

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I. Abstract

The metabolic diversity of microorganisms in nature provides a largely untapped source of important compounds that are difficult or impossible to engineer in the few model systems available. The native ability to condense acetyl-CoA groups to efficiently generate C4 to C8 compounds makes *Megasphaera elsdenii* a compelling platform for the production of fuels and chemicals from lactate, a readily available substrate. *M. elsdenii* produces medium chain organic acids as fermentation products when grown on lactate and glucose, including formation of butyric (four carbon), hexanoic (six carbon), and in some cases octanoic (eight carbon) acids as major fermentation products. To facilitate genetic manipulation, we deleted the uracil phosphoribosyltransferase gene (*upp*, MELS_2191) which converts 5-fluorouracil (5-FU), to the toxic compound fluorodeoxyuridylate. Deletion of *upp* renders cells resistant to 5-FU allowing counter selection of plasmids containing a wild-type allele. That genetic background was used to delete a putative propionyl transferase gene (Mels_0742), that resulted in a shift toward longer fermentation products during growth on lactate. With longer carbon chain compounds as precursors, condensation reactions to generate C14-C16 compounds become more efficient making them an appealing target for aviation fuel, biopolymers and next next-generation gasoline blend stocks beyond ethanol.

II. Introduction

The development of next-generation biofuels and biopolymers is an economic challenge. The enzymatic conversion of fatty acids to alcohols can be performed efficiently in *Megasphaera elsdenii* with the introduction of an *AdhE* gene (90). However, the chemical condensation of fatty acid carbon units in a chain from C2 or C3 to C14 – C16, such as those required for aviation fuel,

has previously been accomplished via condensation reactions that are both low yield and expensive. Using medium chain-length alcohols like butanol (C4) and hexanol (C6) derived from the biological conversion of low cost, sustainable feedstocks as precursors to generate more useful long chain-length alcohols (C14-C16) offers an opportunity to increase the efficiency of this process as well as dramatically reduce costs (91,92). Riley et al. has shown the conversion of butyric acid to butanol in *M. elsdenii* with the introduction of an *AdhE* gene (93). The production of longer-chain organic acids and alcohols has been observed in a variety of organisms, including but not limited to many Clostridia (79,91,94,95), however the yields and titers are low. Carbon chain-elongation pathways from various organisms have been heterologously expressed in *E. coli.* While this strategy has been moderately successful in *E. coli*, the yield of production for C6 and longer chain products remains low, suggesting that extending the chain elongation pathway beyond a single cycle remains a significant challenge (96–98). The ability to generate medium and long chain organic acids biologically, *in vivo*, will reduce the need for condensation of C2 or C4 molecules to longer chain alcohols, an economic barrier. Jet fuels, for example, require C14 alcohols and condensation of ethanol, or even butanol, is not cost efficient.

Megasphaera elsdenii is a ruminal mesophilic obligate anaerobe and a member of the Negativicutes. It natively possesses high-flux metabolic pathways that convert a variety of carbon sources such as lactate, to medium chain fatty acids including butyric acid (C4), hexanoic acid (C6), and even octanoic acid (C8) by some species (99–101). We recently developed a method for DNA transformation of two strains of *Megasphaera elsdenii* (102), opening this organism to advanced physiological studies and bioengineering. These genetic tools for *M. elsdenii* include the development of a counter-selectable marker for targeted gene deletions and insertions to increase flux towards targeted products such as medium and long chain organic acids. This strategy

involves a chromosomal deletion of *upp* (*uracil phosphoribosyltransferase*), which enables counter-selection of integrated vectors using 5-fluorouracil (5-FU). *The* upp gene has been used as a counter-selectable marker in a variety of bacteria (103,104). We have also used existing and newly generated genome sequences to develop a central metabolic reconstruction, transcriptomic and metabolomic analyses to better understand the physiology of *M. elsdenii* and enable predictions for pathway engineering. This work will also lay the foundation for more advanced processing options such as a co-culture or sequential fermentation in which one organism converts sugars to lactate and an engineered *M. elsdenii* converts the lactate to a higher value product.

III. Results

Predicted metabolic pathways *in M. elsdenii*. Relatively little is known about *M. elsdenii* metabolism or the biochemical nature of enzymes involved in its condensation and chain elongation reactions. We used the genome sequences of two strains *M. elsdenii* strains, NCIMB 702410 and ATCC 25940 to build a reconstruction of central carbon metabolism. PacBio and Illumina sequencing platforms were used to obtain the initial genome sequencing data, and complete genome sequences were prepared using the assemblers SPAdes (105) and Canu (106). Each genome was annotated using RAST (107) (Genbank accession numbers CP027569 and CP027570) to investigate phenotypic differences between the two strains. *M. elsdenii* NCIMB 702410 is annotated to contain 2,280 coding sequences whereas ATCC 25940 is predicted to include 2,194 coding sequences. Of the genes belonging to each strain, 1,972 coding sequences share homology with a median percent identity of 99.7%. This result suggests that around 200 or 300 unique coding sequences contribute to phenotypic variation between strains ATCC 25940 and NCIMB 702410, respectively. Several genomic islands are present in each genome. Genomic

islands are typically regions several thousand base pairs in length that encode nonhomologous genes possibly acquired by horizontal gene transfer. In some cases, these islands have been found to encode genes related to organism defense (108,109), evolution (110), pathogenicity (111), antibiotic resistance or even entire metabolic pathways (112). We hypothesize that genomic islands identified within the two *M. elsdenii* strains drive the difference in genome length and number of coding sequence features. Both genomes encode homologous genes for central carbon metabolism including those for glycolysis, a pentose phosphate pathway, and a branched TCA cycle, allowing us to condense the metabolic models for central carbon metabolism into a single model (**Fig. 2.1**). Additional manual curation of the *M. elsdenii* carbon metabolism was conducted using the KEGG database and traditional sequence comparison methods, including the addition of the acrylate pathway for propionate formation as experimentally confirmed (100), (**Fig. 2.2**). Additionally, metabolic pathways in *M. elsdenii* were modeled using the DOE's KBase application Build Metabolic Model (113) and gap-filled within the KBase platform (114) used to generate Figure 2.2. Expansion of this DOE KBase model into a larger framework will enable Flux Balance Analysis and predictions of the effect of single and multiple gene deletions.

Deletion of the *M. elsdenii* uracil phosphoribosyltransferase gene allows counter-selection of the wild type allele using 5-fluorouracil. To expand the genetic toolbox for *M. elsdenii* and enable the construction of gene deletions for further study of *M. elsdenii* metabolism, a counter-selection strategy using the *upp* gene was developed in this study. The *upp* gene encodes uracil phosphoribosyltransferase, an enzyme capable of converting the uracil analogue, 5-fluorouracil (5-FU), to a toxic product, fluorodeoxyuridylate (FdUMP) in *Mycobacterium tuberculosis* (115). To test whether *M. elsdenii* was sensitive to 5-FU, cells were grown in liquid medium with 5, 25, 50, 100, 200 ug/mL of 5FU. The growth of *M. elsdenii* was completely

inhibited in the presence of 50 ug/ml 5-FU. Growth of the wild-type strain on 5-FU selecting resistance resulted in spontaneous mutations in this gene indicating that it is responsible for conversion of 5-FU to FdUMP.

A plasmid (pLAR151) containing the *cat* gene (chloramphenicol acetyltransferase) and ~1000 base pairs of chromosomal homology upstream and downstream of *upp* was cloned in *E. coli*. Plasmid pLAR151 was transformed into wild-type *Megasphaera elsdenii* ATCC 25940. The outgrowth was plated in the rich medium Reinforced Clostridial Media (RCM) and selected with thiamphenicol (5µg/mL) for the presence of the plasmid. A single colony was picked and grown in RCM under 5-FU selection. This resulted in a deletion of the uracil phosphoribosyltransferase gene (*upp*, MELS_2191) in the *M. elsdenii* chromosome (**Fig. 2.3**) creating strain AG5855 (**Table 2.1**), that is resistant to >300 ug/ml 5-FU. This chromosomal deletion allowed for the counter-selection of plasmids containing a copy of the wild-type upp allele. We have used KEGG locus designations for this manuscript. Locus tags were reassigned by NCBI and a table listing both designations for each locus is shown in Table 2.2.

Deletion of a putative propionyl-CoA reductase gene resulted in loss of propionate production, decreased valerate production and increased organic acid production. Acyl-CoA transferase enzymes are common components of various metabolic pathways and are generally responsible for catalyzing the transfer of coenzyme A (CoA) groups from an acyl-CoA donor to carboxylic acids. Nine putative acyl-CoA transferase genes were identified by sequence analysis in the *M. elsdenii* chromosome, yet none of these putative enzymes have been biochemically characterized. Four of the putative acyl-CoA transferases, annotated as propionate-CoA transferases (*pct*), Mels_0742, Mels_0464, Mels_1631 and Mels_1130, potentially transfer the

CoA group from acetyl-CoA to propionate, generating propionyl-CoA. According to our metabolic model, propionyl-CoA is an intermediate in the *M. elsdenii* acrylate pathway and a precursor to valerate (C5) production (**Fig. 2.2**). With lactate used as the growth substrate, RNAseq and proteomic analysis revealed that the most highly expressed putative propionate-CoA transferase is MELS_0742. Expression of MELS_1130 was not detected. To investigate the possible role of MELS_0742 in organic acid production from lactate, a deletion of this gene was constructed in the AG5855 (Δ*upp*) background strain generating strain JWME04 (Δ*upp* Δ*pct*) (**Fig. 2.4**). The plasmid (pLAR179) designed to generate this deletion contained a *cat* gene with upstream and downstream homology to MELS_0742 with ΦC31 attachment sites flanking the *cat* gene. The cat gene allowed selection of the marker replacement event and subsequent engineering would allow the removal of the ΦC31 attachment sites and the *cat* gene by transient expression of the ΦC31 integrase (116,117).

To investigate the effect of the Mels_0742 deletion on organic acid production, HPLC analysis was performed on the supernatant from cells grown in lactate as the primary substrate. As shown in Figure 2.5, while the wild-type and Δ*upp* strains displayed similar levels of propionate and valerate production, a complete loss of propionate production and decrease in valerate production was observed in JWME04 (Δ*upp* Δ*pct*). This result suggests the primary role of MELS_0742 is the conversion of propionyl CoA to propionate in *M. elsdenii*, and that none of the other predicted propionyl-CoA transferases (Mels_0464, Mels_1631, and Mels_1130) can fully substitute for Mels_0742 activity.

Surprisingly, we further observed changes in butyrate and hexanoate production. JWME04 (Δ*upp* Δ*pct*) was observed to produce significantly more butyrate and hexanoate than the wild-

type or Δ*upp* strains, suggesting that the deletion of Mels_0742 leads to increased flux of carbon to the pathways for these longer chain organic acids (**Fig. 2.5**).

To test whether deletion of Mels_0742 affected growth on either glucose or lactate, growth of AG5855 (Δ*upp*) and JWME04 (Δ*upp* Δ*pct*) were compared to the wild-type on both substrates (**Fig. 2.6**). Strain AG5855 (Δ*upp*) was included to examine whether the deletion of upp carried any growth defect. All *M. elsdenii* strains grew to a higher maximum optical density on glucose. Growth of the deletion strains on glucose was indistinguishable from wildtype. All strains reached a lower maximum optical density on lactate. The JWME04 (Δ*pct*) strain took twice as long to reach the same optical density as compared to wild-type and AG5855 when grown on lactate.

IV. Methods and Materials

Deletion of MELS_2191, a uracil phosphoribosyltransferase (*upp*), in the *M. elsdenii* chromosome. To construct a deletion of MELS_2191 in the *M. elsdenii* ATCC 25940 genome, plasmid pLAR151 was constructed by Gibson assembly (**Table 2.1**) to contain 1 kb of DNA sequence upstream and 1 kb downstream of the *upp* gene inserted into the MCS of pMTL85141. The plasmid was transformed into *E. coli* strain AG4157 (**Table 2.1**) which expresses two methyltransferases and their corresponding specificity subunits (MELS_0050-0051, MELS_1615-1616) from *M. elsdenii* cloned into the chromosome (102). Methylated plasmid DNA was subsequently isolated and used to transform *M. elsdenii* ATCC 25940. Transformants were selected on RCM agar plates with 5 μg/mL thiamphenicol (TM) incubated for 72 hours. Colonies were transferred into Reinforced Clostridial Medium (RCM) (BD Difco, Sparks, MD, USA) with 5 μg/mL TM and incubated overnight. The liquid cultures were passaged into RCM (BD Difco) with 50 μg/mL 5-fluorouracil. The plates were incubated overnight, and colonies were plated on

RCM. Single colonies were picked into RCM (BD Difco, Franklin Lakes, NJ, USA) and PCR screened for the chromosomal deletion of *upp*. The deletion was confirmed by PCR amplification of the chromosomal region containing *upp* (**Fig. 2.3**) and resulted in strain AG5855 (Δ*upp*).

Deletion of a putative propionyl coA transferase (MELS_0742) from the *M. elsdenii* chromosome. To construct a deletion mutant of Mels_0742 in AG5855 *M. elsdenii* ATCC 25940 (Δ*upp*) genome, plasmid pLAR179 was constructed by Gibson assembly (**Table 2.1**) to contain 1 kb of DNA sequence upstream and 1 kb downstream of the *Mels_0742* gene. These upstream and downstream sequences included the *cat* gene flanked by two ΦC31 attachment sites between them. This sequence was inserted into the MCS of pMTL85141. The plasmid was transformed into *E. coli* strain AG4157 (**Table 2.1**) using a BioRad Gene Pulser Xcell Electroporation System (BioRad, Hercules, CA, USA) with square wave protocol at 1400V for 1.5 milliseconds, 1 pulse, in a 1mm cuvette. *E. coli* strain AG4157 expresses two methyltransferases and their corresponding specificity subunits (Mels_0050-0051, Mels_1615-1616) from *M. elsdenii* cloned into the *E. coli* chromosome (102). Methylated plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen) according to manufacturer's protocol. Methylated plasmid DNA was then used to transform AG5855 *M. elsdenii* ATCC 25940 (Δ*upp*). Transformants were selected on RCM agar plates with 5 µg/mL thiamphenicol (TM) and incubated for 72 hours. Colonies were picked into RCM (BD Difco) with 5 µg/mL TM and incubated overnight. Cultures were screened for plasmid integration using primers NW065 and NW066. The liquid cultures were plated in RCM with 5 µg/mL TM and 50 µg/mL 5-fluorouracil. Colonies were picked and grown in liquid RCM supplemented with 5 µg/mL TM and 50 µg/mL 5-FU. Cultures were screened via PCR for the chromosomal replacement of Mels_0742 with the ΦC31-*cat*-ΦC31 sequence using primers

NW066 and NW067 (**Fig. 2.4**). The resulting strain was JWME04 (Δ*upp* Δ*Mels_0742*::ΦC31-*cat*-ΦC31).

HPLC analysis of mutants. *M. elsdenii* ATCC 25940 (wild type), *M. elsdenii* Δ*upp* (AG5855), and *M. elsdenii* Δ*upp* ΔMels_0742::ΦC31-*cat*-ΦC31 (JWME04) were grown in 5 mL RCM + 5 μg/mL thiamphenicol, when necessary, overnight. 50 μL of each strain was added to Balch tubes containing 10 mL of modified RCM with lactate and glucose separately. Each strain was cultured in duplicate for 72 hours at 37 degrees C. Samples were taken at 24-hour intervals, optical densities measurements taken, and fermentation products were quantified using High Performance Liquid Chromatography (HPLC). Lactate, glucose, acetic acid, butyric acid, valeric acid, propionic acid, and hexanoic acid were quantified on Agilent 1260 infinity series HPLC with the Aminex-HPX-87H column (Bio-Rad). The mobile phase was 5 mM sulfuric acid. The column was heated at 30 degrees C, the flow rate was 0.5 mL/min, and the chromatograph was visualized using a refractive index (RI) detector.

Optical density measurements and growth curves for all strains on glucose and lactate. All growth curve data were generated with an Agilent BioTek Epoch 2 using a kinetic protocol taking 600 nm measurements every hour for 72 hours at. Each strain was passaged once overnight in the desired substrate immediately before starting growth curve analysis. Prior to inoculating a 48-well plate, all cultures were diluted to achieve a theoretical measurement of 0.05 OD_{600}.

V. Discussion

A deletion of the uracil phosphoribosyltransferase (*upp)* gene in the *M. elsdenii* chromosome resulted in resistance to 5-FU allowing counter selection of plasmids containing a wild-type allele. The ability to counter-select plasmids facilitates the ability to make deletions quickly and accurately. This genetic background was used to delete a putative propionyl-CoA transferase gene (Mels_0742), that resulted in a shift toward longer fermentation products during growth on lactate. The ability to generate medium chain organic acids as substrates for catalytic upgrading to medium chain alcohols *in vivo* allows the elimination of condensation reactions that are costly and reduce yield.

Jet fuels, for example, require C12 to C14 chain length alcohols, and condensation of ethanol is especially challenging at industrial scale. Interestingly, there are 9 putative propionyl-CoA transferases in *M. elsdenii* and deletion of one of them led to an increase in butyric and hexanoic acids specifically. This opens the possibility that targeting propionyl-CoA transferases for deletion may lead to increased production of specific organic acids. While an overall increase in medium chain organic acids is useful, the ability to generate specific chain lengths solves a major problem in separation, also an issue for industrial production. While this work is at an early stage, we have laid the groundwork for genetic manipulation of organic acid synthesis and suggest that *Megasphaera elsdenii* is an excellent target for further production on an industrial scale. Because lactate is a preferred substrate for organic acid synthesis in this organism, the possibility of using lactate or sequential fermentation with organisms that produce lactate to make medium and even potentially long chain alcohols as an industrial process is feasible.

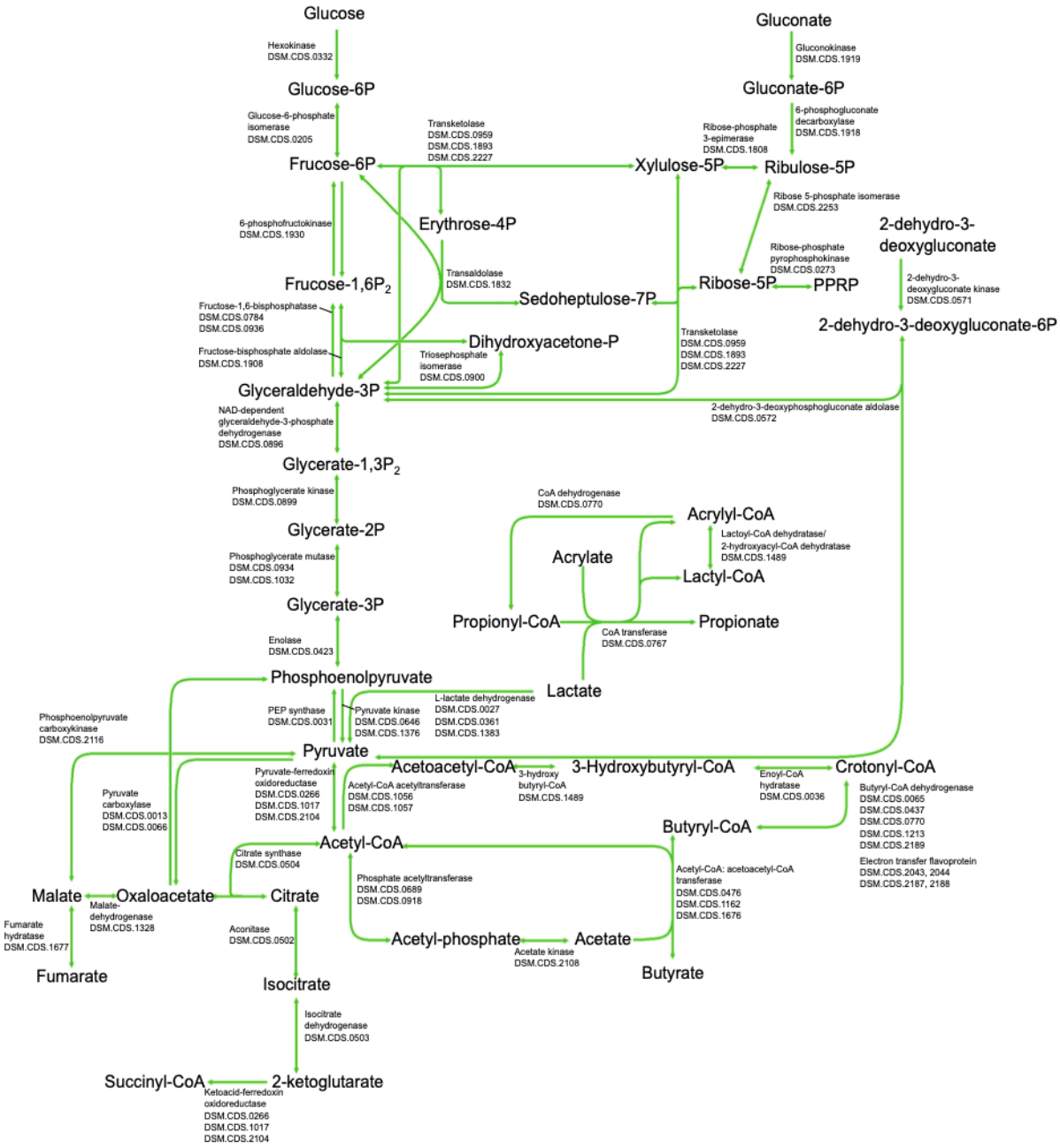

Figure 2.1: Metabolic reconstruction of central carbon metabolism of *M. elsdenii*. Model was constructed using the DOE Kbase (113,114) and represents glucose fermentation to butyrate and lactate fermentation to butyrate and propionate.

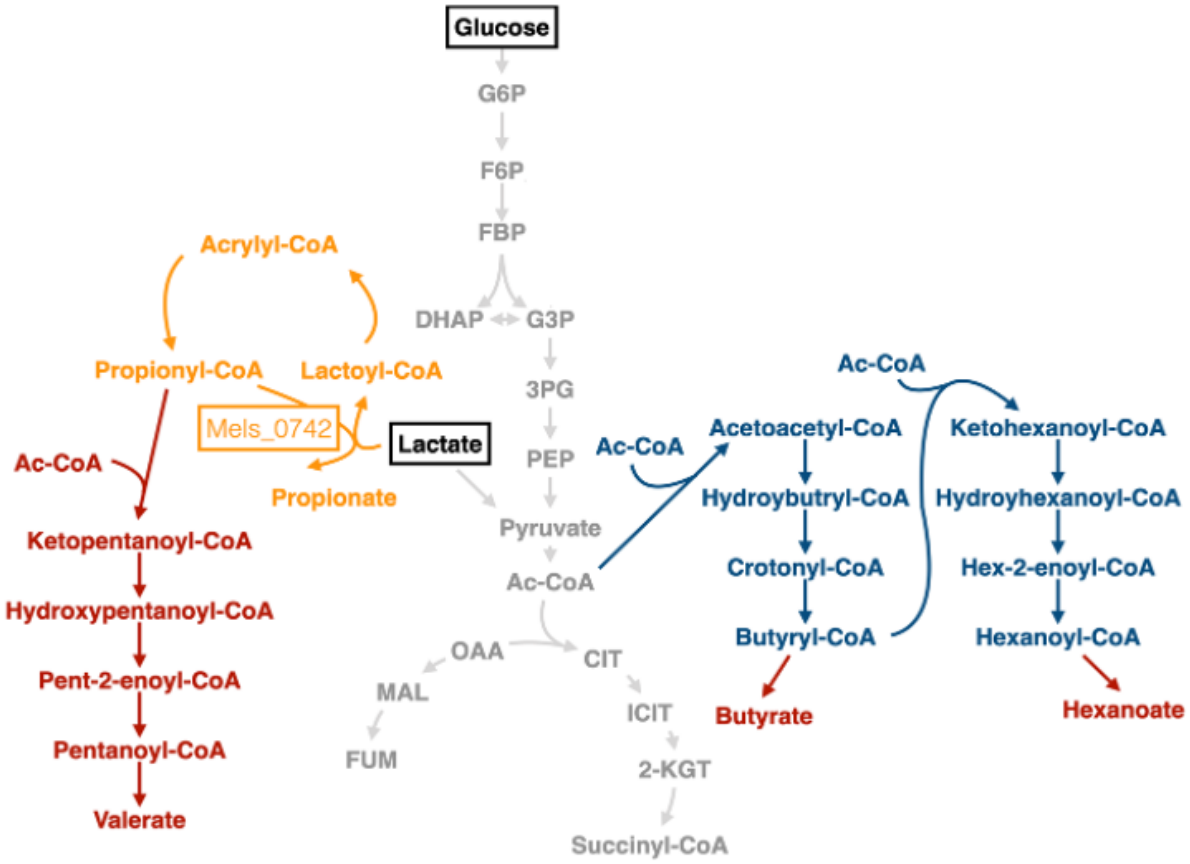

Figure 2.2: Predicted metabolic pathways in *Megasphaera elsdenii.* Gray: glycolysis and branched TCA cycle; Orange: acrylate cycle with the assignment of the Mels_0742 propionyl-CoA transferase; Red: putative chain elongation pathway to delete; Blue: putative chain elongation pathway to retain; Black boxes: growth substrates.

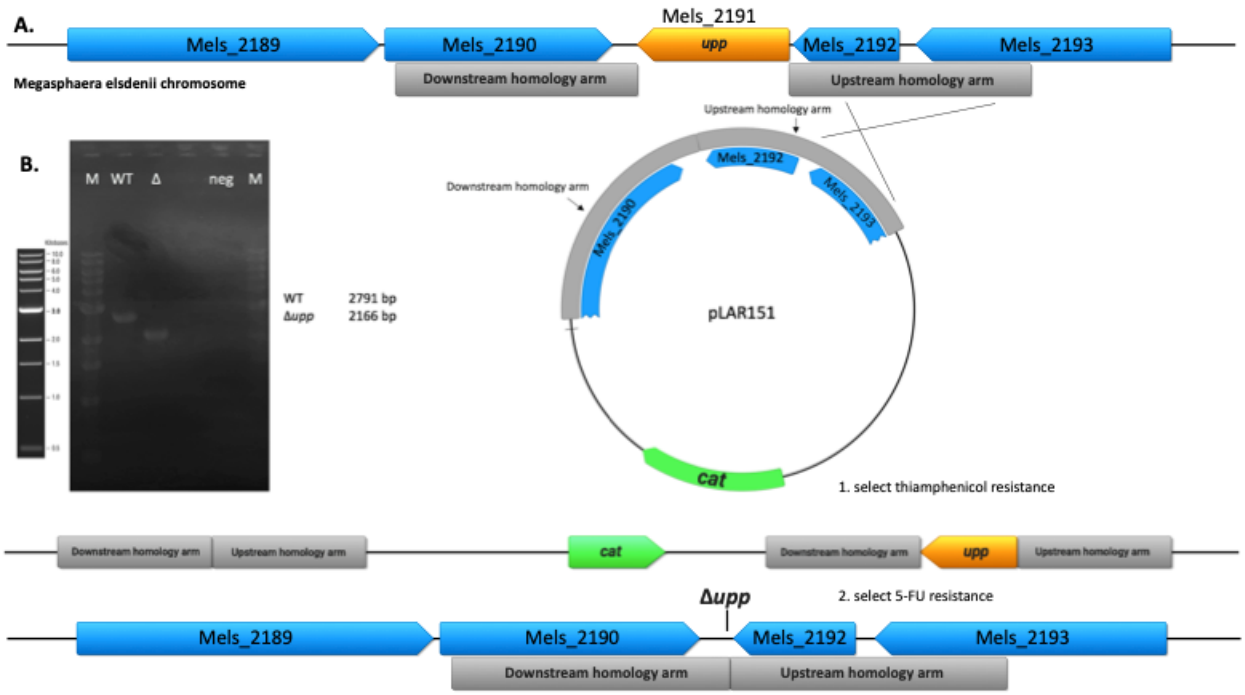

Figure 2.3: Deletion of the *Megasphaera elsdenii uracil phosphoribosyl transferase (upp)* gene. A: depicts the *M. elsdenii* chromosome and pLAR151 with homology to the chromosome, plasmid integration, and the resulting markerless *upp* deletion. B: PCR of *upp* chromosomal region on an agarose gel using primers MR073 and MR074. Mels_2189 O-acetyl homoserine aminocarboxypropyl transferase; Mels_2190 homoserine O-succinyl transferase; Mels_2191 uracil phosphoribosyl transferase; Mels_2192 ribose 5-phosphate isomerase B; Mels_2193 L-threonylcarbamoyladenylate synthase
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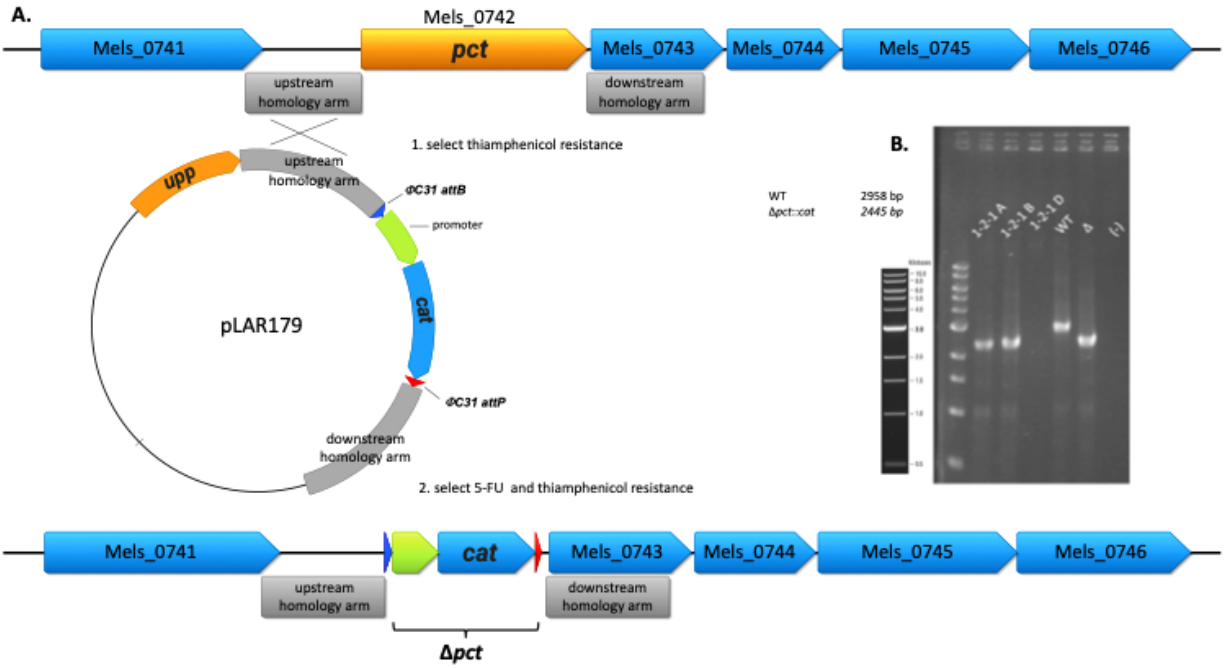

Figure 2.4: Deletion of a putative propionyl-CoA transferase (Mels_0742) in the *M. elsdenii* genome. A. Diagram depicting strategy for replacing propionyl CoA transferase (*pct*) with chloramphenicol transferase (*cat*). **B.** PCR of *pct* chromosomal region using primers NW066 and NW067. Mels_0741 L-lactate permease; Mels_0742 propionyl-CoA transferase (*pct*); Mels_0743 VOC family protein; Mels_0744 acyl-CoA dehydratase activase; Mels_0745 2-hydroxyacyl-CoA dehydratase family protein; Mels_0746 2-hydroxyacyl-CoA dehydratase family protein
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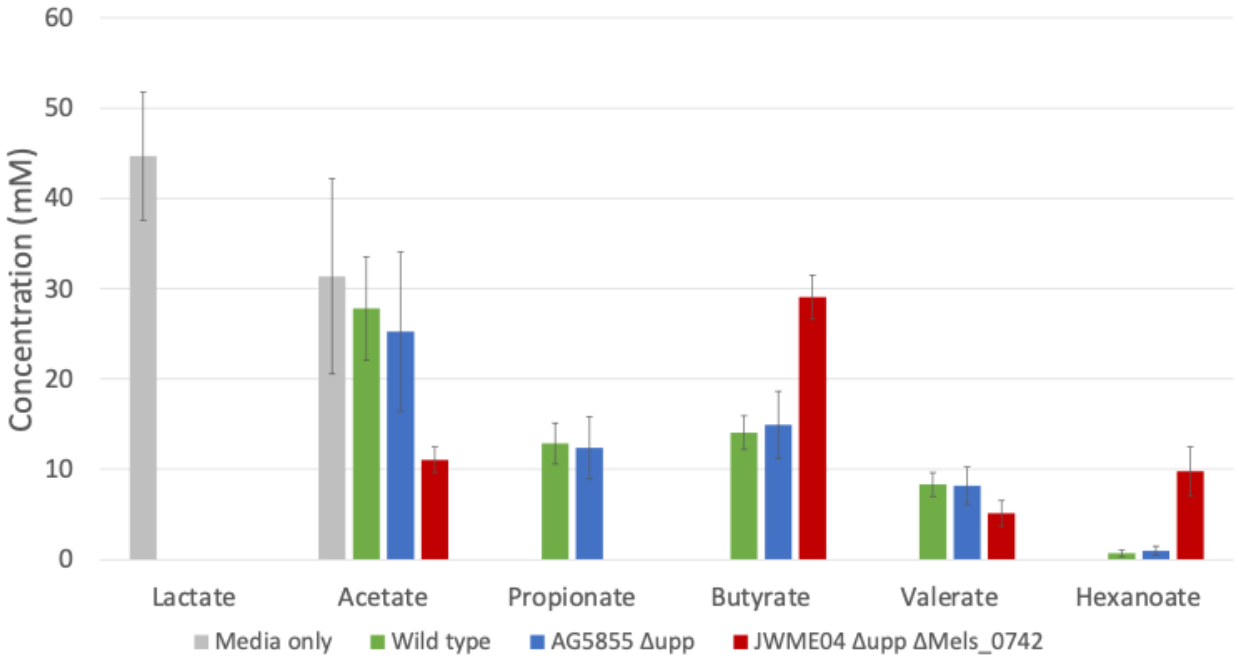

Figure 2.5: Deletion of a putative propionyl-CoA transferase gene resulted in loss of propionate production, decreased valerate production and increased hexanoic acid production. 72-hour cell cultures were pelleted, and supernatant was run on HPLC in triplicate.

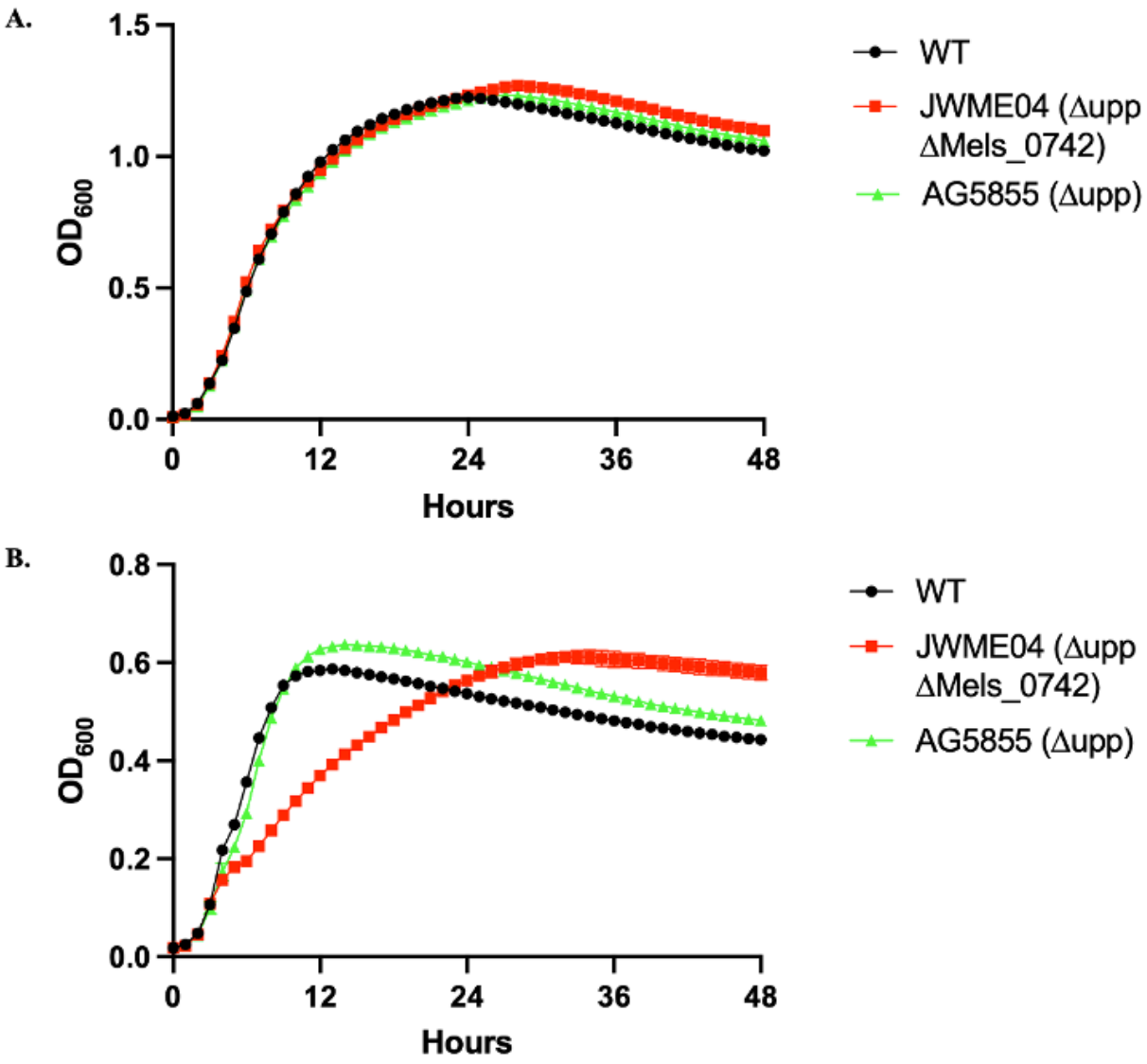

Figure 2.6: *Megasphaera elsdenii* growth curves in rich medium. Cultures grown in triplicate in 96-well plate (orbital shake for 1 minute before each hourly reading) and incubated at 37°C inside an anaerobic chamber. **A.** Growth in rich media with glucose as the sole carbon source. **B.** Growth in rich media with lactate as the sole carbon source. WT: *Megasphaera elsdenii* ATCC 25940 (wild-type). *upp*: uracil phosphoribosyl transferase. *Mels_0742*: *propionyl-CoA transferase* (*pct*)

Table 2.1: Strains and plasmids used in Chapter 2.

Strains/Plasmids	Genotype/Phenotype	Source
E. coli		
AG4157	λtop10 dcm- HK::poly*attB* R4::Mels_0050-51 λ::Mels_1615=16	[Riley 2021]
M. elsdenii		
AG5855	Δ*upp*	this study
JWME04	Δ*upp* Δ*Mels_0742*::ΦC31-*cat*-ΦC31	this study
Plasmids		
pMTL85141	Shuttle vector containing pBC1 ori	[pMTL series]
pLAR151	Mels_2191 (*upp*) deletion vector (Thiamphenicol^R)	this study
pLAR179	Mels_0742 gene replacement vector (Thiamphenicol^R)	this study

Table 2.2: Locus tags used in this study

KEGG locus tags	NCBI locus tags	Gene annotations
Mels_0050	MELS_RS00255	restriction modification system DNA specificity protein type I restriction enzyme, S subunit
Mels_0051	MELS_RS00260	N-6 DNA methylase type I restriction enzyme M protein
Mels_0052	MELS_RS00265	type I restriction enzyme, R subunit
Mels_0464	MELS_RS02450	acetyl-CoA:acetoacetyl-CoA transferase alpha subunit propionate-CoA transferase
Mels_0742	MELS_RS03915	coenzyme A transferase propionate-CoA transferase
Mels_0747	MELS_RS03940	butyryl-CoA dehydrogenase
Mels_0842	MELS_RS04415	orotidine-5'-phosphate decarboxylase (pyrF)
Mels_1130	MELS_RS05920	coenzyme A transferase propionate-CoA transferase
Mels_1615	MELS_RS08375	N-6 DNA methylase type I restriction enzyme M protein
Mels_1616	MELS_RS08380	restriction modification system DNA specificity protein type I restriction enzyme, S subunit
Mels_1617	MELS_RS08385	type I restriction enzyme, R subunit
Mels_1631	MELS_RS08465	coenzyme A transferase propionate-CoA transferase
Mels_2191	MELS_RS11265	uracil phosphoribosyltransferase

CHAPTER 3

DELETION OF *PYRF* PROVIDES A SELECTION AND COUNTERSELECTION FOR STRAIN CONSTRUCTION IN *MEGASPHAERA ELSDENII*

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I. Abstract

The native ability to condense acetyl-CoA groups to efficiently generate C4 to C8 carbon compounds makes *Megasphaera elsdenii* a compelling platform to produce fuels and chemicals from lactate and plant carbohydrates. The long-term objective of this work is to develop *M. elsdenii* as a platform for the conversion of lignocellulosic biomass sugars and organic acids into longer chain alcohols such as hexanol as well as other valuable chemicals. While progress has been made in developing basic genetic tools in this strain, methods for DNA transformation rely on *in vivo* methylation of DNA in a strain of *E. coli* that contains two methyltransferases from *M. elsdenii.* Using that technology, we report the construction of a deletion of *pyrF* that allows counter selection of plasmids containing the wild-type allele. We use this counter-selectable marker in proof of principle experiments to delete each of two methyltransferases in the *M. elsdenii* chromosome.

II. Introduction

The native abilities of non-model bacteria to produce fuels and chemicals at high flux and high yields will be essential for the production of renewable fuels and chemicals. The ability to engineer these organisms relies on the development of facile genetic tools. *Megasphaera elsdenii* is of particular interest because of its ability to efficiently produce medium and longer chain carboxylic acids anaerobically from lactate and glucose at high yield and high titer (66).

Here we constructed a new counter selectable marker, Δ*pyrF*, that will facilitate strain constructions, extending the genetic tools available for engineering *M. elsdenii*. When exposed to 5-fluoroorotic acid (5-FOA), the *pyrE* gene product adds a phosphate group to 5-FOA, converting it into 5'-fluoroorotidine monophosphate, which is then processed by the *pyrF* gene

(118). *pyrF* removes a carboxyl group, resulting in the formation of 5'-fluorouridine monophosphate (5'-FUMP), a toxic analog of uridine monophosphate. This analog inhibits thymidylate synthetase, which prevents the conversion of 5'-fluorouracil to thymidine. The accumulation of this toxic nucleotide disrupts RNA translation, DNA replication, and ultimately leads to cell death. Mutant *pyrF* strains, which are uracil auxotrophs, are resistant to 5-FOA. Selecting uracil prototrophy in defined medium, allows selection for the pyrF gene (119).

We validate the use of this counterselection in the construction of two strains lacking each of one of the restriction enzymes encoded by *M. elsdenii.* Construction of restriction-deficient strains is a tested strategy for increasing transformation efficiencies for many bacteria and we suggest this strategy is especially important for the development of genetic methods in non-model organisms. The data herein confirm the use of *pyrF* as a valuable counter-selectable marker for strain constructions in *M. elsdenii,* based on monitoring the resistance to 5-FOA.

R-M systems are widespread in bacteria and archaea and are well established as a barrier to transformation of DNA from heterologous sources, especially DNA from other genera (120). Almost 90% of bacterial genomes contain R-M systems and 43% contain four or more according to "The Restriction Enzyme Database" (REBASE) (121). R-M systems typically contain pairs of enzymatic activities, a restriction endonuclease and a DNA methyltransferase (122). R-M systems are classified as type I, type II, type IIS, type III and type IV according to enzyme composition, cofactor requirements, recognition sequence symmetry, location of DNA cleavage relative to the recognition site, and mode of action (122). The methyltransferase subunits of R-M systems methylate specific sites in the host DNA preventing cleavage by the cognate restriction endonuclease. Nonmethylated foreign DNA is often cleaved by the host organism [22]. Eliminating restriction endonucleases in a number of host organisms, including *Bacillus subtilis*

(123), *Thermosynechococcus elongatus* (124), *Borrelia afzelii (125)*, *Clostridium acetobutylicum* (126), and *Caldicellulosiruptor bescii* (127) either improved transformation efficiency or, in some cases, allowed DNA transformation to occur at all. In addition, simplified transformation protocols eliminate time-consuming laborious DNA and often inefficient modification steps.

We recently reported methods for efficient DNA transformation of *M. elsdenii*, and the ability to direct marker replacement between non-replicating plasmids and chromosomal genes (128). Restriction of DNA from *E. coli* was found to be a serious, and in some cases an absolute barrier to DNA transformation (102). This barrier but could be overcome by *in vivo* methylation of DNA, which involved passing the relevant plasmid through a strain of *E. coli* that was expressing two methyltransferases from *M. elsdenii* (102). Methylome analysis was performed on *Megasphaera elsdenii* ATCC 25940 using Single Molecule Real-Time sequencing (SMRT) on the PacBio platform and Whole Genome Bisulfite Sequencing (WGBS) using Illumina (102). It was determined that *Megasphaera elsdenii* ATCC 25940 has three active restriction modification systems, two type I systems and one type II system.

Type I systems consist of three polypeptides: a restriction subunit (R), a DNA modification subunit (M), and DNA specificity subunit (S) (129). In contrast, type II systems are comprised of only R and M subunits (129). The type II system was determined to act on the GATC motif which could be methylated by a DAM+ *E. coli* strain (102). Therefore, the M and S subunits from both active type I systems were cloned into *E. coli* strain AG4157 (**Table 3.1**). The corresponding methyltransferases and specificity subunits were expressed in *E. coli* AG4157 and are under the transcriptional control of an inducible arabinose promoter and expression required efficient induction and expression to protect the DNA (102).

III. Results and Discussion

Construction of a *M. elsdenii ΔpyrF* mutant strain. We first tested whether *M. elsdenii* was sensitive to 5-FOA and determined that in Reinforced Clostridium Medium (a complex media likely containing uracil), 1mg/mL and 2.5mg/mL in liquid and solid mediums respectively, were sufficient for inhibiting growth. This indicated that the conversion of 5FOA to 5-fluorouracil occurs at sufficient levels in rich medium to allow selection of resistance.

The *pyrF* gene (Mels_0842) is required for uracil biosynthesis and converts 5-fluoroorotic acid (5-FOA) to the toxic product 5-fluorouracil. Strains deleted for *pyrF* are uracil auxotrophs and resistant to 5FOA. Uracil prototrophy is a useful selectable marker, however it requires a medium without uracil. We have developed a defined medium for *M. elsdenii* that allows the omission of uracil to allow this positive selection.

The region of the *M. elsdenii* chromosome containing the presumed *pyrF* gene is depicted in Figure 3.1A. A vector for targeted deletion of *pyrF* (pMRW003) was constructed by joining 1 kb of the upstream and 1 kb of the downstream region of the *pyrF* open reading frame and lacking the *pyrF* open reading frame itself **(Fig. 3.1B)**. Plasmid pMRW003 also contained a chloramphenicol/thiamphenicol resistance gene (*cat*) for selection in *E. coli* and *M. elsdenii* but no origin of replication for *M. elsdenii.* pMRW003 was transformed into the wild-type strain of *M. elsdenii* and transformants were selected for thiamphenicol resistance. Thiamphenicol resistant colonies were expected to have integrated the plasmid at the *pyrF* locus based on the recombination events schematized in Figure 3.1B. The transformants were plated in medium with 5FOA to counter-select against the presence of the *pyrF* gene. This counter-selection resulted in excision of the plasmid and deletion of the *pyrF* gene in the chromosome (**Fig. 3.1B**). The final deletion was confirmed by PCR. Using primers SS021 and SS022, the *pyrF* region in the wild-type strain produced a 3461 bp band, and as expected, the Δ*pyrF* mutant produced a 2747 bp band (**Fig. 3.1C**)

When inoculated into a defined medium lacking uracil, Modified Amino Acid Medium (MAAD), the Δ*pyrF* mutant strain (JWME03) was found not to grow (**Fig. 3.3**). Additionally, the Δ*pyrF* strain was resistant to 5-FOA when grown in rich media. These results confirmed the predicted function of Mels_0842 and that the appropriate deletion strain had been constructed.

Constructing deletions of the restriction enzyme encoding genes *Mels_0052* and *Mels_1617*. To test whether a deletion of *Mels_0052* or *Mels1617* will alleviate restriction of DNA from *E. coli* in *M. elsdenii* and allow transformation of unmethylated DNA, we constructed a chromosomal deletion of each type II R subunit in JWME03 (Δ*pyrF*) using a targeted marker replacement strategy previously described (**Figure 3.2B, Table 3.1**). The deletion vectors, pMTV486 and pMTV487, contain DNA fragments that include both the 5' (~1000 bp) and 3' (~1000 bp) flanking regions of each restriction enzyme (*Mels_0052* and *Mels_1617* respectively). Each plasmid contains the *cat* resistance gene between the 5' and 3' homology arms (**Fig. 3.2B**) for selection of transformants. The wild-type *pyrF* cassette is also on each plasmid for counter-selection and excision of the plasmid backbone (**Fig. 3.2B**). This non-replicating vector was transformed into JWME03 (*ΔpyrF*) with selection for thiamphenicol resistance followed by an additional counter-selection for 5-fluoroorotic acid (5-FOA) resistance. Initial screening of isolates by PCR revealed merodiploids with a mixture of wild-type and deletion genomes. These were further purified on solid medium containing both thiamphenicol and 5-FOA. Each isolate was further analyzed by PCR amplification of the targeted restriction enzyme gene locus in the chromosome. PCR amplification of each locus from the parent strain JWME03 (*ΔpyrF*) produced the expected wild-type sized bands. PCR amplification from JWME05 (*ΔpyrF ΔMels_1617*) using primers NW130 and MR048 produced a 3403 kb band indicating marker replacement with *chloramphenicol acetyltransferase* (*cat*) (**Fig. 3.2C**). Amplification of JWME06 (*ΔpyrF*

ΔMels_0052) using primers NW152 and NW146 produced a 4640 kb band indicating marker replacement (**Fig. 3.2D**). The site of each deletion will be confirmed by DNA sequence analysis of the PCR product. The resulting strains, JWME05 (*ΔpyrF ΔMels_1617*) and JWME06 (*ΔpyrF ΔMels_0052*) (**Table 3.1**) were used for further analysis.

Growth of these mutants in rich media was not identical to growth of the parent JWME03 or the wild-type strain, however this less robust growth will provide vehicles for further studies (**Fig. 3.4**). These data suggest that loss of the restriction enzymes does not severely compromise cellular fitness. Based on these data, generating a restriction deficient strain is a viable strategy to facilitate further genetic manipulation of *M. elsdenii*.

IV. Methods and Materials

Bacterial strains, media, and culturing conditions: *Megasphaera elsdenii* strains and plasmids were grown under anaerobic conditions at 37°C in liquid Reinforced Clostridial Medium (RCM) and within solid RCM (1.5% agar) supplemented with 5-Fluoroorotic acid (5-FOA) concentrations of 1mg/mL and 2.5mg/mL respectively. Additionally, *M. elsdenii* strains were grown with RCM supplemented with thiamphenicol (5ug/mL). *E. coli* DH5α was used for construction of plasmid DNA and grown in LB liquid or solid medium at 37°C and supplemented with 15μg/mL chloramphenicol. *E. coli* AG4157 was used for preparation of methylated plasmid DNA and grown in LB liquid or solid medium at 37°C and supplemented with 15μg/mL chloramphenicol. Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen) according to manufacturer's protocol. Both *E. coli* DH5α and AG4157 cells were transformed by heat shock at 42°C for 1 minute 30 seconds. *M. elsdenii* cells were transformed by electroporation in 1mm gap cuvettes with a 1200V square wave for 1.5ms and selected for chloramphenicol

resistance. *Megasphaera* chromosomal DNA was extracted using the Quick-gDNA Miniprep (Zymo) as per the manufacturer's instructions.

Construction of the *pyrF* deletion in *M. elsdenii*: pMRW003 was constructed using Q5 High-Fidelity DNA polymerase (New England BioLabs, Ipswich, MA, USA) for PCR, restriction enzymes (New England BioLabs) for digestion, and T4 ligase (New England BioLabs) for sticky-end ligation, all per the manufacturer's instructions. The replicating plasmid for deletion of *pyrF* (Mels_0842) was constructed using *M. elsdenii* ATCC 25940 genomic DNA as a template to amplify both the 5' homologous arm (1,026bp, using primers MR013 and MR014) and the 3' homologous arm (1,026bp, using primers MR015 and MR016). Restriction motifs were added by using the 5' homologous arm PCR product as a template for a subsequent PCR (1,038bp, using primers MR023 w/XmaI and MR017 w/KpnI). Restriction motifs were added by using the 3' homologous arm PCR product as a template for a subsequent PCR (1,038bp, using primers MR018 w/KpnI and MR024 w/XhoI). The products resulting from the two previously described PCRs were digested with KpnI enzyme (NEB) and ligated together with T4 Ligase (NEB), both per the manufacturer's protocol. The resulting ligation product was amplified by PCR with primers MR023 (XmaI) and MR024 (XhoI). Plasmid pMTL85141 was used as a template for PCR (2,669bp) using primers MR021 (XhoI) and MR022 (XmaI), each primer adding a restriction motif to the sequence. Both templates resulting from PCRs using primers MR023/MR024 and MR021/MR022 were each used in a double digest reaction using XhoI and XmaI restriction enzymes (NEB) as per the manufacturer's instructions. The resulting digestion products were ligated with T4 ligase, per instructions. The resulting ligation product (pMRW003) was amplified via transformation into *E. coli* DH5α, generating *E. coli* JW650. pMRW003 was isolated from *E. coli* JW650 and used to transform AG4157, generating *E. coli* JW651. JW651 was grown in LB

supplemented with 1mM arabinose to produce methylated pMRW003. Plasmid DNA was isolated from *E. coli* JW651 and used to transform *M. elsdenii* wild-type. Electroporation was performed with 20uL of competent cells and 2uL of plasmid DNA. After recovering for 4 hours in RCM, cells were plated within RCM supplemented with thiamphenicol. The *M. elsdenii* cells capable of growing in the presence of thiamphenicol were isolated and grown in liquid RCM supplemented with 1mg/mL 5-FOA. The resulting strain, JWME03 (Δ*pyrF*), was confirmed using PCR. Wild-type and JWME03 were amplified using primers SS021 and SS022. The wild-type strain produced the expected band size of 3461 bp, while the mutant strain, JWME03, produced a band size of 2747 bp (**Fig. 3.1)**.

Deletion and complementation of *orotidine-5'-phosphate decarboxylase* (*pyrF*) in *M. elsdenii* in media without uracil. *pyrE* encoding orotate phosphoribosyltransferase (EC 2.4.2.10) is located 1 ORF and 720bp downstream of *pyrF*. In the presence of 5-FOA, *pyrE* adds a phosphate group via phosphoribosyl pyrophosphate to 5-FOA becoming 5'-fluoroorotidine monophosphate and the substrate for the *pyrF* gene. *pyrF* cleaves the carboxylic group from 5'-fluoroorotidine monophosphate creating 5'-fluorouridine monophosphate (5'-FUMP), a toxic analog of uridine monophosphate and a precursor to both the RNA nucleotide uracil and the DNA nucleotide thymine. The addition of fluorine at the 5' carbon becomes a lethal inhibitor to thymidylate synthetase, preventing the methylation and conversion of 5'-fluorouracil to thymidine.[A] These toxic nucleotide analogs prevent the translation of RNA, the replication of DNA, and ultimately cause cell death. Nonconservative *pyrF* mutants are uracil auxotrophs and resistant to 5-FOA. We have established 5-FOA MICs in Reinforced clostridial medium (RCM), a rich complex media, at 1mg/mL and 2.5mg/mL in liquid and solid mediums respectively. 5-FOA allows for counter-selection with sufficient uracil present in the growth medium.

Plasmids containing a wild-type *pyrF* allele from Clostridium spp. being driven by different promoters known to function in *M. elsdenii* were synthesized and tested for growth in MAAD media lacking uracil. Plasmid pMTV2203, with the Mels_2121 promoter was successful in complementation of the *pyrF* deletion, restoring uracil prototrophy (**Fig 3.3**). To investigate the complementation of *pyrF*, we developed a defined medium without uracil - Modified Amino Acid Defined medium (MAAD). The growth curve in MAAD medium was performed in an Agilent BioTek Epoch 2 plate reader placed within a Coy Labs anaerobic chamber with gas mix specified at 85% nitrogen, 10% carbon dioxide, and 5% hydrogen. The growth curve (**Fig. 3.3**) illustrates the *pyrF* mutant's inability to grow in the absence of uracil and the complementation of uracil auxotrophy in JWME14 (Δ*pyrF* + pMTV2203).

V. Discussion

In this study we have generated a *pyrF* mutant strain that has both selection and counterselection potential. As such this work provides the foundation for future efforts to generate a restriction negative strain of *M. elsdenii*. Further work will be performed to determine whether the deletions of the *Megasphaera elsdenii* native restriction enzymes, whether individually or in combination, removes a substantial barrier to routine transformation and chromosomal modification of *M. elsdenii* for both functional analyses of genes as well as metabolic engineering for the production of biofuels and bioproducts from biomass.

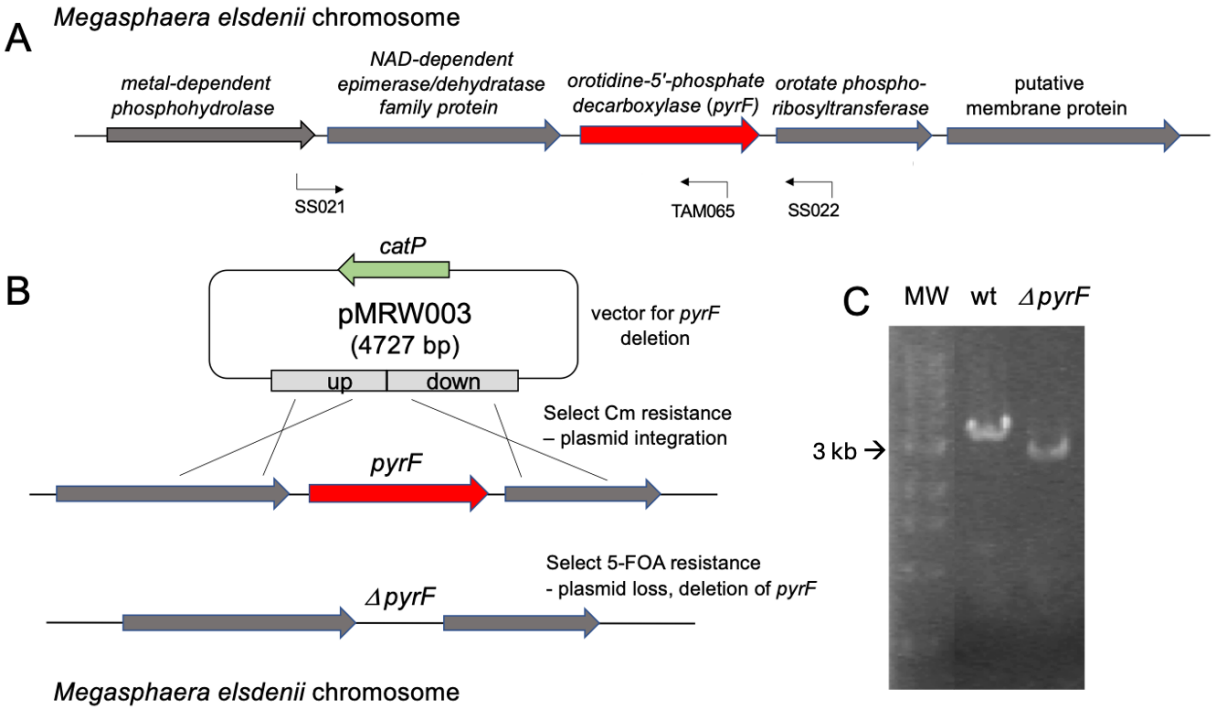

Figure 3.1: Deletion strategy for *pyrF* mutant. A. Illustration of *M. elsdenii* chromosomal *pyrF* region. **B.** Illustration of *pyrF* deletion vector (pMRW003) and illustration of a *pyrF* deletion via homologous recombination and curing of the plasmid. **C.** Agarose gel from PCR with primers SS021 and SS022. The expected WT band size is 3461 bp and the expected band size for the Δ*pyrF* mutant is 2747 bp.

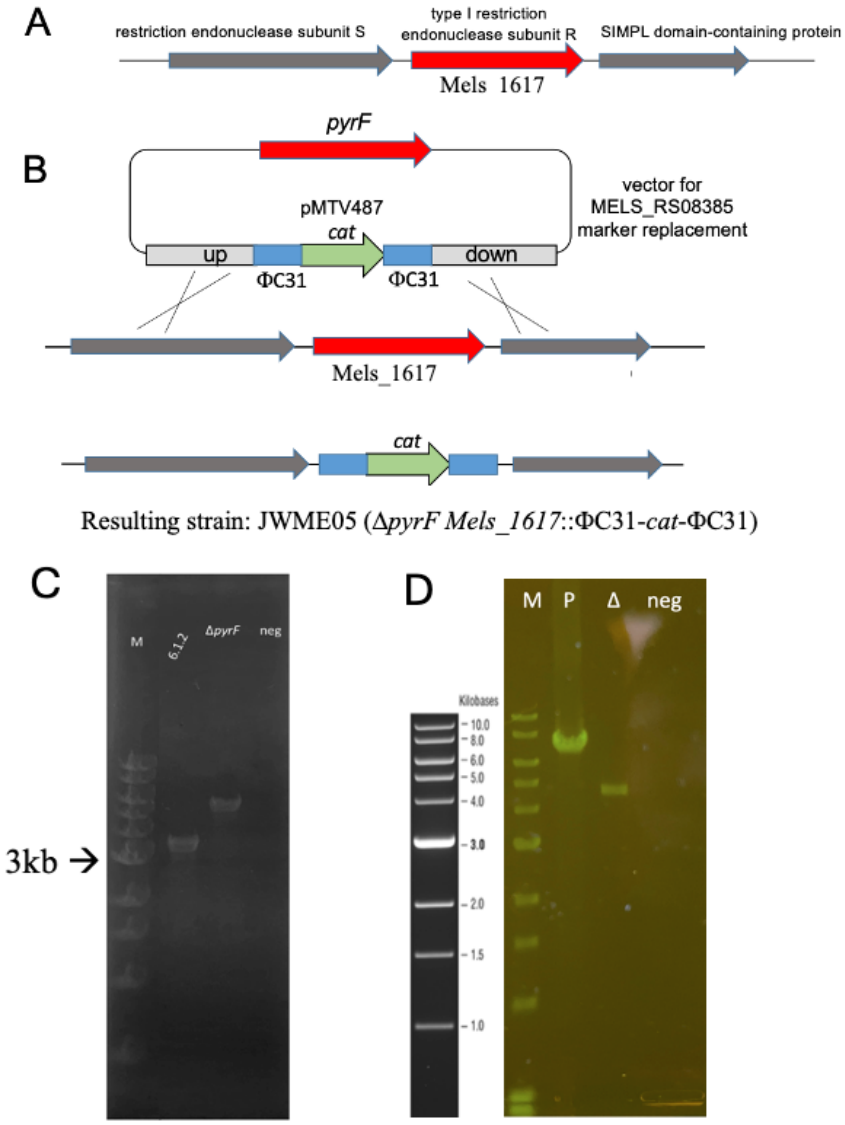

Figure 3.2: Marker replacement strategy for two restriction endonuclease subunits R (Mels_1617 [shown] and Mels_0052). A. Illustration of *M. elsdenii* chromosome. **B.** Illustration of restriction enzyme deletion vector and homologous recombination strategy for marker replacement. **C.** Agarose gel with PCR products of recombinant chromosome. 6.1.2 is the final isolate of the clean JWME05 strain after 3 rounds of selection/counter-selection. Expected band size for parent strain (Δ*pyrF*) – 5404 bp. Expected band size for marker replacement JWME05 (Δ*pyrF Mels_1617*::ΦC31-*cat*-ΦC31) – 3403 bp. **D.** Agarose gel with PCR products of recombinant chromosome. Δ indicates the final isolate of the clean JWME06 strain after 3 rounds of selection/counter-selection. Expected band size for parent strain (Δ*pyrF*) – 6638 bp. Expected band size for marker replacement JWME05 (Δ*pyrF Mels_1617*::ΦC31-*cat*-ΦC31) – 4640 bp.

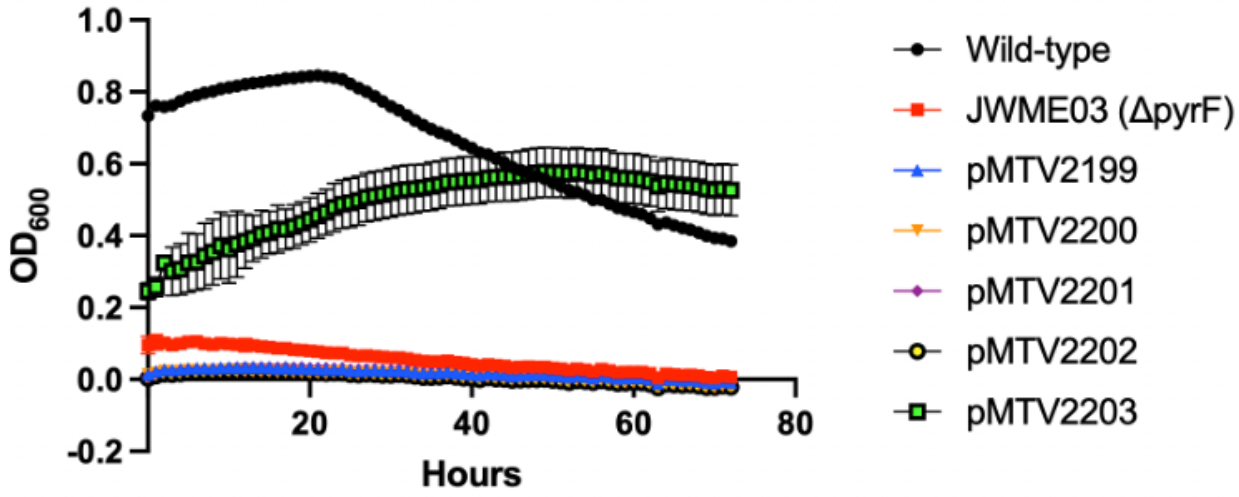

Figure 3.3: Growth curve in defined medium without uracil. Each strain was grown in triplicate in a 96-well plate reader at 37°C inside an anaerobic chamber. pMTV2199-2203 are plasmids with a wild-type *pyrF* driven by different promoters.

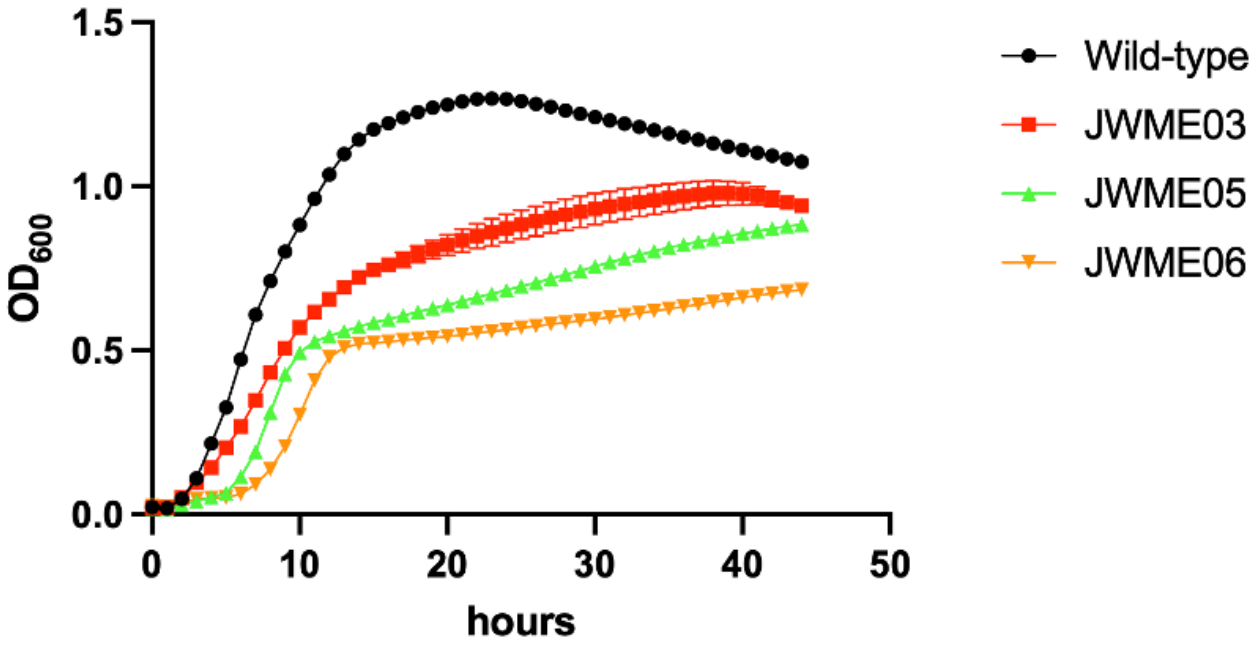

Figure 3.4 *Megasphaera elsdenii* growth curves in rich medium with glucose as sole carbon source. Cultures grown in triplicate in 96-well plate (orbital shake for 1 minute before each hourly reading) and incubated at 37°C inside an anaerobic chamber. JWME03 (Δ*pyrF*), JWME05 (Δ*pyrF Mels_1617*:: ::ΦC31-*cat*-ΦC31, JWME06 (Δ*pyrF Mels_0052* ::ΦC31-*cat*-ΦC31)

Table 3.1: Strains and plasmids used in Chapter 3.

Strains/Plasmids	Genotype/Phenotype	Source
E. coli		
DH5α	F⁻ φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_K^-, m_K^+) *pho*A *sup*E44 λ⁻ *thi*-1 *gyr*A96 *rel*A1	ThermoFisher
AG4157	λtop10 dcm- HK::poly*attB* R4::Mels_0050-51 λ::Mels_1615-16	Guss lab
JW650	DH5α containing pMRW003 (Chloramphenicol^R)	this study
JW651	AG4157 containing pMRW003 (Chloramphenicol^R)	this study
JW711	DH5α containing pMTL85141 (Chloramphenicol^R)	this study
JW712	AG4157 containing pMTL85141 (Chloramphenicol^R)	this study
M. elsdenii		
JWME01	*Megasphaera elsdenii* ATCC 25940	Guss lab
JWME03	Δ*pyrF* (ura$^-$/5-FOA^R)	this study
JWME05	Δ*pyrF* Δ*Mels_1617*::ΦC31-*cat*-ΦC31 (ura$^-$/5-FOA^R) (Chloramphenicol^R)	this study
JWME06	Δ*pyrF* Δ*Mels_0052*::ΦC31-*cat*-ΦC31 (ura$^-$/5-FOA^R) (Chloramphenicol^R)	this study
JWME10	Δ*pyrF* containing pMTV2199 (ura$^-$/5-FOA^R) (Chloramphenicol^R)	this study
JWME11	Δ*pyrF* containing pMTV2200 (ura$^-$/5-FOA^R) (Chloramphenicol^R)	this study
JWME12	Δ*pyrF* containing pMTV2201 (ura$^-$/5-FOA^R) (Chloramphenicol^R)	this study
JWME13	Δ*pyrF* containing pMTV2202 (ura$^-$/5-FOA^R) (Chloramphenicol^R)	this study
JWME14	Δ*pyrF* containing pMTV2203 (ura$^-$/5-FOA^R) (Chloramphenicol^R)	this study
Plasmids		
pMTL85141	Shuttle vector containing pBC1 ori *repL ColE1-RNA-II catP LacZa* (Chloramphenicol^R)	[pMTL series]
pMRW003	Mels_0842 (*pyrF*) deletion vector (Thiamphenicol^R) based on pMTL85141	this study
pMTV2199	*pyrF* complement with Clo1313_1194 promoter	this study
pMTV2200	*pyrF* complement with Clo1313_gapDH promoter	this study
pMTV2201	*pyrF* complement with Clju-ferrodoxin promoter	this study
pMTV2202	*pyrF* complement with Cauto-ferrodoxin promoter	this study
pMTV2203	*pyrF* complement with Mels_2121 promoter	this study

CHAPTER 4

SUMMARY AND FUTURE DIRECTIONS

Microbes have been utilized in various applied processes for millennia, long before a scientific understanding of their characteristics and capabilities emerged. Fermentation, one of the earliest applications, was used by ancient cultures to create wine, beer, and bread around 7000 BCE, with yeasts like *Saccharomyces cerevisiae* playing a key role in these processes (1–3). The ancient Egyptians also utilized bacteria, including Lactobacillus and Streptococcus, for brewing, bread-making, yogurt, and cheese production (4). In total, these processes and applications can be thought of as laying the foundation for the field of biotechnology we are familiar with today.

The 17th century brought advancements in microscopy through Antonie van Leeuwenhoek, who first observed microorganisms and coined the term "animalcules" (6). The 19th century saw Louis Pasteur's groundbreaking work, which linked microbes to fermentation and led to the development of pasteurization (7,8). Alongside Robert Koch, Pasteur's contributions established medical microbiology by associating specific diseases with specific microbes (9).

In the latter half of the 19th century, applied microbiology expanded into agriculture, improving soil fertility through nitrogen-fixing bacteria, and advancing industrial processes via enzymes for antibiotic and pharmaceutical production (10). Research also explored the production biofuels using bacterial processes. These studies led to successfully integrating ethanol from corn to reduce reliance on petroleum.

Today, microbiology and metabolic engineering are promising fields, specifically as they focus on developing petroleum-free energy alternatives and environmentally safe bioremediation

processes. As we discover new applications, we enter a transformative era of large-scale industrial biotechnology, one that builds upon ancient practices while implementing modern scientific methods.

Biofuels are liquid fuels derived from renewable biological sources, primarily plants. Biofuels date back to when humans first burned wood for energy. Ethanol was the main fuel for lamps until the 1850s. In 1895 Rudolf Diesel developed an engine that could run on peanut oil, highlighting the potential of vegetable oils (16). Henry Ford also envisioned biofuels in transportation, designing the Model T to run on ethanol (17). However, the rise of inexpensive fossil fuels in the early 20th century diminished interest in biofuels. Events like the World Wars and the 1970s oil crisis revived the focus on biofuels due to rising fuel prices and concerns over petroleum supply (18).

In the 21st century, there is renewed interest in biofuels as a sustainable alternative to fossil fuels, driven by the need to reduce dependence on non-renewable energy and address greenhouse gas emissions (130). Current biofuel research aims to improve fuel efficiency and economic viability while offering environmental benefits and enhancing national security by promoting energy sources within national borders (131).

For biofuels to be effective and usable, they must meet key requirements, including specific physical and chemical properties, have a minimal environmental impact, prove to be economically feasible, and meet regulatory compliance (53). Most importantly, biofuels must have an energy density high enough to effectively replace fossil fuels in their many applications. A requirement for longer carbon chain alcohols creates a cost barrier. Few organisms have the synthetic ability to generate long-chain alcohols, and bioengineering has often sought to bring pathways in and/or create hybrid pathways.

The unique metabolic chemistry of *Megasphaera elsdenii* offers solutions to cost and energy challenges faced with other microbial systems. The native capability of this organisms to condense acetyl-CoA groups enables the efficient production of C4 to C8 compounds, makes it an attractive platform for generating fuels and chemicals from lactate and plant carbohydrates (66).

Megasphaera elsdenii produces organic acids, primarily butyric, hexanoic, and occasionally octanoic acids when fermenting lactate and glucose (66). These acids are generated through chain elongation using acetyl-CoA, similar to pathways in Clostridium species. As the carbon chain length increases, biofuel properties improve—hexanol, in particular, offers higher energy density and lower hygroscopicity than butanol and ethanol. This makes hexanol a promising candidate for next-generation gasoline blends and bio jet fuel. To realize this potential, *M. elsdenii* must be developed as a bioengineering platform, leveraging both its native and engineered biochemical capabilities to efficiently produce hexanol at high yields.

The acrylate pathway in microorganisms converts simple carbon sources, like sugars or organic acids, into acrylate through a series of enzymatic reactions (100). It begins with the conversion of substrates to propionyl-CoA, followed by the formation of propionate and its oxidation to acrylate. This pathway offers a sustainable alternative to traditional petrochemical methods for acrylate production, utilizing renewable biomass.

Various microorganisms, such as genetically modified strains of Clostridium and *E. coli*, have been engineered to enhance acrylate yields (71). However, challenges remain, including efficient substrate utilization and the managing of by-products. Research is focused on optimizing enzyme performance and scaling up production to make microbial acrylate production commercially viable.

In contrast, the goal of the current study was to disrupt or eliminate the acrylate pathway in *Megasphaera elsdenii*, which will eliminate the production of propionate and valerate. This alteration aimed to redirect metabolic flux to produce longer-chain fatty acids using acyl-CoA transferases, with a focus on achieving specific long-chain fatty acid profiles.

Propionate-CoA transferases are bidirectional enzymes that facilitate the transfer of Coenzyme A (CoA) between propionate and other derivatives, converting propionate into propionyl-CoA and the reverse reaction. This reaction is vital for various metabolic processes, including fatty acid catabolism, energy production, and the synthesis of biofuels and industrial chemicals, particularly in microorganisms like Clostridium (72).

These enzymes help maintain cellular energy balance and metabolic flux, playing a significant role in fermentation processes (75,76). Engineering propionate-CoA transferases for enhanced activity and specificity holds promise for improving bioproduction efficiency from renewable sources, such as lignocellulosic biomass.

Challenges include achieving high enzyme activity and stability in industrial settings, which can be addressed through advances in enzyme engineering. The ongoing research aims to optimize these enzymes and understand their metabolic roles, potentially leading to more efficient biocatalysts for sustainable chemical and fuel production.

Our current hypothesis suggests that deleting the gene for propionyl-CoA transferase (Mels_0742) in *Megasphaera* will compel the organism to use other acetyl-CoA transferases, ultimately promoting the production of longer-chain fatty acids to maintain redox balance.

In Chapter 2, I described the promise of using *Megasphaera elsdenii* for the production of fuels and chemicals due to its native ability to condense acetyl-CoA groups into C4 to C8

compounds. When grown on lactate and glucose, *M. elsdenii* generates medium to long-chain organic acids, including butyric, hexanoic, and occasionally octanoic acids.

To facilitate genetic manipulation, we deleted the uracil phosphoribosyltransferase gene (upp), which confers resistance to the toxic compound 5-fluorouracil (5-FU), allowing for the selection of transformants. This genetic background was then used to delete the putative propionyl-CoA transferase gene (Mels_0742), leading to the production of longer-chain fermentation products from lactate. These longer carbon chain compounds can improve the efficiency of condensation reactions to generate C14-C16 compounds, making them attractive targets for aviation fuel, biopolymers, and next-generation gasoline blends beyond ethanol.

In Chapter 3, I described the progress I made in establishing genetic tools for *Megasphaera elsdenii*, specifically targeting barriers to transformation that are generated by restriction/modification systems. Current methods for DNA transformation rely on in vivo methylation of the relevant DNA by passing it through a specialized *E. coli* strain containing two *M. elsdenii* methyltransferases. As an initial step in deleting the methyltransferase enzymes in *M. elsdenii* I constructed a deletion of the *pyrF* gene in *M. elsdenii.* This mutation enabled the counter-selection of plasmids with the wild-type allele of *pyrF* using 5-FOA, a toxic analog of orotic acid. This counter-selectable marker was used in experiments to delete two methyltransferases from the *M. elsdenii* chromosome. Continuing experiments are required to determine the transformation efficiency of strains lacking one of the methyltransferases. Additional genetic manipulations are being implemented to construct the appropriate strain lacking both methyltransferases. This is the strain that will serve as a vehicle to generate further engineered strains in our effort to exploit *M. elsdenii*'s ability to produce medium to long-chain organic acids.

In total, the continuation of the research described in this dissertation focuses on understanding the genetic and metabolic pathways of *Megasphaera elsdenii*, a bacterium with potential applications for producing valuable organic acids. The gene Mels_0742, which is part of a putative operon related to lactate utilization and the acrylate cycle, was initially suspected of being involved in propionic or valeric acid biosynthesis during growth on lactic acid. However, when the Mels_0742 gene was deleted (JWME04), the strain still grew on lactic acid but produced no propionic acid and less valeric acid than the wild-type strain. Interestingly, it increased acetic acid consumption and produced more hexanoic acid. This finding indicates that *M. elsdenii*'s metabolism is more complex than initially thought, revealing the potential for future metabolic engineering to optimize the production of desired products.

Efforts to explore these results further will involve systematically deleting genes involved in lactic acid utilization and fermentation product formation. Such genes include those involved in substrate uptake, the acrylate pathway, chain elongation, in addition altering the lactate dehydrogenases, and CoA transferases encoded by *M. elsdenii*. Manipulating *M. elsdenii* in this way will result in a better understanding how metabolic flux can be directed toward the production of specific compounds.

Further, *M. elsdenii* encodes several homologs of genes involved in the Clostridial butyrate/butanol production pathway. This finding raises the possibility that the bacterium may have dedicated biosynthetic pathways for producing various fatty acids like butyric, valeric, and hexanoic acids. I hypothesize that this bacterium uses different pathways for each of these acids that are similar in structure. This hypothesis can be tested by deleting genes in the putative chain elongation pathways and monitoring the disruption in carbon flux to various products.

The genome of *M. elsdenii* encodes five lactate dehydrogenases that catalyze the interconversion of pyruvate and lactate, which are key for either lactic acid production or consumption. Deleting these genes individually or in combination, will provide a better understanding of the pathways involved in lactic acid catabolism. These deletion strains can then be screened for successful gene removal through PCR and whole genome sequencing, followed by characterization in growth tests on glucose or lactic acid as the sole carbon source. Substrate consumption and fermentation products can be quantified using High Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC) similar to the process described in Chapter 2.

It is possible that some CoA transferases are functionally redundant, meaning that deleting one gene may not have a detectable phenotype. To explore this, we will create strains with multiple deletions, utilizing recombination techniques to remove selectable markers and stack mutations. This approach will allow the investigation of metabolic redundancy and will be helped by the genetic tools developed in this dissertation work.

Beyond carbon metabolism, the study acknowledges the role of electron transport pathways in balancing redox reactions, which are critical for controlling flux to different fatty acids. *M. elsdenii* encodes three putative hydrogenases and a putative Rnf complex, which could impact redox balance and affect acetic acid consumption. Genes involved in these processes should be deleted to explore their role in metabolic processes and the formation of fermentation products. An additional approach is to overexpress genes that impact electron carrier pools, such as transhydrogenases, to further understand the role of electron flux in the relevant metabolic remodeling.

It is of interest to examine whether butyric acid can contribute to carbon chain extension, potentially increasing the production of hexanoic and octanoic acids. This idea can be tested in 1-liter fermenters under controlled environmental conditions, where different growth-limiting factors (e.g., carbon or nitrogen sources) are manipulated to observe their effects on product distribution. The use of ^{13}C-labeled acetic or butyric acids to trace carbon flux can help refine our understanding of carbon and electron metabolism.

Benchtop fermentation units are an attractive system to test hypotheses related to *M. elsdenii*'s metabolic pathways, enabling real-time monitoring and control of growth conditions like temperature, pH, and oxygen levels. These systems will be used in combination with transcriptomics (RNA sequencing) to explore the impact of organic acid supplementation, such as acetic and butyric acids, on *M. elsdenii*'s metabolic profile. Transcriptomic analysis can also be employed to understand how perturbations in redox metabolism, such as changes in electron availability, affect fermentation product ratios.

To accelerate the genetic engineering of *M. elsdenii*, an ongoing collaboration with Adam Guss at ORNL will continue. Efforts included in this collaboration will employ a method called Serine recombinase-Assisted Genome Engineering (SAGE). This technique, which enables site-specific DNA integration, has been adapted for use in *M. elsdenii* and allows the integration of heterologous plasmid DNA into the chromosome more efficiently than traditional homologous recombination methods. SAGE can also be used to quickly characterize libraries of genetic parts (e.g., promoters and terminators), aiding in metabolic engineering efforts.

Furthermore, use of temperature-sensitive plasmid origins of replication and counter-selectable markers to enable the creation of markerless chromosomal modifications is a valuable direction to explore. This approach could allow more precise genetic manipulation, including

stacking mutations and removing antibiotic resistance genes. Together, these advanced tools will facilitate the development of *M. elsdenii* as a platform for the high-yield production of fatty acids and other valuable compounds derived from the chain elongation pathway.

In summary, the future for the project initiated with this dissertation work will be efforts to deeply investigate the genetic and metabolic processes in *M. elsdenii*, utilizing advanced genetic techniques and fermentation systems to optimize the production of valuable long-chain organic acids. The work will contribute to a better understanding of metabolic pathways and redox balance in *M. elsdenii* and may lead to the development of a robust platform for the sustainable production of biofuel components and bio-based chemicals.

REFERENCES

1. Samuel D. Investigation of Ancient Egyptian Baking and Brewing Methods by Correlative Microscopy. Science. 1996 Jul 26;273(5274):488–90.

2. McGovern PE, Glusker DL, Exner LJ, Voigt MM. Neolithic resinated wine. Nature. 1996 Jun;381(6582):480–1.

3. McGovern PE, Zhang J, Tang J, Zhang Z, Hall GR, Moreau RA, et al. Fermented beverages of pre- and proto-historic China [Internet]. [cited 2025 Apr 1]. Available from: https://www.pnas.org/doi/10.1073/pnas.0407921102

4. Kiple KF, Ornelas KC. The Cambridge World History of Food. Cambridge University Press; 2000. 1068 p.

5. McGovern PE. Uncorking the Past: The Quest for Wine, Beer, and Other Alcoholic Beverages. University of California Press; 2009. 357 p.

6. Jessup AJC, Coroneo MT. Examining the fabric of the eye: Antoni van Leeuwenhoek, the draper and ocular microscopist. Survey of Ophthalmology [Internet]. 2024 Aug 29 [cited 2025 Apr 1]; Available from: https://www.sciencedirect.com/science/article/pii/S0039625724000985

7. Schwartz M. The life and works of Louis Pasteur. Journal of Applied Microbiology. 2001;91(4):597–601.

8. Fleming A. Louis Pasteur. Br Med J. 1947 Apr 19;1(4502):517–22.

9. Gossel PP. Pasteur, Koch and American Bacteriology. History and Philosophy of the Life Sciences. 2000;22(1):81–100.

10. Elmerich C. One Hundred Years Discovery of Nitrogen-Fixing Rhizobacteria. In: Biological Nitrogen Fixation [Internet]. John Wiley & Sons, Ltd; 2015 [cited 2025 Apr 1]. p. 897–912. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1002/9781119053095.ch88

11. Buchholz K, Bornscheuer UT. Enzyme Technology: History and Current Trends. In: Applied Bioengineering [Internet]. John Wiley & Sons, Ltd; 2017 [cited 2025 Apr 1]. p. 11–46. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1002/9783527800599.ch2

12. Shapouri H, Duffield J, Wang M. The Energy Balance of Corn Ethanol Revisited. Transactions of the American Society of Agricultural Engineers. 2003 Jul 1;46:959–68.

13. Wang M, Saricks C, Wu M. Fuel-Cycle Fossil Energy Use and Greenhouse Gas Emissions of Fuel Ethanol Produced from U.S. Midwest Corn [Internet]. 1997 Dec [cited 2025 Apr 1] p. 1218367. Report No.: 1218367. Available from: http://www.osti.gov/servlets/purl/1218367/

14. Saeed MU, Hussain N, Sumrin A, Shahbaz A, Noor S, Bilal M, et al. Microbial bioremediation strategies with wastewater treatment potentialities – A review. Science of The Total Environment. 2022 Apr 20;818:151754.

15. Bhandari S, Poudel DK, Marahatha R, Dawadi S, Khadayat K, Phuyal S, et al. Microbial Enzymes Used in Bioremediation. Journal of Chemistry. 2021;2021(1):8849512.

16. Suresh S, Kumar A, Shukla A, Singh R, Krishna CM, editors. Biofuels and Bioenergy (BICE2016) [Internet]. Cham: Springer International Publishing; 2017 [cited 2025 Apr 1]. (Springer Proceedings in Energy). Available from: http://link.springer.com/10.1007/978-3-319-47257-7

17. Kovarik B. Henry Ford, Charles F. Kettering and the fuel of the future. Automotive History Review. 1998;32:7–27.

18. Hamilton JD. Historical Oil Shocks. National Bureau of Economic Research [Internet]. 2011; Available from: http://www.nber.org/papers/w16790

19. Tirado MC, Cohen MJ, Aberman N, Meerman J, Thompson B. Addressing the challenges of climate change and biofuel production for food and nutrition security. Food Research International. 2010 Aug 1;43(7):1729–44.

20. Huang WD, Zhang YHP. Energy Efficiency Analysis: Biomass-to-Wheel Efficiency Related with Biofuels Production, Fuel Distribution, and Powertrain Systems. Saks V, editor. PLoS ONE. 2011 Jul 13;6(7):e22113.

21. Felix E, Cardona CA, Quintero J. technIcal and economIc vIabIlIty of bIofuel productIon chaIns. 2010 Jan 7;

22. Lippke B, Puettmann ME, Johnson L, Gustafson R, Venditti R, Steele P, et al. Carbon Emission Reduction Impacts from Alternative Biofuels*. Forest Products Journal. 2012 Jul;62(4):296–304.

23. Mara D, Nate S, Stavytskyy A, Kharlamova G. The Place of Energy Security in the National Security Framework: An Assessment Approach. Energies. 2022 Jan;15(2):658.

24. Pettersson K, Grahn M. How much can biofuels reduce greenhouse gas emissions?

25. Knothe G. Biodiesel and renewable diesel: A comparison. Progress in Energy and Combustion Science. 2010 Jun 1;36(3):364–73.

26. Sheehan J, Camobreco V, Duffield J, Graboski M, Shapouri H. An Overview of Biodiesel and Petroleum Diesel Life Cycles.

27. Østergaard PA. Comparing electricity, heat and biogas storages' impacts on renewable energy integration. Energy. 2012 Jan 1;37(1):255–62.

28. Martins das Neves LC, Converti A, Vessoni Penna TC. Biogas Production: New Trends for Alternative Energy Sources in Rural and Urban Zones. Chemical Engineering & Technology. 2009;32(8):1147–53.

29. Themelis NJ, Ulloa PA. Methane generation in landfills. Renewable Energy. 2007 Jun 1;32(7):1243–57.

30. Luque R. Algal biofuels: the eternal promise? Energy Environ Sci. 2010;3(3):254.

31. Chisti Y. Biodiesel from microalgae. Biotechnology Advances. 2007 May 1;25(3):294–306.

32. Wise M, Muratori M, Kyle P. Biojet fuels and emissions mitigation in aviation: An integrated assessment modeling analysis. Transportation Research Part D: Transport and Environment. 2017 May;52:244–53.

33. Mortier RM, Fox MF, Orszulik ST, editors. Chemistry and Technology of Lubricants [Internet]. Dordrecht: Springer Netherlands; 2010 [cited 2025 Apr 1]. Available from: http://link.springer.com/10.1007/978-1-4020-8662-5

34. Searchinger T, Heimlich R, Houghton RA, Dong F, Elobeid A, Fabiosa J, et al. Use of U.S. Croplands for Biofuels Increases Greenhouse Gases Through Emissions from Land-Use Change. Science. 2008 Feb 29;319(5867):1238–40.

35. Singh DP, Trivedi RK. Biofuel from Wastes an Economic and Environmentally Feasible Resource. Energy Procedia. 2014;54:634–41.

36. Kim S, Dale BE, Martinez-Feria R, Basso B, Thelen K, Maravelias CT, et al. Global warming intensity of biofuel derived from switchgrass grown on marginal land in Michigan. GCB Bioenergy. 2023;15(3):319–31.

37. Monti A, Polugodina M. Biofuel and Biogas Policies: Economic, Regulatory, and Sustainability Challenges. In: Leal Filho W, Azul AM, Brandli L, Lange Salvia A, Wall T, editors. Affordable and Clean Energy [Internet]. Cham: Springer International Publishing; 2020 [cited 2025 Apr 1]. p. 1–18. Available from: https://doi.org/10.1007/978-3-319-71057-0_126-1

38. Hassan MHj, Kalam MdA. An Overview of Biofuel as a Renewable Energy Source: Development and Challenges. Procedia Engineering. 2013;56:39–53.

39. Savage N. Fuel options: the ideal biofuel. Nature. 2011;474(7352):S9–11.

40. Hileman JI, Stratton RW, Donohoo PE. Energy Content and Alternative Jet Fuel Viability. Journal of Propulsion and Power. 2010;26(6):1184–96.

41. Dhande DY, Sinaga N, Dahe KB. Study on combustion, performance and exhaust emissions of bioethanol-gasoline blended spark ignition engine. Heliyon. 2021 Mar;7(3):e06380.

42. Christensen E, McCormick RL. Long-term storage stability of biodiesel and biodiesel blends. Fuel Processing Technology. 2014 Dec 1;128:339–48.

43. Elfasakhany Ashraf. Gasoline engine fueled with bioethanol-bio-acetone-gasoline blends: Performance and emissions exploration. Fuel. 2020 Aug 15;274:117825.

44. Thangavelu SK, Ahmed AS, Ani FN. Review on bioethanol as alternative fuel for spark ignition engines. Renewable and Sustainable Energy Reviews. 2016 Apr 1;56:820–35.

45. Groom MJ, Gray EM, Townsend PA. Biofuels and Biodiversity: Principles for Creating Better Policies for Biofuel Production. Conservation Biology. 2008;22(3):602–9.

46. Searchinger T, Heimlich R, Houghton RA, Dong F, Elobeid A, Fabiosa J, et al. Use of U.S. Croplands for Biofuels Increases Greenhouse Gases Through Emissions from Land-Use Change. Science. 2008 Feb 29;319(5867):1238–40.

47. Caldeira C, Freire F, Olivetti EA, Kirchain R, Dias LC. Analysis of cost-environmental trade-offs in biodiesel production incorporating waste feedstocks: A multi-objective programming approach. Journal of Cleaner Production. 2019 Apr 10;216:64–73.

48. Richard TL. Challenges in Scaling Up Biofuels Infrastructure. Science. 2010 Aug 13;329(5993):793–6.

49. Alleman T, Fouts L, Chupka G. Quality Parameters and Chemical Analysis for Biodiesel Produced in the United States in 2011 [Internet]. 2013 Mar [cited 2025 Apr 1] p. NREL/TP--5400-57662, 1071952. Report No.: NREL/TP--5400-57662, 1071952. Available from: https://www.osti.gov/servlets/purl/1071952/

50. Standard Specification for Aviation Turbine Fuel Containing Synthesized Hydrocarbons [Internet]. [cited 2025 Apr 2]. Available from: https://store.astm.org/d7566-22.html

51. Akdeniz HY, Balli O, Caliskan H. Energy, exergy, thermoecologic, environmental, enviroeconomic and sustainability analyses and assessments of the aircraft engine fueled with biofuel and jet fuel. J Therm Anal Calorim. 2023 May 1;148(9):3585–603.

52. Yang J, Xin Z, He Q (Sophia), Corscadden K, Niu H. An overview on performance characteristics of bio-jet fuels. Fuel. 2019 Feb 1;237:916–36.

53. Standard Specification for Aviation Turbine Fuel Containing Synthesized Hydrocarbons [Internet]. [cited 2025 Feb 18]. Available from: https://www.astm.org/d7566-22.html

54. Vozka P, Modereger BA, Park AC, Zhang WTJ, Trice RW, Kenttämaa HI, et al. Jet fuel density via GC × GC-FID. Fuel. 2019 Jan;235:1052–60.

55. D02 Committee. Specification for Aviation Turbine Fuels [Internet]. ASTM International; [cited 2025 Apr 2]. Available from: http://www.astm.org/cgi-bin/resolver.cgi?D1655-22A

56. Standard Test Method for Freezing Point of Aviation Fuels (Automatic Phase Transition Method) [Internet]. [cited 2025 Apr 2]. Available from: https://store.astm.org/d5972-23.html

57. El-Maghraby RM. A Study on Bio-Diesel and Jet Fuel Blending for the Production of Renewable Aviation Fuel. MSF. 2020 Aug;1008:231–44.

58. Barrett SRH, Yim SHL, Gilmore CK, Murray LT, Kuhn SR, Tai APK, et al. Public Health, Climate, and Economic Impacts of Desulfurizing Jet Fuel. Environ Sci Technol. 2012 Apr 17;46(8):4275–82.

59. Jet fuel range hydrocarbon generation from catalytic pyrolysis of lignin and polypropylene with iron-modified activated carbon - ScienceDirect [Internet]. [cited 2025 Apr 2]. Available from: https://www.sciencedirect.com/science/article/abs/pii/S0165237024000135

60. Toward an optimal formulation of alternative jet fuels: Enhanced oxidation and thermal stability by the addition of cyclic molecules - ScienceDirect [Internet]. [cited 2025 Apr 2]. Available from: https://www.sciencedirect.com/science/article/abs/pii/S0016236116000491

61. Love J. Microbial pathways for advanced biofuel production. Biochem Soc Trans. 2022 Apr 29;50(2):987–1001.

62. van Dyk S, Saddler J. Progress in Commercialization of Biojet/Sustainable Aviation Fuels (SAF): Technologies and policies. 2024. (IEA Bioenergy; vol. 39).

63. Cabral LDS, Weimer PJ. Megasphaera elsdenii: Its Role in Ruminant Nutrition and Its Potential Industrial Application for Organic Acid Biosynthesis [Internet]. Biology and Life Sciences; 2023 [cited 2025 Apr 2]. Available from: https://www.preprints.org/manuscript/202312.2261/v1

64. Rogosa M. Transfer of Peptostreptococcus elsdenii Gutierrez et al. to a New Genus, Megasphaera [M. elsdenii (Gutierrez et al.) comb. nov.]. International Journal of Systematic and Evolutionary Microbiology. 1971;21(2):187–9.

65. Mutuyemungu E, Motta-Romero HA, Yang Q, Liu S, Liu S, Singh M, et al. Megasphaera elsdenii, a commensal member of the gut microbiota, is associated with elevated gas production during in vitro fermentation. Gut Microbiome (Camb). 2024;5:e1.

66. Cabral L da S, Weimer PJ. Megasphaera elsdenii: Its Role in Ruminant Nutrition and Its Potential Industrial Application for Organic Acid Biosynthesis. Microorganisms. 2024 Jan 21;12(1):219.

67. Guo X, Li X, Feng J, Yue Z, Fu H, Wang J. Engineering of Clostridium tyrobutyricum for butyric acid and butyl butyrate production from cassava starch. Bioresource Technology. 2024 Jan 1;391:129914.

68. Schoen HR, Hunt KA, Strobel GA, Peyton BM, Carlson RP. Carbon chain length of biofuel- and flavor-relevant volatile organic compounds produced by lignocellulolytic fungal endophytes changes with culture temperature. Mycoscience. 2017 Sep;58(5):338–43.

69. Veith C, Diot-Néant F, Miller SA, Allais F. Synthesis and polymerization of bio-based acrylates: a review. Polym Chem. 2020;11(47):7452–70.

70. Yvonne. Eureka Blog. 2024 [cited 2025 Apr 2]. Acrylic Acid Explained: Key Features and Industrial Uses. Available from: https://eureka.patsnap.com/blog/what-is-acrylic-acid/

71. Kandasamy V, Vaidyanathan H, Djurdjevic I, Jayamani E, Ramachandran KB, Buckel W, et al. Engineering Escherichia coli with acrylate pathway genes for propionic acid synthesis and its impact on mixed-acid fermentation. Appl Microbiol Biotechnol. 2013 Feb 1;97(3):1191–200.

72. Baur T, Wentzel A, Dürre P. Production of propionate using metabolically engineered strains of Clostridium saccharoperbutylacetonicum. Appl Microbiol Biotechnol. 2022 Nov;106(22):7547–62.

73. Xu X, Lin J, Cen P. Advances in the Research and Development of Acrylic Acid Production from Biomass1. Chinese Journal of Chemical Engineering. 2006 Aug 1;14(4):419–27.

74. Becker J, Wittmann C. Advanced Biotechnology: Metabolically Engineered Cells for the Bio-Based Production of Chemicals and Fuels, Materials, and Health-Care Products. Angewandte Chemie International Edition. 2015;54(11):3328–50.

75. Folch PL, Bisschops MMM, Weusthuis RA. Metabolic energy conservation for fermentative product formation. Microbial Biotechnology. 2021;14(3):829–58.

76. Buckel W. Energy Conservation in Fermentations of Anaerobic Bacteria. Front Microbiol [Internet]. 2021 Sep 13 [cited 2025 Apr 2];12. Available from: https://www.frontiersin.org/journals/microbiology/articles/10.3389/fmicb.2021.703525/full

77. Duerre P. Handbook on Clostridia. CRC Press; 2005. 919 p.

78. Srirangan K, Bruder M, Akawi L, Miscevic D, Kilpatrick S, Moo-Young M, et al. Recent advances in engineering propionyl-CoA metabolism for microbial production of value-added chemicals and biofuels. Critical Reviews in Biotechnology. 2017 Aug 18;37(6):701–22.

79. Jang YS, Lee J, Malaviya A, Seung DY, Cho JH, Lee SY. Butanol production from renewable biomass: Rediscovery of metabolic pathways and metabolic engineering. Biotechnology Journal. 2012;7(2):186–98.

80. Yang TH, Kim TW, Kang HO, Lee SH, Lee EJ, Lim SC, et al. Biosynthesis of polylactic acid and its copolymers using evolved propionate CoA transferase and PHA synthase. Biotechnology and Bioengineering. 2010;105(1):150–60.

81. Haller T, Buckel T, Rétey J, Gerlt JA. Discovering New Enzymes and Metabolic Pathways: Conversion of Succinate to Propionate by *Escherichia coli*. Biochemistry. 2000 Apr 1;39(16):4622–9.

82. Hedges S. Hedges, S.B. The origin and evolution of model organisms. Nat. Rev. Genet. 3, 838-849. Nature reviews Genetics. 2002 Dec 1;3:838–49.

83. Parekh S, Vinci VA, Strobel RJ. Improvement of microbial strains and fermentation processes. Appl Microbiol Biotechnol. 2000 Sep 1;54(3):287–301.

84. Russo ML, Mock M, Eiteman MA, Westpheling J. Megasphaera elsdenii carbon balance elucidates Mels_0742 function with defined media and chemostat. Not yet published.

85. Chandel NS. Nucleotide Metabolism. Cold Spring Harb Perspect Biol. 2021 Jul 1;13(7):a040592.

86. Garavito MF, Narváez-Ortiz HY, Zimmermann BH. Pyrimidine Metabolism: Dynamic and Versatile Pathways in Pathogens and Cellular Development. Journal of Genetics and Genomics. 2015 May 20;42(5):195–205.

87. Rose K, Liebergesell M, Steinbüchel A. Molecular analysis of the Aureobasidium pullulans ura3 gene encoding orotidine-5′-phosphate decarboxylase and isolation of mutants defective in this gene. Appl Microbiol Biotechnol. 2000 Mar 1;53(3):296–300.

88. Groom J, Chung D, Young J, Westpheling J. Heterologous complementation of a pyrF deletion in Caldicellulosiruptor hydrothermalisgenerates a new host for the analysis of biomass deconstruction. Biotechnol Biofuels. 2014 Sep 16;7(1):132.

89. Singh V, Brecik M, Mukherjee R, Evans JC, Svetlíková Z, Blaško J, et al. The Complex Mechanism of Antimycobacterial Action of 5-Fluorouracil. Chemistry & Biology. 2015 Jan 22;22(1):63–75.

90. Riley LA, Guss AM. Approaches to genetic tool development for rapid domestication of non-model microorganisms. Biotechnol Biofuels. 2021 Jan 25;14(1):30.

91. Choi YJ, Lee J, Jang YS, Lee SY. Metabolic Engineering of Microorganisms for the Production of Higher Alcohols. mBio. 2014 Sep 2;5(5):e01524-14.

92. Lamsen E, Atsumi S. Recent progress in synthetic biology for microbial production of C3–C10 alcohols. Frontiers in Microbiology [Internet]. 2012 [cited 2022 Nov 15];3. Available from: https://www.frontiersin.org/articles/10.3389/fmicb.2012.00196

93. Riley L. Overcoming DNA Restriction-Modification systems to enable transformation and genetic engineering in non-model organisms. Doctoral Dissertations [Internet]. 2020 Dec 1; Available from: https://trace.tennessee.edu/utk_graddiss/6180

94. Tracy BP, Jones SW, Fast AG, Indurthi DC, Papoutsakis ET. Clostridia: the importance of their exceptional substrate and metabolite diversity for biofuel and biorefinery applications. Current Opinion in Biotechnology. 2012 Jun 1;23(3):364–81.

95. Weimer PJ, Nerdahl M, Brandl DJ. Production of medium-chain volatile fatty acids by mixed ruminal microorganisms is enhanced by ethanol in co-culture with Clostridium kluyveri. Bioresource Technology. 2015 Jan 1;175:97–101.

96. Dekishima Y, Lan EI, Shen CR, Cho KM, Liao JC. Extending Carbon Chain Length of 1-Butanol Pathway for 1-Hexanol Synthesis from Glucose by Engineered Escherichia coli. J Am Chem Soc. 2011 Aug 3;133(30):11399–401.

97. Dellomonaco C, Clomburg JM, Miller EN, Gonzalez R. Engineered reversal of the β-oxidation cycle for the synthesis of fuels and chemicals. Nature. 2011 Aug;476(7360):355–9.

98. Kim S, Clomburg JM, Gonzalez R. Synthesis of medium-chain length (C6–C10) fuels and chemicals via β-oxidation reversal in Escherichia coli. Journal of Industrial Microbiology and Biotechnology. 2015 Mar 1;42(3):465–75.

99. Nelson RS, Peterson DJ, Karp EM, Beckham GT, Salvachúa D. Mixed Carboxylic Acid Production by Megasphaera elsdenii from Glucose and Lignocellulosic Hydrolysate. Fermentation. 2017 Mar;3(1):10.

100. Prabhu R, Altman E, Eiteman MA. Lactate and Acrylate Metabolism by Megasphaera elsdenii under Batch and Steady-State Conditions. Appl Environ Microbiol. 2012 Dec;78(24):8564–70.

101. Kim H, Jeon BS, Sang BI. An Efficient New Process for the Selective Production of Odd-Chain Carboxylic Acids by Simple Carbon Elongation Using Megasphaera hexanoica. Sci Rep. 2019 Aug 19;9(1):11999.

102. Riley L. Overcoming DNA Restriction-Modification systems to enable transformation and genetic engineering in non-model organisms. Doctoral Dissertations [Internet]. 2020 Dec 1; Available from: https://trace.tennessee.edu/utk_graddiss/6180

103. Solem C, Defoor E, Jensen PR, Martinussen J. Plasmid pCS1966, a New Selection/Counterselection Tool for Lactic Acid Bacterium Strain Construction Based on the *oroP* Gene, Encoding an Orotate Transporter from *Lactococcus lactis*. Applied and Environmental Microbiology. 2008;74(15):4772–5.

104. Shi T, Wang G, Wang Z, Fu J, Chen T, Zhao X. Establishment of a Markerless Mutation Delivery System in Bacillus subtilis Stimulated by a Double-Strand Break in the Chromosome. PLOS ONE. 2013 Nov 25;8(11):e81370.

105. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, et al. SPAdes: A New Genome Assembly Algorithm and Its Applications to Single-Cell Sequencing. Journal of Computational Biology. 2012 May;19(5):455–77.

106. Koren S, Walenz BP, Berlin K, Miller JR, Bergman NH, Phillippy AM. Canu: scalable and accurate long-read assembly via adaptive k-mer weighting and repeat separation. Genome Res. 2017 May 1;27(5):722–36.

107. Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, et al. The RAST Server: Rapid Annotations using Subsystems Technology. BMC Genomics. 2008 Feb 8;9(1):75.

108. Makarova KS, Wolf YI, Snir S, Koonin EV. Defense Islands in Bacterial and Archaeal Genomes and Prediction of Novel Defense Systems. Journal of Bacteriology. 2011 Nov;193(21):6039–56.

109. Dobrindt U, Hochhut B, Hentschel U, Hacker J. Genomic islands in pathogenic and environmental microorganisms. Nature Reviews Microbiology. 2004 May;2(5):414–24.

110. Boyd EF, Almagro-Moreno S, Parent MA. Genomic islands are dynamic, ancient integrative elements in bacterial evolution. Trends in Microbiology. 2009 Feb 1;17(2):47–53.

111. Hacker J, Carniel E. Ecological fitness, genomic islands and bacterial pathogenicity. EMBO reports. 2001 May;2(5):376–81.

112. Juhas M, van der Meer JR, Gaillard M, Harding RM, Hood DW, Crook DW. Genomic islands: tools of bacterial horizontal gene transfer and evolution. FEMS Microbiol Rev. 2009 Mar;33(2):376–93.

113. Build metabolic model [Internet]. Available from: https://kbase.us/applist/apps/fba_tools/build_metabolic_model/release

114. Gapfill metabolic model [Internet]. Available from: https://kbase.us/applist/apps/fba_tools/gapfill_metabolic_model/release

115. Singh V, Brecik M, Mukherjee R, Evans JC, Svetlíková Z, Blaško J, et al. The Complex Mechanism of Antimycobacterial Action of 5-Fluorouracil. Chemistry & Biology. 2015 Jan 22;22(1):63–75.

116. Elmore JR, Furches A, Wolff GN, Gorday K, Guss AM. Development of a high efficiency integration system and promoter library for rapid modification of Pseudomonas putida KT2440. Metab Eng Commun. 2017 Apr 15;5:1–8.

117. Elmore JR, Dexter GN, Francis R, Riley L, Huenemann J, Baldino H, et al. The SAGE genetic toolkit enables highly efficient, iterative site-specific genome engineering in bacteria [Internet]. bioRxiv; 2020 [cited 2022 Nov 15]. p. 2020.06.28.176339. Available from: https://www.biorxiv.org/content/10.1101/2020.06.28.176339v1

118. Yamagishi A, Tanimoto T, Suzuki T, Oshima T. Pyrimidine biosynthesis genes (pyrE and pyrF) of an extreme thermophile, Thermus thermophilus. Appl Environ Microbiol. 1996 Jun;62(6):2191–4.

119. Groom J, Chung D, Young J, Westpheling J. Heterologous complementation of a pyrF deletion in Caldicellulosiruptor hydrothermalisgenerates a new host for the analysis of biomass deconstruction. Biotechnol Biofuels. 2014 Sep 16;7(1):132.

120. Thomas CM, Nielsen KM. Mechanisms of, and Barriers to, Horizontal Gene Transfer between Bacteria. Nat Rev Microbiol. 2005 Sep;3(9):711–21.

121. Roberts RJ, Vincze T, Posfai J, Macelis D. REBASE: a database for DNA restriction and modification: enzymes, genes and genomes.

122. Vasu K, Nagaraja V. Diverse Functions of Restriction-Modification Systems in Addition to Cellular Defense. Microbiology and Molecular Biology Reviews. 2013 Mar 7;77(1):53–72.

123. Bron S, Murray K, Trautner TA. Restriction and modification in B. subtilis. Molec Gen Genet. 1975 Jan 1;143(1):13–23.

124. Improved Genetic Transformation of the Thermophilic Cyanobacterium, Thermosynechococcus elongatus BP-1 | Plant and Cell Physiology | Oxford Academic [Internet]. [cited 2025 Apr 2]. Available from: https://academic.oup.com/pcp/article-abstract/45/2/171/1814952

125. Ruivo M, Kovács NZ, Schötta AM, Stelzer T, Hermann L, Mündler V, et al. Optimising Transformation Efficiency in Borrelia: Unravelling the Role of the Restriction-Modification System of Borrelia afzelii and Borrelia garinii. International Journal of Molecular Sciences. 2024 Jan;25(21):11343.

126. Croux C, Nguyen NPT, Lee J, Raynaud C, Saint-Prix F, Gonzalez-Pajuelo M, et al. Construction of a restriction-less, marker-less mutant useful for functional genomic and metabolic engineering of the biofuel producer Clostridium acetobutylicum. Biotechnol Biofuels. 2016 Feb 2;9(1):23.

127. Chung D, Farkas J, Westpheling J. Overcoming restriction as a barrier to DNA transformation in Caldicellulosiruptor species results in efficient marker replacement. Biotechnol Biofuels. 2013 May 29;6(1):82.

128. Russo ML, Riley LA, Wood NM, Eschedor G, Blum D, Guss AM, et al. Deletion of a Putative Acyl-CoA Transferase in Megasphaera elsdenii Eliminates Propionate Production and Increases Medium Chain Organic Acid Production. Not yet published.

129. Vasu K, Nagaraja V. Diverse Functions of Restriction-Modification Systems in Addition to Cellular Defense. Microbiology and Molecular Biology Reviews. 2013 Mar 7;77(1):53–72.

130. Jeswani HK, Chilvers A, Azapagic A. Environmental sustainability of biofuels: a review. Proc Math Phys Eng Sci. 2020 Nov;476(2243):20200351.

131. El-Araby R. Biofuel production: exploring renewable energy solutions for a greener future. Biotechnology for Biofuels and Bioproducts. 2024 Oct 15;17(1):129.

APPENDIX A

Jordan F. Russell, **Matthew L. Russo**, Xuewen Wang, Neal Hengge, Daehwan Chung, Lance Wells, Yannick J. Bomble, Janet Westpheling. (2020) Deletion of a Peptidylprolyl Isomerase Gene Results in the Inability of *Caldicellulosiruptor bescii* To Grow on Crystalline Cellulose without Affecting Protein Glycosylation or Growth on Soluble Substrates. Appl Environ Microbiol 86:e00909-20.

Abstract

Caldicellulosiruptor bescii secretes a large number of complementary multifunctional enzymes with unique activities for biomass deconstruction. The most abundant enzymes in the *C. bescii* secretome are found in a unique gene cluster containing a glycosyl transferase (GT39) and a putative peptidyl prolyl cistrans isomerase. Deletion of the glycosyl transferase in this cluster resulted in loss of detectable protein glycosylation in *C. bescii*, and its activity has been shown to be responsible for the glycosylation of the proline-threonine rich linkers found in many of the multifunctional cellulases. The presence of a putative peptidyl prolyl cis-trans isomerase within this gene cluster suggested that it might also play a role in cellulase modification. Here, we identify this gene as a putative prsA prolyl cis-trans isomerase. Deletion of prsA2 leads to the inability of *C. bescii* to grow on insoluble substrates such as Avicel, the model cellulose substrate, while exhibiting no differences in phenotype with the wild-type strain on soluble substrates. Finally, we provide evidence that the prsA2 gene is likely needed to increase solubility of multifunctional cellulases and that this unique gene cluster was likely acquired by

members of the Caldicellulosiruptor genus with a group of genes to optimize the production and activity of multifunctional cellulases.