Conversion of Cellulose to Ethanol by the Biofuels Microbe Clostridium Phytofermentans: Quantification of Growth and Role of an Rnf-Complex in Energy Conservation

Jesús G. Alvelo-Maurosa

University of Massachusetts Amherst

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CONVERSION OF CELLULOSE TO ETHANOL BY THE BIOFUELS MICROBE
CLOSTRIDIUM PHYTOFERMENTANS: QUANTIFICATION OF GROWTH AND
ROLE OF AN RNF-COMPLEX IN ENERGY CONSERVATION

A Dissertation Presented

by

JESÚS G. ALVELO MAUROSA

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 6, 2016

Department of Microbiology
CONVERSION OF CELLULOSE TO ETHANOL BY THE BIOFUELS MICROBE
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Approved as to style and content by:

________________________________
Susan B. Leschine, Chair

________________________________
James F. Holden, Member

________________________________
Samuel P. Hazen, Member

________________________________
Yasu Morita, Member

Steven J. Sandler, Department Head
Department of Microbiology
DEDICATION

To my parents, Jesús M. Alvelo Santiago and Alba I. Maurosa Colón. To my aunt Norma Maurosa and to my sisters Cristina M. Alvelo and María M. Alvelo. Sin ellos soy nada.
The youth has the right to defend their country with the weapons of knowledge.
Pedro Albizu Campos
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ABSTRACT

CONVERSION OF CELLULOSE TO ETHANOL BY THE BIOFUELS MICROBE
CLOSTRIDIUM PHYTOFERMENTANS: QUANTIFICATION OF GROWTH AND
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MAY 6, 2016

JESÚS G. ALVELO MAUROSA,
B.S., UNIVERSIDAD DE PUERTO RICO, RECINTO DE RÍO PIDERAS
Ph.D., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor Susan B. Leschine

The anaerobic mesophilic bacterium Clostridium phytofermentans grows and ferments multiple plant-based substrates into ethanol as the main product of fermentation. The capacity of C. phytofermentans to convert plant biomass into ethanol, propanol, and short-chain fatty acids is strongly attractive for industry. Specific physiological capabilities of C. phytofermentans allow the microbe to generate high amounts of ethanol compared to acetate. However, little is known about membrane energetics in C. phytofermentans, or its role in energy conservation and production of high levels of ethanol during fermentation of plant biomass substrates.

In the first research project presented in this dissertation, we examined C. phytofermentans growth on three insoluble plant-based substrates: Whatman #1 filter paper, wild-type Sorghum bicolor and the S. bicolor reduced lignin double mutant, bmr-6/bmr-12. Cells were visualized and quantified employing a novel dual-fluorescent staining protocol, combining Fluorescent Brightener 28, a blue fluorescent dye that stains cellulose and chitin, and SYTO 9, a green fluorescent dye that stains nucleic acid. Our results demonstrated this protocol allows the visualization and differentiation of C.
*phytofermentans* cells from plant debris using epifluorescence microscopy. Our results also showed greater cell growth and substrate attachment on reduced lignin substrates. Moreover, ethanol production increased over time while cell numbers decreased when cultured with either sorghum substrate. These results suggested that only a portion of the cell population remained active during growth, possibly due to sporulation and reduced metabolic activity.

The second project presented in this dissertation involved the characterization of a bacterial microcompartment (BMC) in *C. phytofermentans*. Transmission electron microscopy (TEM) of thin sections of *C. phytofermentans* cells grown with fucose or rhamnose as growth substrate showed the presence of polyhedral structures, similar in appearance to known BMCs. Visualization of these intracellular structures was possible due to various modifications of a TEM sample embedding protocol. Use of picric acid and potassium ferricyanide resulted in enhanced contrast of BMC images while stabilizing cell wall structure during sample preparation.

A third and final project described in this dissertation concerned the development of a model for *C. phytofermentans* membrane energetics and energy conservation that might provide an explanation for high levels of ethanol production by this microbe. Genomic and biochemical analyses indicated the presence of a membrane-bound sodium-and proton-translocating pyrophosphatase and three ATPases, including a Na\(^+\) V-type ATPase, a Na\(^+\) F-type ATPase and a possible H\(^+\) ATPase that had not previously been characterized. Furthermore, results showed that Rnf ferredoxin:NAD\(^+\) oxidoreductase activity in inverted membrane vesicles was driven by a proton electrochemical gradient. Supporting data showed that cell growth was inhibited by both sodium and proton ionophores. Taken together, these results suggested that *C. phytofermentans* is capable of conserving energy through ferredoxin oxidoreductase activity of the Rnf-complex, resulting in increased levels of NADH for ethanol production.
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CHAPTER 1

INTRODUCTION

1.1 Central Objectives and Goals

Global temperature has been on the rise due to increased concentrations of atmospheric CO$_2$ (Allen et al. 2009). In order to limit the increase in global temperature to 2°C, 1,200 gigatons (Gt) of CO$_2$ or less needs to be produced from 2015 to 2100 (Fuss et al., 2014). Emissions from use of fossil fuels and cement production have contributed to increased concentrations of CO$_2$ with nearly 40 Gt produced in 2015. Strong public policy and human will are essential if we do not want to go above the 2°C benchmark. Greater effort will be required the longer the wait.

The transportation sector is responsible for 23% of total world CO$_2$ emissions (Fig. 1.1). Bioethanol is an important renewable energy source that could substitute for petroleum-based fuels in land transportation and reduce CO$_2$ emissions. First generation bioethanol from edible food crops, such as corn and sugarcane, has dominated the market but land use management and increased food prices have created controversy (Graham-Rowe, 2011). Second generation bioethanol derived from lignocellulosic biomass has the potential for sustainable production and a decreased impact on global food supply (Somerville et al., 2010). Nonetheless, recalcitrance of lignocellulosic biomaterial, due primarily to its lignin content, presents a challenge for bioethanol production inasmuch as the pretreatment of woody biomass requires hydrolytic enzymes, acid, and heat, which

An alternative approach for the conversion of lignocellulosic biomass to bioethanol is consolidated bioprocessing (CBP), where a microbe capable of producing lignocellulose-decomposing enzymes also ferments the resulting carbohydrates to ethanol as a fermentation product (Lynd 2005). *Clostridium phytofermentans*, which has emerged as a model CBP microorganism (Jin et al., 2011, Tolonen et al., 2011, Zuroff et al., 2013, Yee et al., 2014 and Petit et al., 2015), directly converts all major components of plant biomass (cellulose, hemicellulose, pectin, starch) into fermentation products, primarily ethanol. In order to better appreciate the potential of *C. phytofermentans* as a CBP microbe, strategies to examine the microbe interacting with plant biomass are required. Additionally, a deeper understanding of the physiology of *C. phytofermentans* is needed to evaluate the potential of this microbe for biofuels production; e.g., the role of intracellular protein-bound bacterial microcompartments (BMCs) in fermentation product formation and the function of membrane electrochemical gradients in energy conservation and ethanol production.

The goals of this dissertation are to: 1) develop a method to enable visualization and quantification of microbial cells interacting with plant biomass, 2) generate a method that allows consistent visualization of BMCs to facilitate studies of the function and distribution of BMCs in cells and 3) using structural bioinformatics and biochemical assays, develop a testable model for energy conservation in *C. phytofermentans* involving membrane-bound ATPases, an Rnf complex and ion gradients across the membrane.
This dissertation project addresses the following questions:

i- How does the lignin content of plant biomass feedstocks affect the growth of *C. phytofermentans*?

ii- What preparation procedures are required to visualize bacterial microcompartments using transmission electron microscopy?

iii- What mechanisms are utilized by *C. phytofermentans* to conserve energy, and how is energy conservation related to fermentation product formation?
Figure 1.1 Figure 5.5 Model of the pyrophosphatase protein of *C. phytofermentans*. The model shows conserved amino acids T87 (magenta spheres), F91 (purple spheres), M117 (yellow spheres) and D143 (red spheres) involved in binding H+ and Na+. The model was developed using SWISS MODEL and 3D rendered using VMD 1.9.1.
1.2 Organization of the Dissertation

The research questions described above are addressed as separate chapters in this dissertation. Chapter 3 has appeared as a first-author publication in *Applied and Environmental Microbiology*; some of the research presented in Chapter 4 has been published in *PLoS ONE* as part of a research collaboration; the research described in Chapter 5 has been prepared for publication in *Frontiers in Microbiology*, also as a first-author publication. Chapter 1 describes the objectives of the research. A literature review with background information and the research approach are given in Chapter 2. In Chapter 3 a microscopy method to visualize *C. phytofermentans* cells in the presence of plant biomass is presented along with its application in investigations of cell growth kinetics on insoluble plant biomass substrates which contributed to a co-authored publication (Lee et al., 2012) and it was recently published on another peer reviewed journal (Alvelo-Maurosa et al., 2015). Chapter 4 describes transmission electron microscopy methodology for visualizing bacterial microcompartments in *C. phytofermentans* cells grown with fucose or rhamnose as substrate. Chapter 4 results were a contribution to a peer reviewed publication (Petit et al., 2013) Chapter 5 describes research using structural bioinformatics and biochemical assays to evaluate ion gradients involved in membrane energetics, which are tied to the activities of ATPases and an Rnf complex. A model for membrane energetics in *C. phytofermentans* also is presented in Chapter 5 and will be submitted to a peer review journal. Chapter 6, the final chapter of this dissertation, includes a discussion of the significance of the research and concluding remarks.
CHAPTER 2

LITERATURE REVIEW AND RESEARCH APPROACH

2.1 Microbial Conversion of Plant Biomass into Biofuels

Lignocellulosic plant biomass is composed of proteins, lignin and polysaccharides (Leschine, 2005). The prevalent polysaccharide in plant cell walls is cellulose, a complex chain-like system composed of over 15,000 beta-(1,4)-linked glucose molecules (Brett CT, 2000). Cellulose enforces strength in the cell wall to maintain turgor pressure. Hemicelluloses (glucomannans and xylans) are composed of multiple monomers including D-xylose, L-arabinose, D-mannose, D-glucose, D-galactose and D-glucuronic acid (Leschine, 1995, Leschine, 2005). Three monolignols compose lignin: coniferyl, synapyl, and p-coumaryl alcohols, which form guaiacyl, syringyl, phenylpropanoids and p-hydroxyphenyl units of lignin (Bonawitz and Chapple, 2010). Lignin is cross linked to cellulose and other polymers, such as hemicellulose, which provides hydrophobicity and strength to the plant cell wall, and also reduces accessibility of polysaccharides to enzymatic degradation and penetration by pathogens (Grabber, 2005 and Bonawitz, 2010).

Microbial interactions with plant biomass are central to technologies for the conversion of lignocellulosic feedstocks into useful products. For example, ethanol is an important renewable energy source that may replace petroleum-based transportation fuels and reduce CO₂ emissions (Sticklen 2008). Development of more sustainable processes for second generation biofuels derived from lignocellulosic biomass is limited by the
recalcitrance of the substrate, primarily due to its lignin content, and often requires expensive pretreatment and use of hydrolytic enzymes (Sanderson 2011, Ragauskas et al., 2006 and Chen et al., 2007). An alternative strategy to convert lignocellulosic feedstocks into biofuels is consolidated bioprocessing (CBP), where microbes capable of producing a complex set of glycoside hydrolases decompose plant biomass and ferment the products in a single step, for a more cost-effective process with savings in capital and operational costs (Lyng et al., 2005).

The use of various wildtype and genetically engineered microbes as CBP catalysts has been shown to increase bioethanol yield (Olson et al., 2012, Peralta-Yahya, 2010 and Lynd et al., 2008). Furthermore, reducing the lignin content of lignocellulosic biomass allows greater accessibility of microbes to plant carbohydrates, such as hemicellulose and cellulose, and higher yields of bioethanol (Chen & Dixon 2007, and Himmel, 2007). Studies have shown that microbes are capable of improved CBP performance and greater saccharification of plant biomass with a reduced lignin content, resulting in higher yields of bioethanol and other fermentation products (Lee et al., 2012 and Yee 2012).

2.2 Metabolism of Complex Carbohydrates by Human Gut Microbiota

The human intestine is home to the gut microbiome, composed of approximately 3.9 x 10^{13} bacteria (Sender et al., 2016), with a constantly changing diversity due to the host diet (Bäckhead et al., 2005). The human gut microbiome can be viewed as a large reactor where microbial breakdown of complex polysaccharides occurs. These compounds, referred to as “dietary fiber,” are derived from major components of the human diet such as fruits, cereals and vegetables. Humans and most other animals lack the enzymatic
capacity required to digest cellulose and many other components of plant cell walls, and instead they rely on plant biomass-decomposing microbial communities to provide nutrition from plant fiber. Dietary fiber serves as a source of growth substrates for intestinal microbes that ferment these components of plant biomass to short chain fatty acids, which are tied to colonic and systemic health (Wong et al., 2006, Flint et al., 2012, Sticklen 2008, Sanderson 2011 and Ragauskas et al., 2006).

Plant polysaccharides such as pectin, cellulose, hemicellulose and starches are degraded by carbohydrate-active enzymes (CAZymes,) produced by the gut microbiota (El Kaoutari et al. 2013). CAZymes generated by the microbiome cleave oligosaccharides and polysaccharides into monosaccharides that are then fermented. These anaerobic digestions produce short-chain fatty acids such as butyrate, acetate and propionate that contribute to important physiological processes (Cantarel, Lombard and Henrissat 2012). For example, butyrate is an important energy source for colonocytes and promotes differentiation of colonic regulatory T cells that have multiple functions including the capacity to fight off cancer cells (Furusawa et al., 2013). It has also been found that patients with type-2 diabetes have a decrease in butyrate-producing bacteria due to dysbiosis, which may lead to an increased probability of colon-rectal cancer (Qin et al., 2012).

2.3 *Clostridium phytofermentans*: model organism

2.3.1 Carbohydrate breakdown and ethanol production

*Clostridium phytofermentans* is a cellulolytic anaerobic bacterium isolated from soil near a water reservoir in Massachusetts (Warnick, Methé, and Leschine 2002). The microbe is capable of hydrolyzing and fermenting plant components such as cellulose and
hemicellulose and producing ethanol as its main product. The *C. phytofermentans* genome encodes 161 CAZymes, with 116 glycoside hydrolases, including hemicellulases, pectinases, and amylases among others (Tolonen et al., 2011 and Petit et al., 2015). With its versatility for plant saccharification and high level of ethanol production, *C. phytofermentans* differentiates itself from other characterized microbes as an ideal candidate to better understand the mechanism of decomposition of different types of plant biomass substrates.

It has been determined that *C. phytofermentans* produces different amounts of ethanol depending on the type of plant biomass substrate, pre-treatment method, particle size, pH and temperature (Jin et al., 2011 and Lee et al., 2012). The lignin content of plant biomass plays a significant role in ethanol production. Lee et al. 2012 reported that *C. phytofermentans* produces higher levels of ethanol when grown with three different types of brown midrib sorghum as substrate compared to growth with wildtype sorghum. *Brown midrib* sorghum contains less lignin than wildtype sorghum, suggesting that a reduction in the lignin content of plant biomass allows more accessibility to cellulose and hemicellulose, making it more digestible. However, prior to research described in Chapter 3 of this dissertation, cell growth kinetics on plant biomass substrates, and the impacts of microbe-biomass interactions on biofuel production were unknown due to a lack of methods for visualizing and quantifying microbes in the presence of plant biomass substrates.
2.3.2 Cell enumeration in the presence of insoluble plant biomass substrates

The enumeration of cellulolytic bacteria was first described by Hungate (Hungate RE, 1950) who reported the quantification of cellulolytic colonies on plates of cellulose agar media. Some bacterial strains grew in liquid media with cellulosic substrates but did not grow on plates of agar media; thus, quantification of growth for some strains was not possible. Various other methods yielded inconsistent results, mostly due to the use of different media components rather than the presence of cellulose itself (Bryant, 1959). By 1960, Kistner had developed a water-cooled agar solidification apparatus to generate a uniform film of agar along the walls of rubber-stoppered roll bottles (Kistner, 1960). The surface of the agar was scanned using an “Apparatenfabriek van Dooren” counting device and cellulolytic colonies were quantified with consistency, Kistner being the first researcher to do so. Another research group developed a fluorescent antibody technique, the first researchers to do so with a cellulolytic microbe, to enumerate cellulolytic cocci microbes from high dilution samples from sheep rumen fluid using fluorescence microscopy (Jarvis, 1967). Numbers for cellulolytic cocci in sheep rumen fluid were consistent; however, the researchers did not use cellulosic substrates in their media when quantify cells. Still, the work of Jarvis et al. to develop the first fluorescent technique for visualizing specific microbes in mixed populations was remarkable. By the end of 1960’s two methods for quantifying cellulolytic bacteria were in use: a ‘direct’ method which relied on the growth of cellulolytic bacteria and production of zones of clearing in cellulose-containing agar media, and an ‘indirect’ method in which an agar medium provided growth conditions for multiple species of microbes, and then, colonies were hand-
picked and transferred to specific media. When compared, these methods provided very similar numbers (Gylswyk, 1970).

For over a decade, few advances in methods to enumerate cellulolytic bacteria occurred. Most attempts involved staining a cellulosic substrate in order to detect cellulose breakdown, but none of these techniques focused on direct cell counts (Smith, 1977 and Mahasme, 1980). A method developed by Teather (Teather, 1982) involved staining β-D-glucans in plates of agar media with Congo red. This technique allowed detection of β-D-glucan hydrolysis on plates of agar media, but it was often still complemented by measurements using the roll bottle technique. In 1994, an enzyme-linked immunosorbent assay (ELISA) for detecting Clostridium aldrichii growing on lignocellulosic substrates was reported (Brigmon, 1994). This assay detected cellulolytic microbes in both pure and mixed cultures at cell number of $10^5$ cells ml$^{-1}$ in a period of 36 hours. In 1995, Hendicks et al., developed a new solid medium that contained Noble agar, gelatin and Congo red in order to visualize and quantify cellulolytic microbes on plates of a cellulose agar medium (Hendricks 1995). This method enabled the identification of cellulose-utilizing bacteria on plates of agar media by the appearance of zones of clearing around colonies. (Fig 2.1).

In 2004, Zhang and Lynd reported measurements of the growth of Clostridium thermocellum in batch cultures with Avicel as growth substrate (Zhang et al., 2004). These researchers employed an indirect ELISA method for measurement of concentrations of cellulase protein and distinct C. thermocellum cell mass determinations. Cell mass and cellulase protein concentration increased exponentially through 20 hours of growth. Interestingly, Zhang and Lynd also reported that cell mass concentration was the highest after 22 hours of growth, and then declined. In 2006, this group of researchers measured
cell growth and specific cellulose hydrolysis rates in both batch and continuous cultures and concluded that cellulose hydrolysis is higher in growing cultures of *C. thermocellum* as compared to purified cellulase preparations, demonstrating the importance of “enzyme-microbe synergy.” (Lu, 2006).

Various researchers have used qPCR to estimate the number of cellulolytic bacteria in ruminants, soils and lakes (Picard et al., 1992, Koike and Kobayashi 2001, Mosoni et al., 2007, McDonald et al., 2009 and de Menezes et al., 2012). Cell numbers have been estimated by measuring the abundance of a gene, such as the 16S rRNA gene, *rrs* (Mosoni, 2007; Koike, 2001). In some instances, traditional techniques, such as growth on solid media in roll tubes or most-probable number (MPN) determinations were combined with qPCR results to estimate cell numbers. Yet none of these studies focused on the growth of individual cellulolytic bacteria in media containing cellulose or another lignocellulosic substrate. In 2009, researchers from Italy quantified bacterial biomass in wastewater sludge by using fluorescent dyes and flow cytometry (Foladori et al., 2009). By using dyes, such as Dead/Live Staining Kit, SYBR-Green I, and Propidium Iodide, Foladori was able to visualize microbes in both raw sludge samples and in diluted samples. The ratio of viable to non-viable bacteria was determined using flow cytometry. Cell aggregates were counted as one cell, and total numbers were estimated, but final numbers were calculated using the volume of each particle. The report by Foladori is unique in employing a technique to directly count cells in a medium containing cellulose fibers.

In an effort to solve the problem of quantifying cellulolytic bacteria in media containing insoluble plant biomass substrates such as cellulose, we employed a dual-fluorescent staining technique along with epifluorescence microscopy that allowed us to
differentiate and enumerate *C. phytofermentans* cells (Alvelo-Maurosa et al., 2015), as discussed in Chapter 3 of this dissertation. The protocol involved staining bacteria using SYBR-Green from the Live/Dead Cell Staining Kit and the use of Fluorescent Brightener 28 to stain cellulose. By using a series of dilution ratios and vortex speeds, we quantified growth in media containing cellulose (ball-milled Whatman #1 filter paper), wildtype sorghum and a double-lignin mutant *bmr-6/bmr-12* sorghum. This method facilitated measurements of cell attachment to substrate and cell-ethanol ratios. Also, our research provided 3D images of *C. phytofermentans* cells growing on cellulosic substrates. To our knowledge, this is the first extensive study involving direct cell counts of a microbe growing on insoluble lignocellulosic substrates.
Figure 2.1 Cellulolytic bacterium from soil growing on a plate of Noble Agar medium containing Congo red and supplemented with gelatin. (Henricks, 1995).
2.3.3 Bacterial Microcompartments

Bacterial microcompartments (BMCs) are protein polyhedral bodies that sequester diverse metabolic pathways (Cameron et al., 2013). The structure of BMCs is similar to those of viral capsids, where hexameric and pentameric protein clusters are combined to form a protein shell. Enzymes of the BMCs are encapsulated by shell proteins possibly to prevent exposure to detrimental environmental factors such as oxygen (Fan et al., 2012). However, BMC shells do have pores, 4-7 Å in diameter, which allow passage of metabolites (Kerfeld and Erbilgin 2015). Various types of BMCs with different functions and generating various metabolites are known; e.g., 1,2-propanediol (PDU), ethanolamine (EUT), ethanol (ETU), choline (CUT), glycyl radical propanediol (GRP), planctomycete and verrucomicrobia metabolosome (PVM) and CO₂ fixation (carboxysome) (Kerfeld and Erbilgin 2015).

BMCs have been found in 23 bacterial phyla and their functions vary in different environments (Axen et al., 2014). For example, in humans, some members of the gut microbiome catalyze trimethylamine produced from choline via a glycyl radical enzyme (GRE)-associated microcompartment (Craciun et al., 2012). PDU and EUT metabolosomes are involved in the virulence of some pathogens such as Escherichia coli O157:H7 and Salmonella enterica (Harvey et al., 2011 and Kendall et al, 2012). Members of the Planctomycetes phylum are capable of breaking down fucose, rhamnose and fucoidan in aerobic conditions (Erbilgin et al., 2014).
Genomic, transcriptomic, biochemical and microscopic evidence has indicated the presence of a PDU BMC in *C. phytofermentans*, which is involved in the conversion of 1,2-propanediol to propionate and propanol during growth on the deoxyhexose sugars (also known as methyl-pentoses), fucose or rhamnose (Petit et al., 2015) (Fig. 2.2). The presence in *C. phytofermentans* of a BMC for the production of 1, 2-propanediol and propionate from deoxyhexose sugars, indicates the potential of this microbe for production of multiple biofuels from a broad range of cellulosic substrates.
Figure 2.2 Diagrammatic representation of the metabolic functions associated with the Pud (propanediol utilization) BMC and the conversion of 1, 2-propanediol (red box) into propionate or into propanol via the oxidization of NADH by PduQ. Propanol is short-chain fatty acid with the potential to be an alternate liquid transportation fuel.

(Adapted from Yeates, Crowley, and Tanaka, 2010)

http://people.mbi.ucla.edu/yeates/microcompartments.html
2.3.4 Bioenergetics

A characteristic of *C. phytofermentans*, which is central to its industrial relevance, is its propensity for ethanol production; ethanol is the main product of fermentation of multiple plant biomass substrates (Petit et al. 2015 and Warnick et al., 2002). NADH reduces Acetyl-CoA to form ethanol, generating NAD\(^+\), which is required in the glycolytic pathway. Formation of acetate, another fermentation product, from Acetyl-CoA yields ATP by substrate level phosphorylation. Since yields of ethanol are higher than acetate we hypothesized that the microbe was capable of conserving energy and forming ATP in other ways. Microarray analyses revealed numerous highly expressed genes involved in membrane energetics and energy conservation (Petit et al., 2015). For example, the genome of *C. phytofermentans* encodes three trans-membrane ATPases. A Na\(^+\) F-type (Cphy_3741) and a Na\(^+\) V-type (Cphy_3076) ATPase have been identified by sequence alignment and enzyme assays (as discussed in Chapter 5 of this dissertation). Additionally, a third ATPase (Cphy_3445), with unknown ion specificity, has also been identified through genome and microarray analyses.

ATPases are membrane-bound ion pumping channels that may contribute to the formation of trans-membrane ion gradients or the formation of adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and inorganic phosphate (Pi). Membrane-bound ATPases are essential for cellular energetics and are found in all three domains of life (Mulkidjanian et al., 2007). F-type ATPases can be found in bacteria, and in the chloroplasts and mitochondria of eukaryotic cells. Structural features of F-type ATPases, such as the *c*-ring, a unit involved in ion binding, different from those of the V-type ATPases. ATPases may be capable of pumping H\(^+\), Na\(^+\), K\(^+\) and/or Ca\(^{2+}\), which results in
the generation of an electrochemical gradient (Kühlbrandt, 2004 and Toyoshima, 2004). ATPases are versatile proteins required for the energy conservation in bacteria and may play an important role in fermentative anaerobes.

The genome of *C. phytofermentans* also encodes an Rnf complex (Cphy_0211-0216) with an integral ferredoxin oxidoreductase. The Rnf complex utilizes electrons from ferredoxin and reduces NAD\(^+\) to NADH while translocating either Na\(^+\) or H\(^+\). The Rnf complex was first identified as a protein complex involved in the nitrogen fixation process of *Rhodobacter capsulatus* (Schmehl et al., 1993). In the acetogen *Acetobacterium woodii*, the Rnf complex catalyzes the reduction of NAD\(^+\) to NADH using ferredoxin as electron donor while extruding a sodium ion (Müller et al., 2008). In contrast, the Rnf-complex of *Clostridium ljungdahlii* has been identified as proton-translocating and essential for autotrophic growth. (Tremblay et al., 2012). An Rnf complex has been linked to energy conservation in *A. woodii* during caffeate respiration (Hess et al., 2013). Genomic analyses have revealed the presence of an Rnf-complex in other Clostridiales (*Clostridium, Eubacterium*), in *Thermoanaerobacter, Thermaanaerovibrio* and in some Achaeans (Biegel et al., 2011 and Schlegel et al., 2012).

In an analysis of the genome and transcriptome of *C. phytofermentans*, Petit et al. (2015) proposed a membrane energetics model that includes a sodium-pumping Rnf-complex and three sodium ATPases, but this model was not tested experimentally. We hypothesized that the Rnf complex of *C. phytofermentans* contributes to ethanol formation by producing NADH and that it also contributes the formation of a Na\(^+\) or H\(^+\) ion gradient, which drives trans-membrane ATP synthase(s). Research related to this hypothesis and
characterization of the \textit{C. phytofermentans} Rnf complex and membrane energetics are presented in Chapter 5 of this dissertation.

The genome of \textit{C. phytofermentans} also encodes a membrane-bound pyrophosphatase (PPase) (Cphy\_1812) that couple’s energy of pyrophosphate (PP\textsubscript{i}) hydrolysis to the translocation of Na\textsuperscript{+} or H\textsuperscript{+} across the cell membrane (Luoto et al., 2013). PP\textsubscript{i} is an important energy source for the phosphorylation of fructose-6-phosphate and conversion of phosphoenolpyruvate to pyruvate (Petit et al., 2015). \textit{C. phytofermentans} also possess glycolytic enzymes such an adenylate kinase, which exclusively use PP\textsubscript{i} and can increase ATP yield of glycolysis (Mertens et al., 1993). We hypothesize that the membrane-bound pyrophosphatase in \textit{C. phytofermentans} may be involved in generating a Na\textsuperscript{+}/H\textsuperscript{+} trans-membrane gradient and also may use the Na\textsuperscript{+}/H\textsuperscript{+} gradient to produce PP\textsubscript{i} for glycolysis while reducing the need for acetate-dependent ATP synthesis.

\textbf{2.4 Summary and Research Approach}

This dissertation examines the physiology of \textit{C. phytofermentans} in plant biomass breakdown, the formation of a bacterial microcompartment and the role of membrane energetics in ethanol production. Although it is known that ethanol production by \textit{C. phytofermentans} varies with growth substrate, measurements of cell growth and observations of cell-substrate interactions in plant biomass substrates have not been possible due to the inability to differentiate cells from insoluble plant biomass substrates. In Chapter 3 of this dissertation a dual-fluorescent stain method to visualize \textit{C. phytofermentans} cells in the presence of plant biomass is presented along with the application of this method in investigations of cell growth kinetics on insoluble plant
biomass substrates. Using this staining method and fluorescence microscopy we present results of analyses of cell growth dynamics with three different plant biomass substrates: Whatman #1 filter paper, wildtype *Sorghum bicolor* and a reduced lignin mutant *bmr-6/bmr-12 Sorghum bicolor*. Cell growth kinetics are correlated with ethanol production over a period of 5 days.

Chapter 4 describes transmission electron microscopy methodology for visualizing bacterial microcompartments (PDU metabolosomes) in *C. phytofermentans* cells grown with fucose or rhamnose as substrate. These methods were used to confirm the presence of BMCs in *C. phytofermentans* (Petit et al., 2013).

In Chapter 5 of this dissertation, a membrane energetics model for *C. phytofermentans* is presented. Genomic analyses, structural bioinformatics and biochemical assays were used to identify three membrane-bound ATPases, including two Na⁺-translocating ATPases, an F-type and a V-type, and a third unknown type of ATPase, possibly H⁺-translocating. A dual Na⁺/H⁺ pyrophosphatase was identified by sequence alignment and structural bioinformatics. Finally, a possible H⁺-pumping Rnf complex with ferredoxin oxidoreductase activity was identified. We propose that *C. phytofermentans* utilizes these membrane-bound protein complexes to generate electrochemical gradients, produce ATP (possibly reducing acetate production), generate PPᵢ for glycolysis and NADH for ethanol formation.

Chapter 6, the final chapter of this dissertation, includes a discussion of the significance of the research and concluding remarks.
CHAPTER 3

DIRECT IMAGE-BASED ENUMERATION OF CLOSTRIDIUM PHYTOFERMENTANS CELLS GROWING ON INSOLUBLE PLANT SUBSTRATES

3.1 Abstract

A dual fluorescent dye protocol to visualize and quantify Clostridium phytofermentans ISDg (ATCC 700394) cells growing on insoluble cellulosic substrates was developed by combining Calcofluor White staining of growth substrate with cell staining using the nucleic acid dye, Syto 9. Cell growth, cell-substrate attachment, and fermentation product formation were investigated in cultures containing either Whatman #1 filter paper, wild-type Sorghum bicolor, or an S. bicolor reduced lignin double mutant, bmr-6/bmr-12, as growth substrate. After three days of growth, cell numbers in filter paper cultures were 6.0- and 2.2-fold higher than cell numbers in cultures with wild-type sorghum and lignin double mutant sorghum, respectively. However, cells produced more ethanol per cell when grown with either sorghum substrate than with filter paper as substrate. Ethanol yields of cultures were significantly higher with lignin double mutant than with wild-type sorghum or filter paper as substrate. Moreover, ethanol production correlated with cell attachment in sorghum cultures: 90% of cells were directly attached to the lignin double mutant sorghum substrate, while only 76% of cells were attached to wild-type sorghum substrate. With filter paper as growth substrate, ethanol production was correlated
with cell number; however, with either wild-type or mutant sorghum, ethanol production did not correlate with cell number, suggesting that only a portion of the microbial cell population was active during growth on sorghum. The dual-staining procedure described here may be used to visualize and enumerate cells directly on insoluble cellulosic substrates, enabling in-depth studies of interactions of microbes with plant biomass.

3.2 Introduction

Microbial decomposition of plant biomass is central to nutrient cycling in numerous varied environments and this process plays a key role in the cycling of carbon on the planet. In anoxic environments rich in decaying plant material, diverse communities of interacting microbes are responsible for the degradation of cellulose, complex polysaccharides, and other abundantly produced plant cell wall components. Given that most of these substrates are insoluble, microbial decomposition of plant biomass occurs exocellularly, and breakdown products may become available to other community members forming a basis for multifarious interactions that occur in these environments (Leschine 1995).

Anoxic decomposition of plant biomass is also tied to health and nutrition in animals by way of gastrointestinal tract microbial communities. Most animals lack the enzymatic capacity required to digest cellulose and many other components of plant cell walls, and instead rely on plant biomass-decomposing microbial communities to provide nutrition from plant fiber. Ruminants, a group of herbivorous mammals, degrade forage in a specialized foregut organ, the rumen. The microbial community housed in the rumen plays an essential role in development and health of the ruminant by decomposing and fermenting plant materials, and forming products, such as volatile fatty acids, that serve as
essential nutrients (Jami, Israel et al. 2013). In humans, complex plant carbohydrates, also known as dietary fiber, serve as substrates for intestinal microbes that ferment these components of plant biomass to short chain fatty acids, which are tied to colonic and systemic health (McDougall GJ 1996, Wong et al., 2006, Flint, et al., 2012, Koropatkin, et al., 2012 and Larsbrink et al., 2014).

Microbial interactions with plant biomass are also central to technologies for the conversion of lignocellulosic feedstocks into useful products. For example, ethanol is an important renewable energy source that may replace petroleum-based transportation fuels and reduce CO$_2$ emissions (Sticklen 2008, Slade et al., 2009, Dwivedi et al., 2015). Development of more sustainable processes for second generation biofuels derived from lignocellulosic biomass is limited by the recalcitrance of the substrate, primarily due to its lignin content, and often requires expensive pretreatment and use of hydrolytic enzymes (Ragauskas, Williams et al. 2006, Chen and Dixon 2007, Sanderson 2011). An alternative strategy to convert lignocellulosic feedstocks into biofuels is consolidated bioprocessing, where microbes capable of producing a complex set of glycoside hydrolases decompose plant biomass and ferment the products in a single step, for a more cost-effective process with savings in capital and operational costs (Lynd et al., 2005, Jin, Balan et al., 2011, Tolonen et al., 2011, Zuroff et al., 2013, Yee et al., 2014 and Petit et al., 2015). However, details of microbe-biomass interactions and the impacts on cell growth and fermentation are unknown due to a lack of methods for visualizing and quantifying microbial cells in the presence of plant biomass.
Here we present a simple and effective method for visualizing and quantifying microbes interacting with insoluble plant substrates. The method involves separately staining cells and substrate with two different fluorescent dyes. To evaluate our method, we used the cellulolytic microbe *Clostridium phytofermentans* that produces ethanol as its primary fermentation product and grows using a wide array of simple and complex carbohydrate components of plant biomass as substrates (Warnick et al., 2002 and Petit et al., 2015). We demonstrate that the dual-staining procedure may be used to determine characteristics of *C. phytofermentans* growth and substrate attachment with insoluble cellulosic substrates.

### 3.3 Materials and Methods

#### 3.3.1 Bacterial strain and culture conditions

*C. phytofermentans* ISDg (ATCC 700394) was cultured using the anaerobic techniques of Hungate in a modified form of medium GS-2C (Warnick, Methe et al. 2002) containing the following (g/l): yeast extract, 6.0; urea, 2.1; KH$_2$PO$_4$, 2.9 K$_2$HPO$_4$ 1.5; Na$_2$HPO$_4$, 6.5; trisodium citrate dihydrate, 3.0; L-cysteine hydrochloride monohydrate, 2.0; and resazurin, 1; with pH adjusted to 7.0 using KOH. This basal medium was supplemented with 0.3% (wt/vol) of the specific substrate (Whatman #1 filter paper, wild-type *Sorghum bicolor*, or *brown midrib* (*bmr*) reduced lignin double mutant *S. bicolor* *bmr*-6/*bmr*-12) ball-milled as described by Leschine and Canale-Parola (Leschine and Canale-Parola 1983). Cultures were incubated at 30°C under anaerobic conditions (100% N$_2$) as described by Hungate (Hungate et al., 1966).
3.3.2 Dual-staining procedure

Samples were prepared following a procedure similar to that described previously (Zuroff et al., 2014). Prior to staining, each culture was diluted 1:1 with basal medium by adding 1 ml of culture to 1 ml sterile basal medium and vortexed at maximum speed for 15 seconds. This diluted culture was further diluted 1:1 (50 μl in 50 μl of sterile basal medium) and vortexed. Eight μl of a 10-μg/ml solution of Calcofluor White (Fluorescent Brightener 28, Sigma-Aldrich) were added to the diluted culture sample and incubated for 8 min in the dark. Subsequently, 0.3 μl of SYTO® 9 (LIVE/DEAD BacLight Bacterial Viability Kit, Thermo Fisher Scientific) were added to the sample and incubated for 7 min in the dark. A volume of 16 μl of stained sample was placed on a slide and covered with a 22 x 22 mm cover slip. Figure 1 provides a diagrammatic representation of the dual-staining protocol.

3.3.3 Fluorescence microscopy and image processing

A Nikon Eclipse E600 fluorescence microscope, equipped with a Diagnostic Instruments Spot-RT CCD camera and a 40x objective was used to obtain images. For each sample, differential interference contrast and epifluorescence micrographs were obtained. Images were acquired and processed using SPOT Advanced imaging software. Cellulosic growth substrates stained with Calcofluor White dye was visualized at 385 nm and cells stained with SYTO 9 dye were visualized at 480 nm. For three-dimensional (3D) imaging, multifocal plane images of 1 μM depth were obtained and combined to generate an image
21 µM deep. Subsequently, the Z-stack was de-convolved using Autoquant 2.1 (Media Cybernetics) and the three-dimensional image was rendered using Imaris 6.0 (Bitplane) software.

### 3.3.4 Cell quantification

Micrographs were taken at ten different locations on each slide. A custom-written script applied image dimensions, and cell counts were performed manually using Image J (http://rsb.info.nih.gov/ij/). Substrate-attached cells and free cells were separately counted. Vegetative and sporulating cells were included in cell counts. Cell numbers are the average of triplicate determinations.

### 3.3.5 Determination of fermentation products

Non-gaseous fermentation products were determined by HPLC. Ethanol and acetate concentrations in culture supernatant fluids were measured using a BioRad Aminex HPX-87H 300x7.8 mm column at 30°C with 5 mM H₂SO₄ as mobile phase at a flow rate of 0.60 ml/min in a Shimadzu LC-20AD HPLC with a RID-10A Refractive Index Detector.

### 3.3.6 Data analysis

Data analysis was performed using Prism 6.0 (GraphPad Software) One-way ANOVA with a Tukey’s post-test to measure variance. Error bars represent standard deviation of the mean.
3.4 Results

3.4.1 Visualization of C. phytofermentans cells growing on insoluble substrates using the dual-staining procedure.

Cellulolytic bacteria, such as C. phytofermentans, are known to adhere to their cellulosic substrates and many species form biofilms on this nutritive surface (Alonso et al., 2008, Wang et al., 2011, Young et al., 2012, Dumitrache et al., 2013 and Zuroff et al., 2014). However, differentiating cellulolytic microbial cells from their insoluble growth substrates, enumerating cells, and quantifying cell population growth are major challenges. Therefore, the use of light microscopy and fluorescent dyes to distinguish plant-decomposing microbes from their growth substrates is appealing since it would enable researchers to measure microbial growth on plant biomass in terms of an increase in cell number while also observing microbe-substrate interactions. Representative images showing cell-substrate interactions are presented in Fig. 2. A nucleic acid dye, Syto 9, (LIVE/DEAD BacLight Bacterial Viability Kit; (Boulos et al., 1999) was used to stain bacterial cells and Calcofluor White, a cellulose/chitin dye, stained plant material and filter paper. In order to demonstrate the difficulty of visualizing cells growing on insoluble plant substrates by light microscopy, differential interference contrast (DIC) micrographs were also obtained (Fig. 2A). However, when viewed by fluorescence microscopy at 385-400 nm, the filter paper and plant fibers were readily visualized due to the intense blue fluorescence of Calcofluor White staining, but bacterial cells were not apparent (Fig. 2B). When samples were illuminated at 480 nm, bacterial cells fluoresced green due to Syto 9 staining of their nucleic acids (Fig. 2C). The two fluorescent images (Fig. 2B and 2C) were
merged in Fig. 2D to demonstrate cell-substrate differentiation with bacterial cells stained green by Syto 9 and the paper and plant fiber stained blue with Calcofluor White.

3.4.2 Cell morphology and substrate interactions visualized by 3D imaging.

In order to visualize interactions of *C. phytofermentans* cells with an insoluble growth substrate, cultures in filter paper medium were stained using the dual-staining procedure and three-dimensional images were obtained as illustrated in Figure 3. Green fluorescing rod-shaped cells of varying lengths were observed, some with swellings at one end, presumably due to endospore formation as described previously by Warnick et al. (Warnick, Methe et al. 2002). Some *C. phytofermentans* cells were aligned parallel to cellulose fibers as observed by Zuroff et al. (Zuroff, Gu et al. 2014), while many cells appeared to be attached perpendicularly to finer threads of the cellulose substrate with the putative endospores located opposite the site of attachment as observed in *Clostridium thermocellum* (27). In the XY cross-sectional planes, cells were visualized within the substrate. Such 3D images indicate the potential of the dual staining protocol to image bacterial cells interacting with insoluble plant biomass substrates.

3.4.3 Cell growth and ethanol production during fermentation of insoluble substrates by *C. phytofermentans*.

Measurements of growth of bacteria on their insoluble substrates are problematic for several reasons. Viable counts on plates of agar media (Hungate 1950, Kistner 1960
and Hendricks et al., 1995) are complicated by the propensity of cellulose-decomposing cells to adhere to their substrate, frustrating efforts to accurately dilute cultures. Also, viable counts are time-consuming and challenging for fastidious anaerobes. Our method, which allows rapid, direct enumeration of cellulolytic bacterial cells, is facilitated by the use of two fluorescent dyes that enhance the contrast between microbes and cellulosic substrates.

Growth of *C. phytofermentans* on filter paper, wild-type *S. bicolor*, or lignin double mutant *S. bicolor bmr-6/bmr-12* was measured (Fig. 4A). With filter paper as substrate, cell numbers increased over five days, whereas cell numbers on both wild-type and lignin double mutant sorghum reached a maximum at day 3 with $1.1 \times 10^8$ cells/ml and $2.7 \times 10^8$ cells/ml, respectively (Fig. 4A). However, the ethanol concentration in cultures with all of the three substrates steadily increased for 5 days (Fig. 4B). Consistent with previously reported results (Lee et al., 2012), cultures with lignin double mutant sorghum as substrate produced significantly more ethanol than cultures grown on wild-type sorghum. Interestingly, cultures with filter paper as substrate produced significantly less ethanol than cultures with an equivalent amount of either wild-type or lignin double mutant sorghum (Fig. 4B), indicating the important chemical differences in these substrates. When filter paper served as substrate, after day 2, ethanol production per cell decreased (Fig. 4C) as cell numbers increased (Fig. 4A). Since both vegetative and sporulating cells were included in cell counts, reduced ethanol production per cell may reflect cell differentiation and the onset of sporulation, which would suggest that sporulating cells are less metabolically active. Unexpectedly, ethanol production per cell in cultures grown with either wild-type or lignin double mutant sorghum increased after three days of growth (Fig. 4C) when cell
numbers were decreasing (Fig. 4A). Possibly, with an early onset of sporulation, a portion of the microbial cell population was less metabolically active during the first three days of growth on sorghum, and, upon completion of sporulation, mature spores, which were not counted, were lost from the recorded cell counts. These results also suggest that limiting sporulation may be an effective strategy to increase metabolic activity and ethanol production in *C. phytofermentans*.

Figure 4 also includes a direct comparison of cell number (Fig. 4D), substrate attachment (Fig. 4E) and ethanol production (Fig. 4F) after three days of *C. phytofermentans* growth on the three different biomass substrates. Although cell growth on filter paper exceeded growth on either sorghum substrate (Fig. 4D), there was no statistically significant difference in the percentage of cells attached to filter paper (88%) and to lignin double mutant sorghum (89%) (Fig. 4E). However, cell attachment to wild-type sorghum was significantly lower than to either filter paper or lignin double mutant sorghum (Fig. 4E). The increased microbial cell attachment to the lignin double mutant sorghum as compared to wild-type sorghum may reflect greater access to cellulose and other polysaccharide components of biomass in the lignin double mutant sorghum. While cell numbers in cultures with either sorghum substrate were low compared to cultures growing on filter paper (Fig. 4D), the concentration of ethanol produced with wild-type sorghum (17.5 mM) or lignin double mutant sorghum (20.7 mM) was significantly greater than that produced with filter paper as substrate (13.7 mM) after three days of growth (Fig. 4F), possibly due to a higher content of readily fermentable polysaccharides in the sorghum substrates.
These experiments indicated that the *C. phytofermentans* growth cycle varies greatly depending on the cellulosic substrate. With filter paper as growth substrate, ethanol production was strongly correlated with cell number (Fig. 5A). Surprisingly, little or no correlation was found between cell number and ethanol production with either wild-type sorghum (Fig. 5B) or lignin double mutant sorghum (Fig. 5C) as growth substrate. As shown in Figure 4, ethanol production occurred later in the growth cycle on both sorghum substrates when *C. phytofermentans* cell numbers were decreasing, indicating that some cells remained active while the overall cell population decreased, possibly due to sporulation.

### 3.5 Discussion

The dual-staining procedure described here is a simple and efficient method to visualize and enumerate cells in the presence of insoluble plant biomass substrates. This method provides direct total cell counts, and may replace or complement complex and indirect methods for measuring microbial growth on cellulosic substrates (Yang et al., 2009). Also, growth characteristics of co-cultures and other complex communities may be assessed using this method if community members are morphologically distinguishable. Importantly, the dual staining procedure enables studies of interactions of microbial cells with insoluble substrates such as plant biomass feedstocks. It is possible to employ this method to study cell-substrate interactions using 3D visualization with little impact on the sample. Also, coupling the dual-staining procedure with image processing techniques, cell counts may be rapidly performed enabling high-throughput analyses. A possible limitation of this method might be interference due to extracellular polymeric substances (EPS)
produced by biofilm formation on substrates, making cell visualization more difficult; however, EPS formation did not pose a problem in the current study.

Our observation of higher ethanol production by \textit{C. phytofermentans} during fermentation of lignin double mutant sorghum as compared to wild-type sorghum is consistent with the findings of Lee et al (Lee et al., 2012). Also, correlating with higher ethanol production, we observed higher microbial cell attachment to the lignin double mutant sorghum as compared to wild-type sorghum. This may be attributed to the fact that the lignin content of plant biomass adversely affects substrate attachment, decomposition, fermentation, and subsequently, ethanol production (Lynd et al., 2002, Fu et al., 2011, Lee et al., 2012 and Lacayo et al., 2013).

Using the dual-staining procedure, we examined \textit{C. phytofermentans} growth on different insoluble substrates. According to company literature, Whatman #1 filter paper is prepared from cotton linters that are treated to achieve an alpha cellulose content of at least 98\%, and as such, it is a highly processed, relatively pure form of cellulose. We observed very different growth characteristics on purified cellulose (filter paper) as compared to growth on wild-type and mutant sorghum. Both cell numbers and ethanol concentration increased for five days when filter paper served as substrate. However, with wild-type and mutant sorghum as substrate, cell numbers reached a maximum after three days of growth and then decreased while ethanol concentration continued to increase for five days. With filter paper as growth substrate, ethanol production was correlated with cell number but, unexpectedly, little or no correlation was found between cell number and ethanol production with either wild-type or mutant sorghum as growth substrate. This result indicated that only a portion of the microbial cell population remained active during growth.
on sorghum, possibly due to differentiation and the onset of sporulation in some cells, resulting in reduced metabolic activity and lower ethanol production.

The dual-staining procedure described here is an effective means for quantifying microbial cells growing on plant biomass substrates. Due to its simplicity, quantitative data may be obtained rapidly and efficiently. In the dual-staining procedure, individual fluorescent dyes are added at separate times. Syto 9, a nucleic acid dye, stains cells and emits a green fluorescence that is observed by fluorescence microscopy. Cellulosic biomass substrates are stained by Calcofluor White, which emits a blue fluorescence when exposed to UV light. We demonstrated that visualization and enumeration of microbial cells growing on different cellulosic substrates may be performed by obtaining micrographs and counting cells using Image J. Statistical analyses may be used to evaluate differences in cell growth and substrate attachment, which in turn, may be correlated with fermentation product formation. Finally, use of the dual-staining procedure for three-dimensional visualization of microbes, such as *C. phytofermentans*, growing on insoluble plant substrates is a useful tool for gaining insight into plant-microbe interactions in a broad range of applications from biofuels to human health.
Figure 3.1 Diagrammatic overview of dual-staining protocol.
Figure 3.2 Differential interference contrast (DIC) and epifluorescence micrographs of *C. phytofermentans* cultured for 3 days with filter paper, wild-type *S. bicolor* (WT Sorgum), or lignin double mutant *S. bicolor* (*bmr-6/bmr-12* Sorghum). Cultures were prepared for microscopy using the dual-staining procedure. For each sample, one field is shown imaged by DIC microscopy (A), and fluorescence microscopy at 385–400 nM showing cellulose fibers stained with Calcofluor White (B) and at 480 nM showing Syto 9 stained cells (C). The epifluorescence images in rows B and C are merged in the row D images. Stained culture preparations were diluted with sterile basal culture medium prior to slide preparation: the filter paper culture was dilute 1:3 and sorghum cultures were dilute 1:1.
Figure 3.3 Two-dimentional rendering of a 3D image of *C. phytofermentans* cultured for five days in filter paper medium. The culture was prepared for microscopy using the dual-staining procedure. Multiple focal plane images at a dept of 1 µm were obtained and used to generate a 3D images.
Figure 3.4 Cell growth (A) and ethanol production (B, C) by *C. phytofermentans*, and cell numbers (D), cell attachment to substrate (E) and ethanol concentration (F) after three days of growth with filter paper (□), wild-type *S. bicolor* (Δ), or lignin double mutant *S. bicolor bmr-6/bmr-12* (○) as growth substrate. The concentration of ethanol in cultures is expressed as mM (B, F) and the amount of ethanol produced per cell is expressed as nmoles per cell (C). Error bars represent standard deviation of the mean (n=3). Statistically significant differences are represented with asterisks; one asterisk for $P \leq 0.05$, two asterisks for $P \leq 0.01$, and three asterisks for $P \leq 0.001$. 
Figure 3.5 Correlation of ethanol production with cell number during growth. Ethanol production is given as a function of the cell number in cultures with filter paper (A), wild-type *S. bicolor* (B) and lignin double mutant *S. bicolor bmr-6/bmr-12* (C) as growth substrate.
CHAPTER 4

IMAGING INTRACELLULAR PROTEIN-BOUND MICROCOMPARTMENTS IN BACTERIAL CELLS

4.1 Abstract:

Bacterial microcompartments (BMCs) are polyhedral organelles that harbor metabolic enzymes and are bounded by a protein shell. Three loci with genes encoding shell proteins of BMCs were identified in the genome of *Clostridium phytofermentans*, a soil bacterium that directly converts biomass to biofuels. One of the BMC loci was homologous to a BMC-encoding locus in the human gut commensal microbe *Roseburia inulinivorans* and was hypothesized to be involved in the conversion of fucose and rhamnose to propanol and propionate in *C. phytofermentans*. In order to demonstrate the presence of BMCs within cells of *C. phytofermentans* using transmission electron microscopy (TEM), it was necessary to develop a fixation, embedding and staining protocol that would image BMCs while preserving the structure of the bacterial cell envelope, the cell membrane and wall. A procedure involving cell fixation with sodium cacodylate buffer containing glutaraldehyde and picric acid and staining with osmium tetroxide and potassium ferrocyanide was developed. Thin sections of *C. phytofermentans* cells examined using TEM revealed the presence of polyhedral structures, presumably BMCs, in *C. phytofermentans* cells grown with fucose or rhamnose but not with glucose as growth substrate.
4.2 Introduction:

Bacterial microcompartments (BMCs) are polyhedral organelles with protein shells that harbor metabolic enzymes. The first BMCs to be identified were the carboxysomes (Shively et al., 1973). Carboxysomes contain enzymes that fix CO$_2$ including ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO). Other varieties of BMCs are found in multiple microbes; some are implicated in human health such as 1,2-propanediol- and ethanolamine-producing BMCs involved in the pathogenicity of *Salmonella enterica* (Joseph et al., 2006, Klumpp et al., 2007, Sriramulu et al., 2008, and Harvey et al., 2011). Moreover, it has been found that microbes in the *Planctomycetes* phylum are capable of encoding BMCs capable of breaking down rhamnose, fucose and fucoidan in the presence of oxygen (Lage et al., 2014).

*Clostridium phytofermentans* is a soil bacterium that directly converts all major components of plant biomass to ethanol as its primary product of fermentation (ref: Warnick et al., 2002). Analysis of the genome sequence of *C. phytofermentans* revealed the presence of three loci with genes encoding shell proteins of BMCs (Petit et al., 2013 & 2015). Transcriptional profiling analyses indicated that one of the three loci is expressed during growth of *C. phytofermentans* on fucose or rhamnose (Petit et al., 2013). This BMC locus has homology to a BMC-encoding locus in the human gut microbe, *Roseburia inulinivorans*, a member of the Lachnospiraceae family (phylum Firmicutes, class Clostridia), which also includes *C. phytofermentans* (Scott et al., 2006 & 2011). In *R. inulinivorans*, BMCs encoded by this locus are believed to be involved in the metabolism of 1,2-propanediol, an intermediate in the conversion of fucose to propionate and propanol (Scott et al., 2006 and Petit et al., 2013). In *S. enterica* 1,2-propanediol is produced during
growth on either fucose or rhamnose and secreted, but may be taken up and converted into propionate and propanol by enzymes encoded by genes within a BMC locus (Obradors et al., 1988, Baldona et al., 1988, Buan et al., 2004, Penrod and Roth 2006, Cheng et al., 2011, Crowley et al., 2010, Harvey et al., 2011 and Chen et al., 2012).

Fucose and rhamnose are deoxyhexose sugars (also known as methyl-pentoses) found in plant cell walls, in bacterial cells, in some insect species, and on the surface of mammalian cells as components of glycans and glycopeptides. In plant cell walls, fucose and rhamnose form a component of pectin, highly hydrated, branched, heterogeneous polysaccharides, notably in rhamnogalacturonans (Leschine et al., 2005). Fucose is commonly found on plant and mammalian cell surfaces as N- and O-linked glycosylated proteins (glycans); for example, fucose is highly abundant in the intestinal tracts of mammals (Robbe et al., 2003) where it plays an important role in signaling of and colonization by members of the gut microbiome. (Pacheco et al., 2012). Fucosylated proteins of the intestinal epithelium also play a role in maintaining the intestinal microbiome during illness in the host (Pickard et al., 2014).

*C. phytofermentans* is a microbe capable of converting multiple components of plant biomass into ethanol, making it an attractive tool for the bioethanol industry. In this study, we developed a procedure for visualizing BMCs within cells of *C. phytofermentans* grown with fucose or rhamnose as substrate. A TEM embedding protocol was developed for stabilizing the cell wall and improving BMC detection in transmission electron micrographs. Visualization of BMCs within cells of *C. phytofermentans* correlated with the expression of several genes homologous to those found in the BMC locus of *R. inulinivorans*, and also with expression of fucose dissimilatory enzymes and an ATP-
binding cassette transporter as dominant transcripts. Taken together, the data are consistent with the conclusion that the *C. phytofermentans* BMC locus encodes a microcompartment involved in the fermentation of fucose and rhamnose (Petit et al., 2013).

### 4.3 Materials and Methods:

#### 4.3.1 Bacterial strain and conditions:

*C. phytofermentans* was cultured in a modified form of an anaerobic medium containing the following (g/l): yeast extract, 6.0; urea, 2.1; KH₂PO₄, 4.0; Na₂HPO₄, 6.5; trisodium citrate dihydrate, 3.0; L-cysteine hydrochloride monohydrate, 2.0; and resazurin, 1; with pH adjusted to 7.0 using KOH. This medium was supplemented with 0.3% (wt/vol) of the specific substrate (glucose, fucose or rhamnose) added as a filter-sterilized solution to the sterile medium. Triplicate liquid cultures were incubated at 30°C under anaerobic conditions (100% N₂) as described by Hungate (Hungate et al., 1950). Growth was determined spectrophotometrically by monitoring changes in optical density at 660 nm.

#### 4.3.2 Transmission Electron Microscopy:

*C. phytofermentans* cells, cultured with fucose or rhamnose as growth substrate, were grown until mid-exponential phase, fixed for 1 hr at room temperature with 2.5% glutaraldehyde, 0.03% picric acid, and 0.5% NaCl in 0.05 M cacodylate buffer and washed three times in 0.05 M cacodylate buffer containing 0.5% NaCl. Gel pellets were formed by adding 3% agarose, incubated in ice for 15 min followed by post-fixation with 1% osmium tetroxide, and 1.5% potassium ferrocyanide in 0.05 M cacodylate buffer for 2 hrs. The gel
pellets were washed with water and stained by submerging in 2% uranyl acetate for 1 hr, followed by three additional water washes and subsequent dehydration by increasing ethanol concentration. Samples were embedded in 26% Epon-812, 15.5% Araldite-502, 57% dodecenyl succinic anhydride and 1.5% epoxy accelerator DMP-30, and polymerized at 60°C overnight. Thin sections (70 nm) were cut using a Reicher-Jung Ultracut E microtome; sections were stained with lead citrate, and observed using a JEOL 100CX transmission electron microscope at 100 kV.

4.4 Results and Discussion:

Cell wall structure was stabilized by using a cell fixation and embedding protocol that incorporated picric acid and potassium ferrocyanide. As a control, thin sections of *C. phytofermentans* cells grown using glucose as substrate were prepared; BMCs were not observed within cells cultured on glucose (Fig. 4.1). However, BMCs structures were visualized in *C. phytofermentans* cells grown in a culture medium containing fucose (Fig. 4.2) or rhamnose (Fig. 4.3). The diameter of the BMCs in cells grown with either fucose or rhamnose ranged from 30 to 50 nm. Although potassium ferrocyanide and osmium tetroxide enhanced the visualization of BMCs and the overall appearance of *C. phytofermentans* cells in micrographs, the mechanisms behind the enhanced contrast of proteins are unknown. It is suspected that reduction of osmium by potassium ferrocyanide plays a role (Bozzola and Russell, 1998). The addition of picric acid at the initial fixation step most likely enhanced of the preservation of cell envelope structure. The embedding protocol developed for visualizing BMCs efficiently and effectively enhanced the contrast of samples while preserving cell wall structure.
The protocol developed in this study enabled visualization of BMCs within cells of *C. phytofermentans* grown with fucose or rhamnose as substrate. The presence of BMCs within cells of *C. phytofermentans* correlated with the expression of several genes homologous to those found in the BMC locus of *R. inulinivorans*. Also, the appearance of BMCs in *C. phytofermentans* cells cultured on fucose correlated with expression of fucose dissimilatory enzymes and an ATP-binding cassette transporter as dominant transcripts. Results presented here, along with findings presented in Petit et al., 2013 (Petit et al., 2013), indicate that the *C. phytofermentans* BMC locus encodes a microcompartment involved in the fermentation of fucose and rhamnose.
Figure 4.1 Transmission electron micrograph of *C. phytofermentans* cells from cultures grown with glucose as growth substrate. Polyhedral structures were not observed in cells from glucose-grown cultures.
Figure 4.2 Transmission electron micrograph of *C. phytofermentans* cells from cultures grown in fucose as growth substrate. Some cells contained polyhedral structures (arrow), presumably bacterial microcompartments.
Figure 4.3 Transmission electron micrograph of *C. phytofermentans* cells from cultures grown in rhamnose as growth substrate. Multiple polyhedral structures, presumably bacterial microcompartments, were observed in thin sections of *C. phytofermentans* grown in rhamnose culture medium.
CHAPTER 5

MEMBRANE ENERGETICS AND ENERGY CONSERVATION IN THE
CELLULOLYTIC ETHANOLOGEN CLOSTRIDIUM PHYTOFERMENTANS

5.1 Abstract:

_Clostridium phytofermentans_ is a microbe from forest soil that ferments a wide array of plant polysaccharides and produces ethanol as its primary fermentation product. Realizing the industrial potential of this microbe as a catalyst for biofuels production requires a full understanding of the metabolic pathways and associated membrane energetic mechanisms related to ethanol production. Genome and transcriptome analyses of _C. phytofermentans_ have revealed numerous highly expressed genes involved in membrane energetics and energy conservation that are potentially related to ethanol production, including an Rnf complex (Cphy_0211-0216), a membrane-bound sodium- and proton-translocating pyrophosphatase (Cphy_1812), and three ATPases. Identification of sodium-translocating F-type (Cphy_3735-3742) and V-type (Cphy_3070-3077) ATPases was confirmed by the presence of conserved sodium binding motifs in the c subunits of these enzymes. In support of this annotation, ATP synthase activity was inhibited by the sodium ionophore, ETH 2120. Structural information for the third ATPase (V-type ATPase; Cphy_3440-3447) was not available and its ion specificity was not directly determined. Integral ferredoxin oxidoreductase activity of the Rnf-complex was assayed by using inverted membrane vesicle preparations and following reduction of ferredoxin by NADH. Activity required ATP, and was inhibited by the proton ionophore TCS, but not by the sodium ionophore ETH 2120, indicating that the reaction was driven by a proton electrochemical gradient, generated
through the activity of the previously uncharacterized V-type ATPase. These results indicate that *C. phytofermentans* conserves energy by generating both sodium and proton electrochemical gradients across the membrane. In support of this conclusion, *C. phytofermentans* growth was inhibited by both the proton ionophore TCS and the sodium ionophore ETH 2120. Based on these observations, we conclude that *C. phytofermentans* is able to conserve energy through ferredoxin oxidoreductase activity of the Rnf-complex, resulting in increased levels of NADH for ethanol production.

5.2 Introduction:

*Clostridium phytofermentans* is a mesophilic anaerobe that ferments multiple plant substrates, including cellulose, hemicellulose, pectin, and starch, and produces ethanol as its primary fermentation product (Warnick et al. 2002). Additionally, fermentation of fucose and rhamnose into propanol and propionate makes *C. phytofermentans* a strong candidate for the industrial conversion of plant biomass into various bioproducts including liquid transportation fuels (Petit et al., 2013; Petit et al., 2015). *C. phytofermentans* also has been deployed as a catalyst in an assay to detect plant biomass phenotypic variations as a measure of plant feedstock quality (Lee et al., 2012). The effectiveness of this assay is tied to a property of the microbe, that ethanol production is proportional to feedstock quality (digestibility), and recognition of the ease and accuracy of ethanol concentration measurements. Technologies for improved ethanol productivity also have been developed, including a symbiotic bioprocessing of cellulose to ethanol involving coculturing *C. phytofermentans* with *Saccharomyces cerevisiae* (Zuroff et al., 2013) and a metabolic engineering strategy to improve ethanol tolerance (Tolonen et al., 2015)
Genomic and transcriptomic analyses have indicated that *C. phytofermentans* utilizes the Embden-Meyerhof-Parnas pathway to metabolize sugars to pyruvate, which is converted to Acetyl-CoA, the precursor for acetate and ethanol formation. (Petit et al., 2015). Acetyl-CoA may be reduced to ethanol in reactions coupled to the oxidation of NADH, or converted to acetate by phosphate acetyl transferase and acetate kinase, along with formation of ATP. However, production of ethanol as the primary product of fermentation suggests that *C. phytofermentans* may also use a chemiosmotic mechanism to meet energy (ATP) requirements. We hypothesized that the ferredoxin:NAD\(^+\) oxidoreductase activity of an Rnf complex, which had been identified in analyses of the *C. phytofermentans* genome, transcriptome, and proteome (Petit et al., 2015 and Tolonen et al., 2011 & 2013), contributes to a trans-membrane electrochemical gradient supplying energy for ATP synthesis while also replenishing the pool of NADH, providing reductant for ethanol production.

The specific ion dependence of the *C. phytofermentans* Rnf oxidoreductase activity has not previously been reported. In *Clostridium ljungdahlii*, autotrophic growth is dependent on an Rnf–driven proton gradient (Tremblay et al., 2012), while the acetogens *Acetobacterium woodii* and *Eubacterium limosum*, along with several other bacteria, use an Rnf complex to generate a sodium gradient, which contributes to ATP synthesis in these microbes (Hess et al., 2013; Hess et al., 2016; Jeong et al., 2015; Müller et al., 2008).

The genome and transcriptome study mentioned above (Petit et al., 2015) also identified genes for three ATPases among highly expressed genes likely to participate in energy conservation in *C. phytofermentans*. Based on sequence alignment comparisons, an F-type (Cphy_3735-3742) and a V-type (Cphy_3070-3077) ATPase were predicted to be sodium-translocating membrane
complexes. A third ATPase (V-type ATPase cluster Cphy_3440-3447) was not highly similar to any characterized ATPase complexes and its ion specificity was not predicted (Petit et al., 2015). Additionally, highly expressed pyrophosphate-dependent glycolytic enzymes also have been detected in *C. phytofermentans*, and it was hypothesized that utilization of these energetically efficient enzymes (pyrophosphate- rather than ATP-dependent) may indirectly contribute to ethanol production by increasing the ATP yield of glycolysis and decreasing the demand for ATP generation via acetate production (Petit et al., 2015). Interestingly, a membrane-bound sodium-and proton-translocating pyrophosphatase (Cphy_1812) has been identified in the genome of *C. phytofermentans* (Luoto et al., 2012). This enzyme may couple the energy of pyrophosphate hydrolysis to the formation of sodium and proton electrochemical gradients across the membrane to drive ATP synthesis. Conversely, this membrane-integral enzyme may catalyze the synthesis of pyrophosphate, reducing the demand for ATP, by using the energy of an electrochemical gradient, for example, the gradient generated through the activity of the Rnf complex.

The objective of this study is to advance understanding of the metabolic pathways and associated membrane energetic mechanisms related to ethanol production by *C. phytofermentans*. Recent genomic and transcriptomic analyses have identified several proteins that are likely involved in membrane energetics and energy conservation in this microbe (Petite et al., 2015). Structural bioinformatics and biochemical assays were used to characterize the ATPases and Rnf complex. Based on the results of these studies, we generated a metabolic model for energy conservation in *C. phytofermentans* involving both a sodium and proton electrochemical gradient.
5.3 Materials and Methods

5.3.1 Protein sequence alignment, comparison and structure modeling

The website Uniprot (UniProt, 2015) was used for protein sequence identification. Protein sequence alignments were performed using Clustal Omega (Sievers et al., 2011, Goujon et al 2010 and McWilliam et al., 2013) and PROSMAL 3D (Pei et al., 2008). Protein structure homology-modelling was done using SWISS-MODEL (Bordoli et al.,2009). Molecular visualization of the modelled proteins was visualized and rendered using Visual Molecular Dynamics (Humphrey et al., 1996).

5.3.2 Bacterial strain and growth conditions

*C. phytofermentans* ISDg (ATCC 700394) was cultured in a defined medium, M6 (2.0 g/l NaH₂PO₄, 10.0 g/l K₂HPO₄, 1.0 g/l (NH₄)₂SO₄, 1.0 g/l L-cysteine hydrochloride monohydrate, 20 ml/l XT solution (5.0 g/l xanthine and 5.0 g/l thymine in 0.06 N NaOH) 10 ml/l AA1 solution (5.0 g/l of each of the following amino acids: alanine, arginine, histidine, isoleucine, leucine, methionine, proline and valine), and 10 ml/l Bach's trace element (BTE) solution [49]. Resazurin (1 mg/l) was added as an oxidation/reduction indicator. After autoclaving, 30 ml/l CPV5 solution (20 mg/l p-aminobenzoic acid, 1 mg/l biotin, 30 mg/l folinic acid, 80 mg/l nicotinamide, 5 mg/l pantethine, 2 mg/l pyridoxal hydrochloride, 30 mg/l riboflavin, and 10 mg/l thiamine) was added. This medium was supplemented with 0.5% (wt/vol) of glucose. Cultures were incubated at 30°C under anaerobic conditions (100% N₂) as described by Hungate (Hungate 1950).
5.3.3 Effects of ionophores on cell growth

Cells were inoculated into anaerobic culture tubes containing 10 ml M6 media, 25 µM final concentration of the Na⁺ ionophore ETH 2120 or the protonophore 3,3',4',5-tetrachlorosalicylanilide (TCS). Cell growth was measured every 12 hours by optical density at 600 nm.

5.3.4 Preparation of inverted membrane vesicles

Ten milliliters of cell culture were inoculated into 1 L of M6 medium, and incubated at 30°C for 5 days. Cells were harvested by centrifugation at 10,000 x g at 8°C for 45 minutes. After centrifugation, cell pellets were sonicated for 45 minutes and homogenized using a glass tissue grinder. Cell homogenates were resuspended in 5 ml of Buffer A (50 mM Tris-HCl with 2 mM dithiothreitol (DT) at pH 8.0 in N₂), centrifuged at 100,000 x g at 8°C for 45 minutes. Supernatant fluid was removed; cell pellets were resuspended in 2 ml of Buffer A, followed by homogenization using a glass tissue grinder. Washing steps were performed three times. After the final wash, the membrane-associated fraction was resuspended in 2 ml of Buffer A, frozen in liquid N₂ and stored at -80°C.

5.3.5 ATP hydrolysis and ATP synthesis determinations

For ATP hydrolysis, 15 µl of a preparation of C. phytofermentans inverted membrane vesicles (IMVs) was added in 1 ml of Buffer B (100 mM MES buffer, 100 mM Tris-HCl buffer, 5 mM MgCl₂, 200 mM KCl, pH 7.8 in N₂) and the reaction was initiated by the addition of Na₂ATP (2.5 mM, final concentration) at 30°C. Phosphate levels were measured using Abcam’s Phosphate Assay Kit (Colorimetric). ATP synthesis was carried in the same conditions as indicated above.
IMVs were added to Buffer B and the reaction was initiated by the addition of Na₂ADP (2.5mM, final concentration). ATP was measured using a luminometer via the luciferin-luciferase assay at 560 nm excitation and operated at 30°C.

5.3.6 Rnf-associated ferredoxin oxidoreductase activity

Reactions were performed at 30°C in a N₂ atmosphere in anaerobic cuvettes sealed with rubber stoppers. Reaction mixtures contained 1 ml of Buffer C (100 mM Tris-HCl, pH 7.8), 5 mM MgCl₂, 2 mM NaCl, 2 mM dithiothreitol (DT), 30 µM ferredoxin, 5 mM NADH and 10 µl IMVs). The reaction was started by the addition of 2 mM of ATP final concentration. The ionophores ETH 2120 and 3,3’,4’,5-tetrachlorosalicylanilide (TCS) were added at a final concentration of 10 µM each. Ferredoxin reduction was measured at 430 nm.

5.4 Results

5.4.1 Predicted structures of the Na⁺ binding sites of the F-type and V-type ATPase c-rings

To gain insight into the ion specificity of the C. phytofermentans ATPases, structural models of the F-type ATPase and V-type ATPase c-rings were created by homology modeling. The structure of the F-type c subunits from Fusobacterium nucleatum was used as a template to compare with the c-ring from Cphy_3741. The c-ring of Enterococcus hirae, which contains V-type c subunits, was used as a template to compare with the Cphy_3076 c ring. The resulting model for Cphy_3741 consists of 11 c-subunits, with an ion-binding site in between each pair of subunits. The C. phytofermentans F-type c-ring sequence was aligned with Ilyobacter tartaricus, F. nucleatum and Clostridium paradoxum c-rings (Fig. 3). Each binding site contains the specific site
for Na\textsuperscript{+} ion binding: Gln32, Glu65 and Val63. The fourth binding site for Na\textsuperscript{+}, Ser66, is replaced by threonine in the \textit{C. phytofermentans} F-type c-ring sequence. The Cphy_3076 3D protein model consists of 10 c-subunits and has a 53.12\% identity with \textit{E. hirae} (Mizutani et al., 2011). The \textit{C. phytofermentans} V-type c-ring sequence was aligned with \textit{E. hirae}, \textit{E. faecalis}, \textit{Clostridium aspargiforme} and \textit{Clostridium bolteae} (Fig. 4). Results of this analysis indicate that the \textit{C. phytofermentans} V-type c-ring ion-binding groups are highly conserved and consistent with other microbes that contain sodium-binding groups in V-type ATPases. Each binding site contains specific amino acid sequences that bind Na\textsuperscript{+}: Leu61, Thr64, Gln65, Gln110 and Glu139.

5.4.2 Structural model of the pyrophosphatase Na\textsuperscript{+}, H\textsuperscript{+} binding sites

A \textit{C. phytofermentans} membrane-bound pyrophosphatase (-PPase) structural model was generated by homology modeling. The structure of the -PPase dimer from \textit{Bacteroides vulgatus} was used as a template to compare with \textit{C. phytofermentans} ion binding subunits. The resulting model consists of a dimer from the cytosolic side of the membrane. In order to identify the ion-binding sites, the \textit{C. phytofermentans} -PPase sequence was aligned with that of \textit{B. vulgatus}, \textit{Clostridium lentocellum}, \textit{Prevotella dentalis} and \textit{Prevotella melaninogenica}. Results indicate that, in the \textit{C. phytofermentans} -PPase, each binding site contains the specific amino acid subunits involved in the binding of Na\textsuperscript{+} and H\textsuperscript{+}, Thr87, Phe91, Met117 and Asp143.

5.4.3 Effects of ionophores on \textit{C. phytofermentans} growth

In order to identify the specific ion(s) involved in generating an electrochemical gradient in \textit{C. phytofermentans}, we examined growth in the presence of sodium and proton ionophores.
Growth in a defined medium supplemented with glucose as an energy source was inhibited by both the Na\(^+\) ionophore ETH 2120 and the H\(^+\) ionophore TCS. The ionophores inhibited cell growth during the initial phases of exponential growth. Cell morphology did not change and cell lysis did not occur when cells were exposed to the ionophores (Data not shown).

**5.4.4 ATP synthesis in inverted membrane vesicles is driven by a Na\(^+\) gradient**

ATP synthesis in inverted membrane vesicles was examined with and without the Na\(^+\) ionophore ETH 2120 or the H\(^+\) ionophore TCS. ATP synthesis was initiated by the addition of ADP to reaction mixtures. A control sample generated ATP at a rate of 17 µmol/min. The rate of ATP synthesis in the presence of TCS (23 µmol/min) was similar to, or greater than that in control samples. In the presence of ETH 2120, the rate of ATP synthesis was significantly reduced to 3 µmol/min. No ATP synthesis was observed in the absence of ADP or PO\(_4\).

**5.4.5 An ATP driven proton gradient supports NADH-dependent ferredoxin reduction**

In order to determine the specific ion dependence of the *C. phytofermentans* Rnf oxidoreductase activity, NADH-dependant ferredoxin reduction was measured in inverted membrane vesicles. Energy for this reaction was supplied by an electrochemical ion gradient driven by ATP hydrolysis, catalyzed by a membrane-bound ATPase. Thus, we assayed Rnf-complex activity “in reverse” as described by Hess et al., 2013.

Inverted membrane vesicles, ferredoxin and NADH were added to reaction mixtures and activity was initiated by the addition of ATP. Ferredoxin reduction did not occur in the absence of ATP. Interestingly, ferredoxin reduction was significantly reduced when the protonophore TCS
was added to reaction mixtures; however, when the sodium ionophore ETH 2120 was added, ferredoxin reduction was significantly enhanced. These results indicated that the integral ferredoxin oxidoreductase activity of the Rnf-complex is driven by a proton electrochemical gradient, which was generated through the activity of the previously uncharacterized V-type ATPase.

5.5 Discussion

Analysis of the genome of *C. phytofermentans* revealed genes that encode various membrane-bound proteins, which were hypothesized to play a role in membrane energetics and energy conservation (Petit et al., 2015). In order to test the hypothesis, we first analyzed the sequence alignments and modeled one of the V-type ATPases, the F-type ATPase, and the membrane-bound pyrophosphatase. A Na⁺-binding V-type ATPase c-ring protein model for Cphy_3076 was generated using the *E. hirae* c-ring homologue (Fig. 5.1). The *C. phytofermentans* Na⁺ V-type ATPase c-ring shares 53.12% sequence identity with a Na⁺ V-type ATPase from *E. hirae*. The model generated a c-ring with 10 c-subunits including an ion-binding site for each subunit (Fig. 5.1) (Mizutani et al., 2011). The c-ring features include conserved amino acid residues, Leu61, Thr64, Gln65, Gln110 and Glu139, which bind Na⁺ and were perfectly aligned with *E. hirae, E. faecalis, C. aspargiforme* and *C. bolteae* c-rings (Fig 5.2) (Murata et al., 2005). The sequence of the c-ring of *C. phytofermentans* F-type ATPase was modeled using the Na⁺ F-type ATPase c-ring (Cphy_3741) of *Fusobacterium nucleatum*, with a sequence identity of 63.10%, as a template (Schulz et al., 2013) (Fig. 5.3). The model yielded an 11-subunit c-ring with an ion-binding site in each subunit (Fig 5.3). Sequence alignment of the F-type ATPase of *C. phytofermentans* with *I. tartaricus, F. nucleatum, and C. paradoxum* revealed three of four amino
acids that bind Na⁺: Gln32, Glu65 and Val63 (Fig 5.4). The fourth binding ion Ser66 is replaced by threonine, which retains the same hydroxyl groups of serine that binds Na⁺. The acetogen A. woodii contains an F/V-hybrid rotor ring that translocates Na⁺ (Matthies et al., 2014). Interestingly, the c2/3 subunit of the hybrid rotor that binds Na⁺ in A. woodii, contains the same amino acid sequence as C. phytofermentans F-type ATPase. Thus, we conclude that C. phytofermentans has a Na⁺ F-type ATPase. In Petit et al there is a mention of a possible second V-type ATPase cluster (Cphy_3440-3447) however it sequences does not match any known sequence of ATPases.

A membrane-bound ion-pumping pyrophosphatase (PPase) (Cphy_1812) that was a moderately expressed in C. phytofermentans (Petit et al., 2015 and Luoto et al., 2012) was also modeled using a H⁺ PPase from the plant Vigna radiata as template (Lin et al., 2012) (Fig 5.6). Results indicate a 49.13% sequence identity between the proteins. C. phytofermentans -PPase sequence was aligned with B. vulgatus, P. dentallis, C. lentocellum, and P. melaninogenica. Results indicate that in the C. phytofermentans -PPase each binding site contains the specific amino acid subunits involved in binding both Na⁺ and H⁺, Thr87, Phe91, Met117 and Asp143 (Fig 6). Membrane-integral PPases translocate H⁺, Na⁺ or both during pyrophosphate (PPI) hydrolysis. C. phytofermentans possess glycolytic enzymes such an adenylate kinase, which exclusively use PPI, increasing the ATP yield of glycolysis (Mertens, 1993). The membrane-bound pyrophosphatase of C. phytofermentans may use a Na⁺ and H⁺ electrochemical gradient to generate PPI, reducing the demand for ATP generation via acetate production.

Experiments aimed at examining the effects of ionophores on C. phytofermentans growth yielded the surprising result that growth with glucose as substrate was inhibited by both the sodium ionophore ETH 2120 and the protonophore TCS. When ionophores were added at the time of inoculum or at mid exponential phase (Fig. 5.7). In C. ljungdahlii cell growth is inhibited by TCS.
but not ETH 2120 (Tremblay et al., 2012). The opposite occurs in Clostridium paradoxum, which possess a Na\(^+\) F-type ATPase; a sodium ionophore affects growth while the protonophore TCS does not (Ferguson et al., 2006). Inhibition of cell growth by ionophores follows a pattern: C. ljungdahlii possesses an H\(^+\) Rnf complex and a H\(^+\) F-type ATPase, while C. paradoxum has a Na\(^+\) F-type ATPase. In contrast, C. phytofermentans relies on both a H\(^+\) and Na\(^+\) electrochemical gradient for growth, as occurs in F. nucleatum (Schulz et al., 2013).

To determine whether a H\(^+\) or Na\(^+\) electrochemical gradient is involved in ATP synthesis, we measured the ATP synthase activity of inverted membrane vesicles. Activity was not inhibited by the protonophore TCS but was significantly reduced by the sodium ionophore ETH 2120 (Fig. 5.8). This result indicates that C. phytofermentans possess a sodium-pumping V-type ATPase and/or F-type ATPase.

Since a protein structure for the Rnf-complex is not available, we were unable to make a protein model comparison of the A. woodii, and C. ljungdahlii Rnf complexes with that of C. phytofermentans. Previous studies have determined that the C. phytofermentans Rnf-complex genes (Cphy_0211-0216) are 44-61\% similar to A. woodii (Petit et al., 2015). To investigate the activity of the C. phytofermentans Rnf-complex, we measured ATP- and NADH-dependent reduction of ferredoxin using inverted membrane vesicles. In this assay, activity of the Rnf-complex is the reverse of that in cells, using electrons from NADH to reduce ferredoxin. The reaction was initiated in the addition of ATP and ferredoxin reduction was measured at 430 nm (Fig 5.9). Results showed an ATP dependence for ferredoxin reduction. Interestingly, Ferredoxin reduction was significantly reduced in the presence of the protonophore TCS and enhanced in the presence of sodium ionophore ETH 2120, suggesting a proton electrochemical gradient coupling between an ATPase and the Rnf-complex. Based on these results, we conclude that the integral
ferredoxin oxidoreductase activity of the Rnf-complex is driven by a proton electrochemical gradient, which, in our assay, was generated through the activity of the previously uncharacterized H⁺ V-type ATPase (Cphy_3440-3447) (Hess et al., 2012 and Tremblay et al., 2012).

With protein modeling and experimental results reported here, we developed a metabolic model to illustrate the role of membrane energetics in energy conservation in C. phytofermentans, and to offer an explanation for how this microbe is able to generate high levels of ethanol while maintaining adequate pools of ATP (Fig 5.10). The Rnf complex utilizes electrons from reduced ferredoxin to reduce NAD+ to NADH while translocating H⁺ across the membrane, generating an electrochemical gradient that may be used by the previously uncharacterized H⁺ V-type ATPase. A membrane-bound pyrophosphatase (PPase) contributes to ATP formation by augmenting H⁺ and Na⁺ electrochemical gradients that may drive ATP synthesis by the Na⁺ V-type and Na⁺ F-type ATPases. We propose that the Rnf complex contributes to ethanol production by producing NADH required to reduce Acetyl-CoA to ethanol.
Figure 5.1 Model of the *C. phytofermentans* Na\(^+\) F-type ATPase c-ring. An 11 c-ring homo-mer was developed including the location of amino acids involved in Na\(^+\) binding. The model was developed using SWISS MODEL and 3D rendered using VMD 1.9.1.
Figure 5.2 Alignment of F-type ATPase amino acid sequences. Conserved amino acids involved in Na⁺ are marked with an asterisk.

The *C. phytofermentans* protein contains the same conserved amino acids found in other F-type ATPases, V63, E65 and Q32 with the exception of T66. The sequence alignment was performed with microbes known to possess Na⁺ F-type ATPases: *I. tartaricus*, *F. nucleatum* and *C. paradoxum*. Sequences were aligned using PROSMAL3D and visualized by JalView.
Figure 5.3 A model of the *C. phytofermentans* Na\(^+\) V-type ATPase c-ring protein. A 10-c-ring homo-mer was developed containing the location of amino acids involved in Na\(^+\) binding. The model was developed using SWISS MODEL and 3D rendered using VMD 1.9.1.
Figure 5.4 Alignment of V-type ATPase amino acid sequences. Conserved amino acids involved in binding Na\(^+\) are marked with an asterisk. *C. phytofermentans* contains the same amino acids as known microbes with Na\(^+\) V-type ATPases: *E. hirae* and *E. faecalis*. *C. asparagiforme* and *C. bolteae* proteins have the greatest similarity to that of *C. phytofermentans*. Sequences were aligned using PROSMAL3D and visualized by JalView.
Figure 5.5 Model of the pyrophosphatase protein of *C. phytofermentans*. The model shows conserved amino acids T87 (magenta spheres), F91 (purple spheres), M117 (yellow spheres) and D143 (red spheres) involved in binding H+ and Na+. The model was developed using SWISS MODEL and 3D rendered using VMD 1.9.1.
Figure 5.6 Alignment of the *C. phytofermentans* pyrophosphatase amino acid sequence with other pyrophosphatase sequences.
Figure 5.7 Effect of ionophores on growth of *C. phytofermentans* in defined medium M6 with glucose as substrate. Cell growth was inhibited by both ETH 2120 and TCS when added at the time of inoculation or at mid-exponential phase of growth.
Figure 5.8 Effect of ionophores on ATP synthesis in inverted membrane vesicles. ATP synthesis was initiated by the addition of ADP to reaction mixtures. ATP was measured by luminescence using a luciferin-luciferase assay kit. ATP synthesis was significantly reduced in reactions containing the Na⁺ ionophore ETH 2120.
Figure 5.9 NADH-dependent ferredoxin reduction assay of Rnf oxidoreductase activity in inverted membrane vesicles. Reaction mixtures contained ferredoxin, NADH, and inverted membrane vesicles in buffered solution. Reactions were initiated by addition of ATP at 60 seconds. In some reactions, ETH 2120 or TCS was added at a concentration of 10 µM. Ferredoxin reduction was measured at 430 nM.
Figure 5.10 Metabolic model illustrating the role of membrane energetics in energy conservation in *C. phytofermentans*. The Rnf complex utilizes electrons from reduced ferredoxin to reduce NAD+ to NADH while translocating H+ across the membrane generating an electrochemical gradient used by a H+ V-type ATPase. A pyrophosphatase (PPase) augments the H+ and Na+ electrochemical gradients driving ATP synthesis by the Na+ V-type and Na+ F-type ATPases,
CHAPTER 6

DISCUSSION AND FUTURE DIRECTIONS

This dissertation has presented three research projects with the goal of 1) whether a correlation between cell growth and ethanol production existed in the presence of different plant biomass substrates, 2) visualizing and characterizing bacterial microcompartments produced by *C. phytofermentans* when fucose and rhamnose gets fermented, and 3) generating a bioenergetics model of *C. phytofermentans* using genomic and biochemical techniques that links energy conservation of membrane proteins and their role in ethanol production.

The first research project described the development of a dual-fluorescent staining protocol in order to visualize and quantify *C. phytofermentans* cells in plant biomass using epifluorescent microscopy. After the protocol was developed, we analyzed the correlation of cell growth and ethanol production in different plant feedstocks. We found that *C. phytofermentans* cell growth cycle varies depending on the cellulose substrate. With filter paper, cell growth and ethanol production was strongly correlated. However, no correlation was found between cell growth and ethanol production in either wild-type sorghum or the reduced lignin double mutant strain production in the presence of lignocellulosic substrate. The dual-staining protocol allows for a simple and efficient method for visualizing and quantifying microbial growth on cellulosic substrate.

The second research project was the first to characterize and visualize the bacterial microcompartments (BMCs) when a microbe is grown in fucose and rhamnose and produces 1,2-propanediol (PDU) and propionate. BMCs are protein polyhedral bodies that encapsulate different
types of metabolic pathways. Their functions vary depending on the type of environment the microorganism is found. We were able to visualize BMCs inside of *C. phytofermentans* by doing thin-section transmission electron micrograph. In the dehydration and polymerization process we modified the typical TEM embedding protocol by adding potassium ferrycianide and picrid acid. These compounds, although it is not known exactly how, were able to enhance the contrast of BMCs and stability the cell membrane when the thin-section occurred. Understanding different BMCs will increase our knowledge of the different metabolites produced and their role. This project also determined that the employment of potassium ferrycianide and picrid acid allows for higher image contrast and a better preservation of the integrity of cell membrane.

The third research project generated a bioenergetics model of membrane energy conservation by characterizing membrane bound ATPases, a ferredoxin oxidoreductase Rnf-complex and a membrane bound pyrophosphatase. Using protein homologs and sequence alignments, we were able to generate 3D representative structures of a Na\(^+\) F-type ATPase, a Na\(^+\) V-type ATPase and a Na\(^+\)/H\(^+\) pumping pyrophosphatase along with their predicted ion binding sites. We also found that *C. phytofermentans* cell growth gets inhibited in the presence of either proton or sodium ionophores, indicating that the microbe relies on both ions for cell growth. We also employed a ferredoxin reduction assay to generate a coupling system an ATPase and the Rnf-complex. The coupling assay was inhibited in the presence of the proton ionophore but it was enhanced in the presence of sodium ionophore. Our results indicate that *C. phytofermentans* uses both H\(^+\) and Na\(^+\) ions for the conservation of energy through the membrane. This research is the first one every that studies the energy conservation through the membrane of a biofuels microbe and proposes an energy pathway through the membrane that is link to the production of ethanol.
I demonstrated that visualization and quantification of a microbe growing in different plant biomass substrates can be achieved with the use of two fluorescent dyes. To my knowledge, this is the first time direct cell count of a microbe in plant biomass substrate. However, this method has certain limitations. Future versions of this project will be developed to include a transgenic cell line of *C. phytofermentans* with a GPF tag. With this process we will only have to rely on one fluorescent dye, calcofluor white, and minimize photo bleaching effects and dye interaction with biofilm, making cell quantification and visualization faster. This project would also expand into examining *C. phytofermentans* growth in different types of plant biomass such as sugarcane bagasse, different temperatures and in an agitated environment.

With the modification of the fixation, dehydration and embedding protocol from a TEM biological sample we achieved the first high contrast imaging of BMCs generated in the presence of fucose and rhmanose. While precise visualization of BMCs is achieved with this method, it is very difficult to quantify and visualize BMCs inside of a cell due to two-dimensional limitations of TEM micrographs. In order to better understand the development of BMCs inside of *C. phytofermentans* a future project will consist in generating a transgenic cell line of *C. phytofermentans* where a fluorescent tag can be placed in the BMCs. This process will allow us to further examine the dynamics of BMC generation during cell growth phases and to quantify the amount of BMCs per cell. The present *C. phytofermentans* BMC project can also be expanded to modify certain pathways within the BMCs to achieve higher production of 1,2-propanediol and propionate.

The development of a bioenergetics model is a highly iterative process. The model was built using both genomic and biochemical data and it has shed light on how different membrane bound proteins and its ions contribute to the formation of ethanol. While the model was built with
complementary genomic and biochemical data, additional information of other proteins and their genes is necessary to improve its accuracy. Future versions of the model should include biochemical analysis of the H\(^+\)/Na\(^+\) pyrophosphatase in different pH conditions. Moreover, a modification in the rnfB section that can hinder the electron transfer of the Rnf-complex should provide us a better understanding of its NADH production contributes to ethanol formation.
REFERENCES


