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CONVERSION OF ACIDS TO ALCOHOLS BY *CLOSTRIDIUM RAGSDALEI* STRAIN P11: PROCESS OPTIMIZATION AND BIOCHEMISTRY

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CATHERINE E. ISOM
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CONVERSION OF ACIDS TO ALCOHOLS BY *CLOSTRIDIUM RAGSDALEI* STRAIN P11: PROCESS OPTIMIZATION AND BIOCHEMISTRY

A DISSERTATION APPROVED FOR
THE DEPARTMENT OF MICROBIOLOGY AND PLANT BIOLOGY

BY

_____
Dr. Ralph S. Tanner, Chair

_____
Dr. Elizabeth A. Karr

_____
Dr. Michael J. McInerney

_____
Dr. Boris Wawrik

_____
Dr. Mark A. Nanny

To Kay, Chuck, and Travis.

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ABSTRACT

General objectives. In this dissertation, focus is directed toward the development of a biocatalyst that can be used to produce chemicals and fuel from volatile fatty acids ubiquitous in waste biomass. *Clostridium ragsdalei* is introduced to serve as an exemplar carboxidotrophic acetogen that reduces VFAs to alcohols of the same carbon structure with only acetate and ethanol as by-products of the fermentation. This dissertation aims to develop a better understanding of this process in *C. ragsdalei* and, in turn, other similar bacteria and to support previous discoveries as they relate to carboxylate reduction in acetogens. Additionally, pure culture studies allow for a more detailed study of the biochemical behavior response to different compounds without skewing the results due to the influence of other species.

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Broad hypothesis. Based on preliminary observations, *C. ragsdalei* will serve as a viable biocatalyst for the direct reduction of carboxlyates to alcohols.

CHAPTER 1

Conversion of carboxylates to alcohols by *Clostridium ragsdalei*: Introduction of biocatalyst and proof of concept

ABSTRACT

Commodity chemicals, such as ethanol and butanol, are produced from nonrenewable petrochemicals or via microbial catalysts using fermentable sugars and starches. *Clostridium ragsdalei* strain P11 is a carboxidotrophic acetogen that ferments a synthesis gas analog (H_2:CO_2:CO) to ethanol. Previous research has shown that some anaerobic bacteria similar to *C. ragsdalei* can reduce volatile fatty acids (VFAs) to alcohols. *C. ragsdalei* appeared to consume acetate produced earlier in the fermentation and continue to produce ethanol during stationary phase of growth. Thus, investigations into the potential reduction of exemplar VFAs found in organic waste streams to the corresponding alcohols coupled to syngas fermentation in *C. ragsdalei* were conducted. Sodium salts of propionate, butyrate, valerate, hexanoate, lactate, and isobutyrate were added to the medium with carbon monoxide as the substrate and the production of the corresponding solvents was quantified. *C. ragsdalei* converted close to 100% of the consumed propionate and valerate to 1-propanol and 1-pentanol, respectively. Approximately 75% of the consumed butyrate and 50% of the consumed hexanoate were converted to 1-butanol and 1-hexanol, respectively. These results show that *C. ragsdalei* can reduce a range of VFAs commonly associated with organic waste streams, to the corresponding alcohols using syngas analog (H_2:CO_2:CO) as growth substrate and reductant with no substantial effect on growth, ethanol, or acetate production.

INTRODUCTION

Requirements for sustainably supplementing the petroleum industry. World-wide energy consumption has been consistently increasing since the Industrial Revolution. Due to this increase in energy consumption, issues have become apparent regarding the negative impacts of petroleum combustion on the global climate and the potential depletion of petroleum reserves. The transportation industry consumes approximately 74% of refined crude oil (EIA, 2012). The demand for petroleum products from the transportation industry will only grow with the population and is estimated to require 0.6 million barrels per day more refined crude oil from 2010 to 2035 (EIA, 2012). The chemical products industry consumes 16.8% of petroleum products (IEA, 2009); while this is less than the transportation sector, this demand is also likely to increase with growing populations that demand more petroleum-based products. These are likely the industries that will suffer the most from a depleted or more strictly regulated oil supply and are also industries that are required of an advancing society. Therefore, a need exists to, at the very least, supplement the petroleum industry with sustainable (ie. carbon neutral) alternatives to oil. In fact, legislation, both foreign and domestic, has been proposed to stimulate research and development for the production of fuels and commodity chemicals from renewable biomass.

First generation biofuels. Initially, focus was placed on food-based biofuel production, specifically sugar or starch fermentation to ethanol by yeast using corn as a feedstock. These direct fermentation methods have been well-studied and were easily accepted as a way to address growing energy concerns. A few pitfalls arise with direct fermentation of food resources to ethanol: 1) The use of cash crops as feedstocks and shortage of arable land makes the food-to-fuel model unsustainable (Service 2007); 2) Lignocellulosic feedstocks cannot be entirely consumed by microbial catalysts (Cardona and Sánchez 2007); and, 3) Ethanol has a lower energy output than gasoline and can be corrosive to existing infrastructure (Balat and Balat 2009; MacLean and Lave 2003). Another petrol-alternative that is produced on a larger scale is biodiesel. This primarily plant-based bioproduct can be produced by the trans-esterification of vegetable oils or animal fat (Ma and Hanna 1999). As with bioethanol production, the use of food-crops (ie. plant seeds) for the production of biodiesel is not a sustainable process.

Sustainable feedstocks. As mentioned above, the production of ethanol and biodiesel from sugar-rich food crops and plant seeds, respectively, is not an entirely sustainable process. The two main issues are: 1) The difficulty in distinguishing between biofuel crops and food crops for both land management and crop distribution purposes, and 2) a reduction in plant biodiversity. Bacteria that produce fuels and commodity chemicals from more sustainable feedstocks are being explored. Legislation is beginning to include more defined directives not just for

generating these biologically produced fuels, but also for doing so using sustainable feedstocks and existing infrastructure (Energy Independence and Security Act of 2007). Sustainable feedstocks have been outlined previously and include perennials grown on agriculturally abandoned land, crop residues, sustainably harvested woody crops, crop rotation/mixed crop systems, and municipal/industrial waste (Tilman et al. 2009).

In this dissertation, focus is placed on the volatile fatty acids (VFAs) produced during the acidification of municipal and industrial waste biomass. Anaerobic digestion of waste products at municipalities and industrial sites is comprised of three main acidification processes—hydrolysis, acidogenesis, and acetogenesis. The products of these processes depend on the microbial population active during anaerobic digestion. Key products of anaerobic digestion that can be used as model feedstocks for biological fuel production are acetate, propionate, butyrate, valerate, and hexanoate, which can be hydrogenated to ethanol, propanol, butanol, pentanol, and hexanol, respectively.

Production of energy-rich biofuels and commodity chemicals. In any attempt to supplement the petroleum industry with comparable biofuels, the energy density of those products must be comparable to the current transportation fuel (Table 1.1). VFAs are energy-rich, but the high oxygen to carbon ratio makes VFAs less suitable for fuel applications. Converting energy-dense VFAs to alcohols yields a product more applicable to the fuel industry (Coyle 2007). Biological produced C_2-C_6

alcohols can be blended with gasoline as fuel oxygenates (Coyle 2007). Additonally, these solvents are used in a variety of industrial processes. Some of the current uses for propanol are as solvents in the pharmaceutical, paint, and cosmetics industries. Propanol can also be found in brake fluids and some ink. As of 2006, the butanol industry was an approximately 7 billion dollar expanding market (Kirschner 2006). Like propanol, butanol is also used in industry as a diluting agent and extraction solvent. According to the PubChem database, pentanol (CID 6276) can be used as a solvent for coatings in the electronics industry. Hexanol is primarily used in the perfume and food industry for its fragrant properties.

Biocatalysts. Previous research has demonstrated the reduction of fatty acids to the corresponding alcohols by anaerobic bacteria. *Clostridium acetobutylicum* was first reported to reduce propionate and propionaldehyde to 1-propanol by Blanchard and MacDonald in 1935. Not long after this discovery, Langlykke et al. (1937) demonstrated that *Clostridium butylicum* produced amounts of isopropyl alcohol equivalent to the amount of acetone added to the fermentation broth. The reduction of propionate to 1-propanol by *C. acetobutylicum* led Hartmanis et al. (1984) to examine the substrate specificity of *C. acetobutylicum* to a variety of carboxylates. Of the substrates examined *C. acetobutylicum* reduced C_1-C_7 straight chain acids, isobutyrate, DL-2-methylbutyrate, isovalerate, isohexanoate, vinylacetate, and crotonate (Hartmanis et al. 1984). This work, along with the research of Jewell et

al. (1986) suggested that *C. acetobutylicum* could be a useful biocatalyst for the reduction of acids to industrially valuable alcohols. During this time, *Clostridium kluyveri* was shown to produce hexanol in the presence of succinate after accumulation of hexanoate, which suggested that *C. kluyveri* could also reduce acids to alcohols (Kenealy and Waselefsky 1985). Cell-free extracts of *Clostridium formicoaceticum* and resting cells of *Clostridium thermoaceticum*, both grown on fructose, reduced C_2-C_6, C_8, and C_{10} straight chain fatty acids, benzoate, 2-phenylpropionate, and E-2-methylbutenoate to the corresponding alcohols (Fraisse and Simon 1988; Simon et al. 1987). All of the aforementioned studies were conducted with organisms that produced cell mass from fermentable sugars, though some (ie. *Moorella thermoacetica*) are capable of carboxidotrophic growth (Drake and Daniel 2004). In fact, at the time this dissertation was being drafted, Perez et al. (2013) demonstrated the conversion of propionate and buytrate to 1-propanol and 1-butanol, respectively, by *Clostridium ljungdahlii*, *Clostridium ragsdalei* strain P11—the focus of the work presented herein—and *Clostridium coskatii*.

Recently, the application of the microbial conversion of acids to alcohols as a waste treatment practice was described for mixed bacterial consortia that did not require fermentable sugars or starches (Steinbusch et al. 2008). Researchers focused on the conversion of the most dominant acids found in acidified granular distillery sludge—acetate, propionate, and butyrate—to their corresponding alcohols with hydrogen as the electron donor (Steibusch et al. 2008). The major pitfall of using these mixed cultures for the production of biosolvents was the increased pH

due to activated sludge preparation, which led to the prevalence of methanogens (Steinbusch et al. 2008, Steinbusch et al. 2009).

***Clostridium ragsdalei* strain P11.** *Clostridium ragsdalei* strain P11 is an obligately anaerobic, Gram-positive acetogen that was isolated from anoxic freshwater sediment with the ability to produce ethanol from $CO:CO_2$. Similar to other *Clostridia* spp., *C. ragsdalei* produces acetate from sugars and produces acetate, CO_2 and ethanol from C_1 substrates (Allen et al. 2010, Liou et al. 2005 and Tanner et al. 1993). The 16S rRNA gene sequence analysis indicated that *C. ragsdalei* was closely related to *C. ljungdahlii* (99.9 %) within Clostridial cluster I. *C. ragsdalei* is capable of autotrophic growth on CO and $H_2:CO_2$. This is important in that *C. ragsdalei* grows and produces acetate and ethanol from a synthesis gas (syngas) analog. Syngas is a viable biofuel feedstock composed of primarily $H_2:CO_2$:CO and produced via the pyrolization of waste biomass (Datar et al. 2004). Yeast extract is also required for growth. Acetic acid and/or ethanol are the major fermentation products from all utilized substrates.

Postulated energy conservation in *Clostridium ragsdalei*. The Wood-Ljungdahl Pathway (Figure 1.1) is the accepted mechanism for energy conservation and carbon fixation in acetogenic bacteria (Müller 2003). The activities of key enzymes in the Wood-Ljungdahl Pathway, formate dehydrogenase (FDH), carbon monoxide dehydrogenase (CODH), and alcohol dehydrogenase (ADH) and/or aldehyde

dehydrogenase (ALDH), were measured previously in *C. ragsdalei* (Saxena and Tanner 2010). In the methyl branch of the Wood-Ljungdahl Pathway, CO_2 is reduced to formate (Figure 1.1) by FDH. A methylated corrinoid iron sulfur protein is generated via the methyltetrahydrafolate synthetase reaction at the expense of one ATP. This provides the methyl carbon of acetyl-CoA. The carbonyl carbon is generated when CO_2 is reduced to CO by CODH, or CO is directly utilized if readily available, in the carbonyl branch of the Wood-Ljungdahl Pathway (Figure 1.1). Ethanol could be produced from the acetyl-CoA intermediate or be a product of acetate reduction. This latter hypothesis coupled with the ability of *C. ragsdalei* to fix carbon from synthesis gas components makes *C. ragsdalei* a viable biocatalyst for VFA reduction to alcohols.

Research objective. The goal of this preliminary research is to assess the hypothesis that *C. ragsdalei* will convert C_2-C_6 fatty acids to the corresponding alcohols based on the observation that *C. ragsdalei* consumes acetate while still producing ethanol.

Table 1.1 Energy content of exemplar alcohols compared to existing petroleum-derived fuels and biodiesel (EIA 2011).

Product	1,000 BTU[a] per gallon
Gasoline	125
Biodiesel	117
Butanol	110
Propanol	100
Ethanol	76
Liquefied Natural Gas	75

[a]British Thermal Units

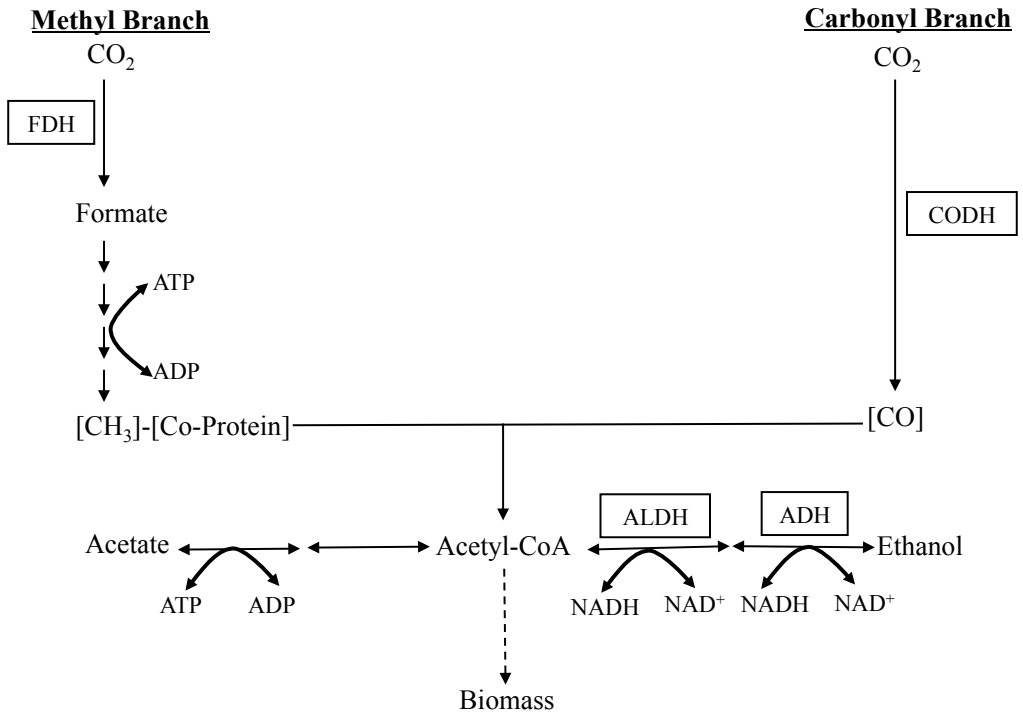

Figure 1.1 Wood-Ljungdahl Pathway with empirically supported enzymes in *Clostridium ragsdalei* strain P11 highlighted as follows: FDH (formate dehydrogenase), CODH (carbon monoxide dehydrogenase), ALDH (aldehyde dehydrogenase), and ADH (alcohol dehydrogenase).

MATERIALS AND METHODS

Bacteria, media, and syngas analog. *Clostridium ragsdalei* strain P11 (ATCC BAA-622) was maintained on acetogen medium (ATCC medium no. 1754). Medium was prepared using strict anoxic technique (Balch and Wolfe 1976). *C. ragsdalei* medium contained (L^{-1}): 20 ml mineral solution (Tanner 2007), modified by the elimination of sodium; 10 ml vitamin solution (Tanner 2007); 10 ml optimized trace metal solution (Saxena and Tanner 2010); 1 g yeast extract (Difco, Becton Dickinson, Sparks, MD); 10 g MES, with the pH adjusted to 6.1 using KOH; and 3 ml cysteine sulfide as a reducing agent (Tanner 2007). Inoculation vessels contained a headspace of 1 atm H_2:CO_2 (80:20) and were over-pressurized with CO (207 kPa gauge) as a syngas analog. Incubations were at 37°C. All chemicals used in this research were obtained from Sigma-Aldrich unless otherwise stated (Sigma-Aldrich Corp.).

Reduction of acids to alcohols. Anoxic, sterile stocks of VFAs were individually added to prepared medium at a concentration of 30 mM prior to inoculation with *C. ragsdalei*. Solventogenic cells (7 d old culture) of *C. ragsdalei* were added to acid-amended media with an initial headspace of H_2:CO_2 (80:20) at 104 kPa gauge fed CO (207 kPa gauge). Samples were collected after inoculation and after 10 d incubation. Experiments were performed in triplicate and repeat experiments were

conducted to determine reproducibility. Additionally, carboxylate-free and abiotic controls were implemented.

Analytical methods. Acid and alcohol concentrations were quantified by gas chromatography (GC) using the Shimadzu GC-8A (Shimadzu Scientific Instruments, Columbia, MD) equipped with a flame ionization detector (FID). Samples were injected into a glass column (2 m x 5 mm x 2.6 mm) packed with 4 percent Carbowax 20M TPA on Carbopack B 80/120 mesh (Supelco Analytical, Bellefonte, PA). The inlet and detector were both set at 200°C. Column temperature was held at 155°C for alcohols containing ≤ 5 carbons and acids ≤ 3 carbons in length. Column temperature was held at 185°C for $>C_5$ alcohols and $>C_3$ acids. Data was analyzed using a C-R8A Chromatopac Integrator (Shimadzu Scientific Instruments, Columbia, MD). Growth was analyzed by measuring the absorbance at 600 nm (A_{600}) using the Spectronic 20D spectrophotometer (Milton Roy, Ivyland, PA). Sample pH was determined using the Fisher Accumet Basic pH Meter (Fisher Scientific, Pittsburgh, PA).

RESULTS AND DISCUSSION

Evidence for the uptake of acetate. The production of acetate and ethanol in growing cells of *Clostridium ragsdalei* strain P11 on a general acetogen medium (Tanner 2007) and syngas analog (H_2:CO_2:CO) was monitored over 10 days incubation at 37°C (Figure 1.2). Changes in acetate concentration suggested that during late log phase of growth (days 3-4), *C. ragsdalei* consumed some of the acetate produced earlier in the fermentation. In conjunction with this acetate consumption, was the continued production of ethanol during stationary phase. This suggested that ethanol production, at least past day 6, does not appear to be growth dependent and may proceed via the reduction of acetate.

Conversion of carboxylates to alcohols by *Clostridium ragsdalei*. To further test the hypothesis that *C. ragsdalei* can reduce other VFAs to the corresponding alcohols, anoxic sterile stocks of VFAs were individually added to prepared acetogen medium prior to inoculation with *C. ragsdalei*. Cultures were fed H_2:CO_2:CO and incubated at 37°C. Incubation times varied for the different carboxylates; therefore, end-point samples were analyzed approximately 24 h after cells reached stationary phase (Table 1.2). *C. ragsdalei* consumed 44, 66, 11, and 61% of the added propionate, butyrate, valerate, and hexanoate, respectively and converted them to 1-propanol, 1-butanol, 1-pentanol, and 1-hexanol, respectively (Table 1.2). Methanol, propanediol, and isobutanol were not produced from added

formate, lactate, and isobutyrate, respectively (Table 1.2). Lactate and isobutyrate had a significant negative impact on growth ($p>0.05$). Lactate-amended cultures were still active, producing 157 ± 6.5 mM acetate and 41.1 ± 4.8 mM ethanol, where as isobutyrate-ammended cultures produced <5 mM of both end products above background concentrations and growth was not observed spectrophotometrically. In a recent study, *C. ljungdahlii* was able to consume approximately 50% of the added isobutyrate and convert about 40% to corresponding branched-chain alcohol (Perez et al. 2013). It was noted, however, that less of the branched chain alcohol was produced from consumed isobutyrate relative to short chain linear fatty acids (C_3-C_6) (Perez et al. 2013). Inhibition of metabolic processes and/or growth by α-hydroxy and branched chain acids is not uncommon among the Clostridia, though concentrations corresponding to this inhibition are typically >100 mM (Russell 1992). C. *ragsdalei* converted close to 100% of the consumed propionate and valerate to the corresponding alcohol. Approximately, 75% of the consumed butyrate and 50% of the consumed hexanoate were converted to 1-butanol and 1-hexanol, respectively. The added carboxylate was not completely consumed for any of those tested. An average decrease in pH of 1.4 was detected for all tested carboxylates, except for isobutyrate (decrease in pH of 0.2). Growth (A_{600}) did not differ significantly among *C. ragsdalei* cultures amended with carboxylates and an unamended control ($p<0.05$) except for those mentioned above. The ethanol to acetate ratio (EtOH:Ac) gives some insight into the acidogenic or solventogenic state of fermentation. An EtOH:Ac ratio < 1 means

that the bacterium is still primarily acidogenic and still in the growth phase, producing <1 ethanol per acetate. An EtOH:Ac ratio > 1 indicates that the bacterium is solventogenic, producing >1 ethanol per acetate. Ideally, the EtOH:Ac ratio should be >1 for *C. ragsdalei* fermentations. The low EtOH:Ac ratio demonstrated in these initial studies (Table 1.2) suggests a need for further process development.

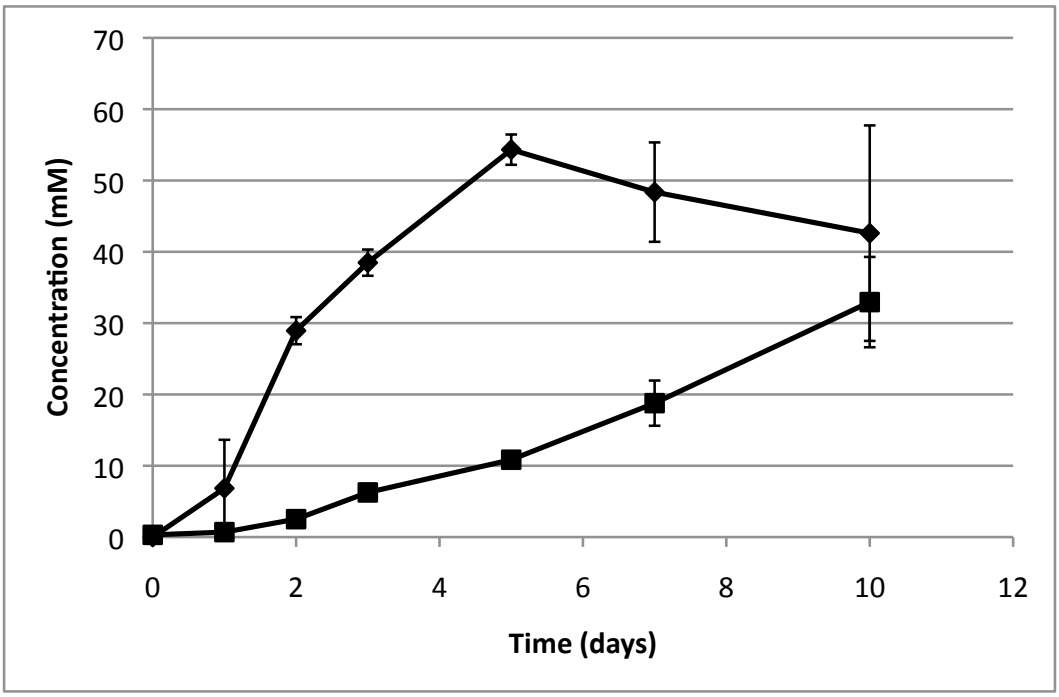

Figure 1.2 Decrease in measured acetate (◆) concomitant with the continued production of ethanol (■) by *Clostridium ragsdalei* at day 5 of incubation at 37°C with H_2:CO_2:CO as the growth substrate. Experiments were performed in triplicate.

Table 1.2 Conversion of carboxylates to corresponding alcohols by *Clostridium ragsdalei* strain P11, final ratio of ethanol to acetate (EtOH:Ac), final growth as optical density (A_{600}) and final pH.

Carboxyate Consumed	Alcohol Produced	Carboxylate Consumed (mM)	Alcohol Produced (mM)	EtOH:Ac	A_{600}	Final pH
Formate	Methanol	30.0 ± 0.0	ND[a]	0.11 ± 0.1	0.601 ± 0.2	4.9 ± 0.2
Propionate	1-propanol	13.1 ± 2.7	13.1 ± 0.2	0.22 ± 0.0	0.760 ± 0.1	5.1 ± 0.1
Butyrate	1-butanol	19.8 ± 1.3	14.1 ± 1.0	0.17 ± 0.1	0.743 ± 0.1	4.5 ± 0.0
Valerate	1-pentanol	3.31 ± 0.7	2.77 ± 0.1	0.04 ± 0.0	0.782 ± 0.0	4.3 ± 0.0
Hexanoate	1-hexanol	18.3 ± 2.1	9.07 ± 2.4	0.18 ± 0.0	0.741 ± 0.2	4.7 ± 0.0
Lactate	Propanediol	12.6 ± 0.3	ND	0.26 ± 0.1	0.282 ± 0.0	5.1 ± 0.8
Isobutyrate	Iso-butanol	0.00 ± 0.0	ND	0.01 ± 0.0	0.171 ± 0.1	5.9 ± 0.3
Unamended				0.22 ± 0.1	0.921 ± 0.1	4.5 ± 0.0

[a]Not detected.

CONCLUSIONS

Clostridium ragsdalei strain P11 converted a range of volatile fatty acids (VFAs), which can be found in organic waste streams to the corresponding alcohols. Preliminary batch experiments showed that acetate, propionate, butyrate, valerate, and hexanoate could be converted to alcohols of the same carbon skeleton with a syngas analog (H_2:CO_2, 80:20, 107 kPa gauge and CO, 207 kPa gauge) as the electron donor and growth substrate. The highest measured alcohol concentrations were 13.1 ± 0.2 mM of 1-propanol, 14.1 ± 1.0 of 1-butanol, 2.77 ± 0.1 of 1-pentanol, and 9.07 ± 2.4 of 1-hexanol produced from the added acid substrates with an efficiency (% alcohol produced from added acid) of approximately 44%, 47%, 9%, and 30% respectively. No effect on growth or ethanol to acetate ratio was noted. Acetate was the largest by-product of fermentation.

C. ragsdalei generated ethanol to acetate ratios up to 10 with an ethanol concentration of 187.80 ± 8.56 mM and acetate concentration of 18.56 ± 2.36 mM in previous studies (Saxena and Tanner 2010). This suggests that the production of ethanol can be dramatically improved. The production of alcohols from consumed acids could occur via the same or a similar mechanism to ethanol production. This indicates that the reduction of acids to alcohols in *C. ragsdalei* could reach 100% conversion efficiency if the fermentation environment becomes more suitable for ethanol production.

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CHAPTER 2

Optimization of the conversion of acids to alcohols by *Clostridium ragsdalei* and demonstration of the process by related bacteria

ABSTRACT

The majority of biologically produced fuels and other chemicals require the use of fermentable sugars and starches as growth and fermentation substrates. A sustainable alternative to these processes is the indirect fermentation of biomass, via synthesis gas (syngas) by solventogenic acetogens. *Clostridium ragsdalei*, *Clostridium carboxidivorans*, and *Alkalibaculum bacchi* were isolated for the ability to grow autotrophically and generate ethanol from syngas components (H_2:CO_2:CO). Some anaerobic bacteria convert fatty acids to alcohols though most of these organisms require sugar for growth. *C. ragsdalei*, a carboxidotrophic acetogen, converts C_2-C_6 linear fatty acids and acetone to the corresponding alcohols even though *C. ragsdalei* was primarily acidogenic during initial studies. In an effort to improve the fatty acid conversion efficiency, the conversion of propionate, butyrate, and valerate to alcohols by *C. ragsdalei* was developed by using an optimized acetogen medium (initial pH 5.3-5.5), refeeding CO every 48 h, shaking incubations, and conducting incubations in vessels that allow for a larger head space. *C. ragsdalei* consumed almost 100% of the propionate, butyrate, and valerate and converted an average of 90% of the consumed acid to the corresponding alcohol post-optimization. ^{13}C NMR studies suggested that this is a direct reduction of the added acid to alcohol. *C. carboxidivorans* and *A. bacchi* converted 77 and 10% of the added propionate to 1-propanol, respectively. Renewable wastes, such as biomass derived synthesis gas and municipal/industrial

organic waste streams are prevalent sources that could provide sustainable feedstocks for biologically derived fuels and commodity chemicals. This research provides evidence that adjusting key fermentation parameters toward solventogenesis, such as initial pH and reductant availability, increases the conversion effiency of *C. ragsdalei.* The conversion of propionate to 1-propanol for other carboxidotrophic acetogens was also demonstrated.

INTRODUCTION

Demand for sustainable fuels and commodity chemicals. Microbially produced fuels and commodity chemicals are currently of interest due to the continued depletion of oil reserves and the negative impacts of petroleum combustion on the global climate. Initially, focus was placed on food-based biofuel production, specifically sugar or starch fermentation to ethanol by yeast using corn as a feedstock. The use of cash crops as feedstocks and shortage of arable land (Service 2007), low energy output and corrosiveness of ethanol (Balat and Balat 2009, Maclean and Lave 2003), and lack of total lignocellulosic feedstock consumption (Cardona and Sánchez 2007) are a few pitfalls that arise with direct fermentation of sugars to ethanol. Alternatively, waste products can be converted into important compounds. Three major platforms for generating bioproducts from waste are the conversion of biomass into sugars via enzyme pretreatment steps (Cardona and Sánchez 2007), indirect fermentation using synthesis gas (syngas) generated via pyrolization of organic waste in a gasifier (Datar et al. 2004, McKendry 2002), and, the recently proposed, carboxylate platform (Agler et al. 2010). The carboxylate platform is broadly defined by the microbial production of liquid fuels and industrial solvents from carboxylates ubiquitous in waste streams (Agler et al. 2010).

Some acetogenic bacteria produce acetate, ethanol, and biomass from syngas components (H_2:CO_2:CO) (Drake et al. 2006). While ethanol was and, to an extent, remains a valuable supplement to the petroleum industry, the aforementioned

pitfalls that arise with ethanol institute a need for higher energy yield and less corrosive biofuels and bioproducts. Previous research has shown that some sugar-fermenting bacteria can reduce volatile fatty acids (VFAs) to alcohols (Fraisse and Simon 1988; Hartmanis et al. 1984; Simon et al. 1987; Tashiro et al. 2007). As mentioned in Chapter 1 of this dissertation, *Clostridium formicoaceticum, Clostridium thermoaceticum, Clostridium acetobutylicum*, *Clostridium saccharoperbutylacetonicum*, *Clostridium ljungdahlii*, and mixed anaerobic cultures reduced VFAs to the corresponding alcohols (Fraisse and Simon 1988; Hartmanis et al. 1984; Simon et al. 1987; Steinbusch et al. 2008; Tashiro et al. 2007). VFAs are ubiquitous in municipal and industrial waste streams providing sustainable feedstocks for biosolvent production (Angenent et al. 2004; Maune 2011; Zacharof and Lovitt 2013). Previous work suggests that bacteria that convert carboxylates to alcohols can consume micromolar (Fraisse and Simon 1988) and up to 20 mM of carboxylates as demonstrated in Chapter 1 of this dissertation.

Production of energy-rich biofuels from sustainable feedstocks. Recently, application of the microbial conversion of acetate, propionate, and butyrate to alcohols as a waste treatment practice was described for mixed bacterial consortia without the requirement of fermentable sugars or starches for growth (Steinbusch et al. 2008). Hydrogen was used as the electron donor (Steibusch et al. 2008). Hydrogen is produced during anaerobic digestion, which reinforces the waste-based feedstock model of this work (Li and Fang 2007). However, the majority of

hydrogen produced during anaerobic digestion is consumed by hydrogenotrophic methanogens (Steinbusch et al. 2009).

Alternatively, some acetogens can generate cell mass from syngas rather than sugars and starches (Drake et al. 2006). In this study, *Clostridium ragsdalei* strain P11, *Clostridium carboxidivorans* strain $P7^T$ and *Alkalibaculum bacchi* strain $CP11^T$ were used to demonstrate the reduction of VFAs to alcohols in acetogens that grow on C_1 substrates. Both *C. ragsdalei* and *C. carboxidivorans* have been shown to consume acids produced in early to late log phase of growth and generate alcohol of the same carbon structure. During $CO:CO_2$ fermentation *C. ragsdalei* appears to take up acetate produced during acidogenesis and continues to produce ethanol (Figure 1.2). Bruant et al. 2008 found *C. carboxidivorans* to perform similarly. However, a more thorough investigation is necessary to determine whether these bacteria are, in fact, generating ethanol from acetate and can reduce other VFAs to the corresponding alcohols, which will be addressed in the research presented here. Additionally, the recently isolated acetogen, *A. bacchi* was tested to highlight the difference between using a well-defined medium versus a newly isolated strain on undefined medium. Examining the carboxylate consumption process in these other carboxidotrophic bacteria could further support the universal ability of acetogens to reduce acids to alcohols.

Energetics of acid reduction. Acetogens growing on C_1 substrates generate energy via substrate level phosphorylation (SLP), whereby 1 mole of ATP is generated per

acetate produced (Drake et al. 2006). This suggests that it is not energetically favorable for *C. ragsdalei, C. carboxidivorans* or related bacteria to consume the acids produced during acidogenesis when grown on C_1 substrates if only considering substrate level phosphorylation as the energy conservation mechanism. This does not imply, however, that these bacteria do not use other mechanisms to conserve energy (ie. oxidative phosphorylation coupled to pyridine nucleotide and/or ferredoxin electron transfer), which will be discussed in Chapter 3 of this dissertation. Research suggests that these and similar bacteria consume acids and produce alcohols in order to maintain an optimum pH (Costa 1981). The free energy for the biological conversion of acids to alcohols, though exothermic, is much less favorable than for glucose fermentation (-225.5 kJ) (Steinbusch et al. 2008). The energetics of acetate reduction to ethanol (Equation 1), propionate to 1-propanol (Equation 2), and butyrate to 1-butanol (Equation 3) with hydrogen as the electron donor are shown below at STP and pH 7 (Thauer et al. 1977):

$$C_2H_3O_2^- + H^+ + 2H_2 \rightarrow C_2H_6O + H_2O \qquad \Delta G^{0'} = -9.6 \text{ kJ mol}^{-1} \quad (1)$$

$$C_3H_5O_2^- + H^+ + 2H_2 \rightarrow C_3H_8O + H_2O \qquad \Delta G^{0'} = -12.1 \text{ kJ mol}^{-1} \quad (2)$$

$$C_4H_7O_2^- + H^+ + 2H_2 \rightarrow C_4H_{10}O + H_2O \qquad \Delta G^{0'} = -16.3 \text{ kJ mol}^{-1} \quad (3)$$

The thermodynamics of acetate reduction (Equation 4) do not change significantly when CO replaces hydrogen as the reductant at STP (pH 7). However, the reaction

becomes more favorable for the reduction of propionate (Equation 5) and butyrate (Equation 6) when CO is used in place of H_2 (Thauer et al. 1977).

$$C_2H_3O_2^- + H^+ + CO \rightarrow C_2H_6O + CO_2 \qquad \Delta G^{0'} = -9.0 \text{ kJ mol}^{-1} \quad (4)$$

$$C_3H_5O_2^- + H^+ + CO \rightarrow C_3H_8O + CO_2 \qquad \Delta G^{0'} = -32.1 \text{ kJ mol}^{-1} \quad (5)$$

$$C_4H_7O_2^- + H^+ + CO \rightarrow C_4H_{10}O + CO_2 \qquad \Delta G^{0'} = -36.5 \text{ kJ mol}^{-1} \quad (6)$$

Although the above thermodynamic calculations are at STP and pH 7, it is indicated that the reaction will be more favorable with CO as the reductant rather than hydrogen. In fact, research has shown that acetogens grown on glucose or fructose will reduce VFAs to the corresponding alcohols with CO as the reductant (Blanchard and MacDonald in 1935, Langlykke et al. 1937, Hartmanis et al. 1984, Jewell et al. 1986, Kenealy and Waselefsky 1985, Simon et al. 1987, Fraisse and Simon 1988).

Research objectives. The goal of this research is to asses the hypothesis that *C. ragsdalei* and other acetogens that grow on C_1 substrates will function as beneficial biocatalysts for generating the desired biosolvents from VFAs and syngas rather than using unsustainable feedstocks. The research objectives are as follows:

1. Demonstrate the conversion of acetate to ethanol in *C. ragsdalei* using ^{13}C Nuclear Magnetic Resonance (NMR) Spectroscopy.

2. In addition to acetate, other VFAs ubiquitous in waste streams will be added to *C. ragsdalei* medium for conversion to corresponding alcohols.
3. Alcohol production will be evaluated on final alcohols concentration, alcohol production rate and reaction efficiency based on theoretical yield of alcohols from consumed acids.
4. *C. ragsdalei* will be grown with H_2 or CO to verify the assumption, based on the thermodynamic calculations, that CO will provide a more energetically favorable environment for the conversion of VFAs to alcohols.
5. Process development experiments will continue to determine the ideal conditions (initial pH, trace element adjustment, scale of fermentation) for total consumption of added fatty acids and conversion to the corresponding alcohols in *C. ragsdalei*.
6. Conversion of propionate in beer wastewater and valerate in raw sewage by growing cells of *C. ragsdalei* will be examined for production of 1-propanol and 1-pentanol following process development.
7. Finally, this process will be demonstrated in the related solventogenic, carboxidotrophic acetogens *Clostridium carboxidivorans* and *Alkalibaculum bacchi*.

MATERIALS AND METHODS

Bacteria, media, and syngas analog. *Clostridium ragsdalei* strain P11 (ATCC BAA-622) and *Clostridium carboxidivorans* strain $P7^T$ (ATCC BAA-624) were maintained on acetogen medium (ATCC medium no. 1754). *Alkalibaculum bacchi* strain $CP11^T$ (ATCC BAA-1772) was maintained on modified acetogen medium (pH 8.0) (Allen et al. 2010). Media were prepared using strict anoxic technique (Balch and Wolfe 1976). *C. ragsdalei* medium contained (L^{-1}): 20 ml mineral solution (Tanner 2007), modified by the elimination of sodium; 10 ml vitamin solution (Tanner 2007); 10 ml optimized trace metal solution (Saxena and Tanner 2010); 1 g yeast extract (Difco, Becton Dickinson, Sparks, MD); 10 g MES, with the pH adjusted to 6.1 using KOH; and 3 ml cysteine sulfide (0.4 g L^{-1} stock) as a reducing agent (Tanner 2007). *C. carboxidivorans* medium was prepared in the same manner as *C. ragsdalei* medium using unmodified trace metal solution (Tanner 2007). *A. bacchi* medium was prepared according to Allen et al. (2010). Inoculation vessels contained a headspace of 1 atm N_2:CO_2 (80:20) and were over-pressurized with CO (207 kPa gauge) as a syngas analog. Incubations were at 37°C. All chemicals used in this research were obtained from Sigma-Aldrich unless otherwise stated (Sigma-Aldrich Corp.).

Nuclear magnetic resonance (NMR) study. *C. ragsdalei* medium was amended with 18 mM 2-^{13}C-acetate. Medium was dispensed in 20 ml aliquots into 160 ml

bottles (Wheaton) in triplicate, sealed with butyl rubber stoppers (Wheaton), inoculated with late log phase *C. ragsdalei* (2.5% v/v) and pressurized with CO (207 kPa gauge). Cultures were incubated at 37°C for 30 d. Abiotic and ^{12}C-acetate controls were implemented. Samples (1.2 ml) were treated with 20 µl washed Chelex 100 Resin according to the manufacturers instructions (Bio-Rad Laboratores), vortexed for 30 sec and centrifuged at 6,000 rpm. Supernatant was stored at -80°C until analysis.

Samples were diluted 1:1 with D_2O for ^{13}C NMR for a total volume of 1 ml. Chemical shift values were confirmed by adding sodium 3-trimethylsilylpropionate-2,2,3,3,-d_4 (MSD Isotopic Products, Merck Sharp & Dohme of Canada Limited) as an internal standard. Spectra were obtained on a VNMRS 400 MHz spectrometer at a frequency of 100.5577 MHz using an indirect direction probe. Spectra were collected at 25°C using a single pulse C^{13} experiment with a 45° pulse width (2.95 µs), a delay time of 2 s, an acquisition time of 1.28 s, a spectral width of 25510.2 Hz and 256 acquisitions. Proton decoupling was achieved using a WALTZ-16 pulse sequence. Acetate and ethanol were quantified by GC-FID as discussed below.

Comparison of a general acetogen medium to optimized acetogen medium.

Acid consumption in *C. ragsdalei* was evaluated on general acetogen medium (Tanner 2007) and optimized acetogen medium (Saxena and Tanner 2010). The optimized medium contained 10X Ni and W, 5X Zn and Se, and no Cu as compared

to the acetogen medium described by Tanner (2007). All other factors were kept the same as for general culture maintenance described above.

Analysis of reductants, CO and H_2. A resting cell experiment was conducted to assess the conversion of propionate to 1-propanol in *C. ragsdalei* with CO or H_2 as the reductant. *C. ragsdalei* cells in the stationary phase of growth cultivated with either CO or hydrogen as the reductant were harvested prior to centrifugation in an anaerobic chamber, washed in anoxic MES buffer (pH 6.0), and resuspended in minimal medium without yeast extract containing 30 mM propionate and amended with H_2 (207 kPa gauge) or CO (207 kPa gauge). Minimal medium contained (L^{-1}): 20 ml mineral solution (Tanner 2007), modified by the elimination of sodium; 10 ml vitamin solution (Tanner 2007); 10 ml optimized trace metal solution (Saxena and Tanner 2010); and 10 g MES, with the pH adjusted to 6.0 using 1 N KOH. Experiments were conducted in quadruplicate and end-point samples were taken after 5 days shaking incubation at 37 °C. H_2:CO_2:CO (H_2:CO_2 80:20 at 100 kPa gauge and CO at 207 kPa gauge) controls were implemented.

Initial biocatalysis of acid conversion. Anoxic sterile stocks (2 M) of VFAs were individually added to prepared medium to a concentration of 30 mM prior to inoculation. Solventogenic cells (7 d old culture) of *C. ragsdalei* (4% v/v) were added to acid-amended media with an initial headspace of H_2:CO_2 (80:20) at 104 kPa gauge fed CO (207 kPa gauge). Samples were collected after inoculation and

after 10 d incubation at 37 °C. Experiments were performed in triplicate and repeat experiments were conducted to determine reproducibility. Additionally, carboxylate-free and heat-killed controls were implemented.

pH optimum assay. The pH range and optimum for ethanol production of *C. ragsdalei* strain P11 was examined from pH 4 to 8 with $CO:CO_2$ as the substrate and a Good's buffer (1 g L^{-1}) as follows: HOMOPIPES (pK_a 4.6), MES (pK_a 6.0), TES (pK_a 7.2), or TAPS (pK_a 8.1) as appropriate for the desired pH. Cultures were incubated at 37°C in Balch tubes (Wheaton) with 5 ml optimized acetogen medium (Saxena and Tanner 2010). Each condition was conducted in triplicate and the entire experiment was repeated to show reproducibility.

Analysis of fed-batch versus batch fermentation. Propionate-amended (30 mM) optimized acetogen medium (pH 5.0) was added to Balch tubes in 5 ml aliquots. Tubes initially contained $N_2:CO_2$ (80:20) vented to 1 atm and were fed, post-inoculation, CO (207 kPa gauge). Fed-batch fermentations were vented and repressurized with CO (207 kPa gauge) every 48 h post inoculation; batch fermentations were not. Each condition was conducted in quadruplicate and analyzed for conversion of propionate to 1-propanol over a 10 d incubation period.

Biocatalysis of carboxylate consumption under optimized conditions. A final experiment was conducted in 500 ml serum bottles containing 50 ml optimized

acetogen medium (pH 5.0) amended with 30 mM propionate, butyrate, or valerate and refed CO (207 kPa gauge) and fresh medium every 48 h. Cultures were incubated at 37°C on a shaking platform to promote gas-liquid mixing. Each condition was conducted in quadruplicate and analyzed for conversion of propionate to 1-propanol.

Consumption of carboxylates in brewer's wastewater and raw sewage in growing cells of *Clostridium ragsdalei*. Raw sewage was obtained from the Norman Wastewater Treatment Plant, Norman, OK. The initial pH (6.9) was adjusted to 5.5 with 1 N HCl. Sterile anoxic stock solutions were aliquoted into 160 ml sterile, acid washed glass bottles sealed with butyl rubber stoppers and kept under a 100% N_2 headspace. Beer wastewater was collected after primary fermentation had occurred. This wastewater consisted of yeast cells, remaining nutrients, remaining carbohydrates, coagulant and remnant particulates of the brewing process. The beer wastewater was acidified to a pH of 1 with HCl and autoclaved at 121 °C for 20 min to hydrolyze the yeast cells. After autoclaving, the cooled hydrolyzed beer wastewater pH was then adjusted to 5.5 with 10 N KOH. Feedstocks were stored at -20 °C.

Wastewater feedstocks were added to anoxic sterile 160 ml bottles in 20 ml aliquots and gassed out with N_2:CO_2 (80:20). A 1:1 ratio of wastewater feedstock to minimal medium with yeast extract (MMYE) was prepared by adding 10 ml of feedstock to 10 ml of MMYE. A MMYE control was also prepared. All conditions

were set up in triplicate. Each bottle was inoculated with *C. ragsdalei* (5% v/v), fed CO (207 kPa gauge) and incubated at 37°C. Growth was monitored visually, due to particulate matter in the beer wastewater. Samples were taken every 3-5 d and monitored for acid consumption and alcohol production as described below.

Analytical methods. Acid and alcohol concentrations were quantified by gas chromatography (GC) using the Shimadzu GC-8A (Shimadzu Scientific Instruments, Columbia, MD) equipped with a flame ionization detector (FID). Samples were injected into a glass column (2 m x 5 mm x 2.6 mm) packed with 4 percent Carbowax 20M TPA on Carbopack B 80/120 mesh (Supelco Analytical, Bellefonte, PA). The inlet and detector were both set at 200°C. Column temperature was held at 155°C for alcohols containing ≤ 5 carbons and acids ≤ 3 carbons in length. Column temperature was held at 185°C for $>C_5$ alcohols and $>C_3$ acids. Data was analyzed using a C-R8A Chromatopac Integrator (Shimadzu Scientific Instruments, Columbia, MD). Quantitative analysis of fatty acids was carried out in some cases using a Beckman HPLC fitted with a Beckman System Gold 166 Detector Module with NEC PC-8300 Controller (Beckman Coulter, Inc., Fullerton, CA) set at a wavelength of 214 nm. The column used was an Aminex® 300 mm x 7.8 mm HPX-87H Ion Exclusion Column (BIO-RAD, Hercules, CA). The system was operated at a flow rate of 0.9 mL/min with 0.002 N H_2SO_4 as the eluent. Growth was analyzed by measuring the absorbance at 600 nm (A_{600}) using the Spectronic 20D spectrophotometer (Milton Roy, Ivyland, PA). Sample pH was

determined using the Fisher Accumet Basic pH Meter (Fisher Scientific, Pittsburgh, PA).

RESULTS AND DISCUSSION

Demonstration of acetate conversion to ethanol by *Clostridium ragsdalei*. *C. ragsdalei* excretes acetate and ethanol when grown on H_2:CO_2:CO. Analysis of fermentation end products over a month incubation period with added acetate revealed that *C. ragsdalei* not only produces acetate, but consumed acetate between 5 and 10 days incubation. Ethanol production and acetate consumption appeared to be negatively correlated (Figure 2.1). A conclusion could not be drawn from GC-FID analysis alone since *C. ragsdalei* produces both acetate and ethanol from C_1 substrates. Thus, labeled 2-^{13}C-acetate was added to medium to verify that some of the ethanol produced by *C. ragsdalei* contained carbons from acetate. ^{13}C NMR analysis clearly demonstrated that the only labeled end product from *C. ragsdalei* in incubations with 2-^{13}C-acetate (23.5 ppm) was 2-^{13}C-ethanol (16.5 ppm) (Figure 2.2).

Survey of substrate range for carboxylate consumption by *Clostridium ragsdalei*. Initially, a survey of C_3-C_8 VFAs at a concentration of 3 g L^{-1} was conducted. *C. ragsdalei* did not grow on most of the VFAs at that concentration. Thus, the starting concentration of VFAs was scaled down to 1 g L^{-1}. *C. ragsdalei* growth yields were still low when VFAs were present even at the lower concentration. After subsequent transfers of low growth yield *C. ragsdalei*, on C_3-C_8 straight chain fatty acids, growth rates were comparable to an acid-free control

(Figure 2.3). The average growth rate for all incubations in Figure 2.3 was 0.243 ± 0.02 d^{-1}. *C. ragsdalei* did not grow on heptanoate or octanoate. Cell lysis was indicated based on visual analysis. Herrero et al. (1985) reported an increase in toxicity as carbon chain length of fatty acids increased; however, the LD50 for these experiments were upward of 100 mM fatty acids. At 1 g L^{-1} the acid concentrations for results presented in Figure 2.3 were 16.6, 13.5, 11.4, 9.79, 8.61, 7.68, and 6.93 mM for acetate, propionate, butyrate, valerate, hexanoate, heptanoate, and octanoate, respectively. This does not eliminate the possibility that as the carbon chain of the carboxylate increases the negative effects on growth, albeit through uncoupling and/or anion accumulation (Russell 1992), increases. With this in mind, research aimed toward process development was continued with the shorter chain fatty acids.

Effect of optimized medium on conversion of propionate to 1-propanol. Since the main focus of this research was improving the conversion of VFAs to alcohols using *C. ragsdalei* as a biocatalyst, it was important not only to focus on growth in the presence of VFAs, but also on solvent production. Saxena and Tanner (2010) enhanced solvent production in *C. ragsdalei* by optimizing the metals in acetogen medium for the metalloenzymes used during ethanol production. The optimized medium (Saxena and Tanner 2010) contained 10X Ni and W, 5X Zn and Se, and no Cu as compared to the acetogen medium described by Tanner (2007). Solventogenesis was assessed based on the ratio of ethanol produced per acetate

produced (EtOH:Ac) and the amount of 1-propanol produced from added propionate. The EtOH:Ac ratio for *C. ragsdalei* grown in acetogen medium was slightly lower (0.17 ± 0.0) than for *C. ragsdalei* grown on optimized acetogen medium (0.19 ± 0.0) (Table 2.1). Additionally, *C. ragsdalei* grown on optimized medium produced 11.9 ± 0.1 mM 1-propanol from the 12.9 ± 1.1 mM propionate consumed, greater than 90% conversion. In contrast, *C. ragsdalei* converted <30% of the consumed propionate to 1-propanol when grown in the general acetogen medium (Table 2.1). The optimized acetogen medium was used from this point in the research forward due to an over two-fold increase in 1-propanol production from consumed propionate when general acetogen medium was replaced with optimized medium.

The production of alcohols from the added carboxylates was examined by amending *C. ragsdalei* medium with 30 mM propionate, butyrate, or valerate. Alcohol production from carboxylates was evaluated on a percent alcohol produced from consumed acid basis. Additionally, the alcohol production rate (μM alcohol generated from added VFA per hour) and final solvent concentration (mM ethanol produced combined with mM alcohol generated from added VFAs) were used to evaluate the ability of *C. ragsdalei* to reduce VFAs to alcohols (Table 2.2). Cells were grown on H_2:CO_2:CO in batch fermentations in Balch tubes (5 ml total optimized acetogen medium and 21 ml CO headspace). *C. ragsdalei* reduced >90% percent of the consumed propionate to 1-propanol (Table 2.2). Approximately 60% of the consumed butyrate and valerate were converted to 1-butanol and 1-pentanol,

respectively (Table 2.2). Growth and ΔpH did not vary significantly among acid-amended cultures ($p<0.05$). While this demonstrated that *C. ragsdalei* will consume added carboxylates and reduce them to the corresponding alcohols, the EtOH:Ac was still less than 1 and final total solvent concentrations were less than 100 mM. This suggested that further process development was needed.

Improving the conversion of carboxylates to alcohols by *Clostridium ragsdalei.* A comparison of H_2 versus CO as the reductant for carboxylate conversion in *C. ragsdalei* was examined. The free energy for the conversion of propionate to 1-propanol with H_2 as the electron donor at STP ($\Delta G^{0\prime}$ = -12.1 kJ mol^{-1}) is less favorable than the free energy for the same reaction under the same conditions using CO as the reductant ($\Delta G^{0\prime}$ = -32.1 kJ mol^{-1}) (Thauer et al. 1977). Additionally, the redox potential provided by CO (CO/CO_2, E_0'=-560 mV) is much closer to the redox potential required for the conversion of carboxylates to aldehydes (carboxylate/aldehyde, E_0'=-550 mV) than is provided by H_2 (H_2/H_+, E_0'=-420 mV). CO-fed cultures converted almost 100% of the consumed propionate to 1-propanol and generating an EtOH;Ac ratio of 0.18 ± 0.0, which is almost 10-fold higher than the EtOH:Ac ratio generated by H_2-fed *C. ragsdalei* (0.01 ± 0.0) (Table 2.3). Growth yields were also 10-fold higher in CO-fed incubations (Table 2.3). Research was continued using optimized acetogen medium under an atompshere of $CO:CO_2$.

Another fermentation parameter that can be manipulated to increase alcohol production in solventogenic Clostridia is initial pH of culture medium. Fermentation profiles of *Clostridium acetobutylicum* have shown that decreasing the initial pH or a lowering in pH during fermentation enhances or even initiates alcohol production (Bahl et al. 1982, Want et al. 2011). The optimal pH for ethanol production was assessed for *C. ragsdalei* on optimized acetogen medium with CO as the reductant. *C. ragsdalei* showed the highest EtOH:Ac (0.43 ± 0.0) at an initial pH of 5.0 (Table 2.4). Thus, process development continued using the optimized medium at an initial pH of 5.0 to 5.3.

Initial observations indicated that fed-batch incubations (vented and repressurized with 207 kPa gauge CO and 1% fresh medium every 48 h) performed better than batch incubations receiving one initial feeding of CO and no fresh medium. Fed-batch incubations produced over 2 times the amount of 1-propanol from added propionate and 6 times the EtOH:Ac (Table 2.5). Conversion efficiency, however, was actually higher for batch than fed-batch fermentations (93% and 71%, respectively). However, batch fermentations stopped consuming propionate and producing 1-propanol between days 4 and 7. Fed-batch fermentations were actively producing 1-propanol up to the final sample collection (day 10). Process development studies were continued using optimized acetogen medium at an initial pH of 5.0 to 5.3 with venting and refeeding of CO every 48 hours.

The final process optimization parameter examined was the effect of scale of fermentation on alcohol production from added propionate (Table 2.6). A direct relationship was shown between increasing the volume of headspace in the reaction vessel and an increase in EtOH:Ac, acid consumption, and alcohol produced. Growth and ΔpH were unaffected (Table 2.6). The production of alcohol from consumed substrate was not affected, since approximately 100% of the consumed acid was converted to the corresponding alcohol under the different headspace volumes examined. However, the amount of acid consumed was almost 3-fold greater in the largest volume (headspace to culture volume ratio of 9) compared to the smallest volume (headspace to culture volume of 3.5). This did not appear to be due to a limitation of CO_2 since increasing the headspace to culture volume ratio for fermentations that were fed H_2:CO_2 did not seem to have a positive affect on alcohol production. This could, however, be due to the role CO plays in generating a redox environment suitable for carboxylate consumption.

Conversion of carboxylates to alcohols under optimized conditions. All of the previous work led to an optimized cultivation regime. These unified parameters were tested in 500 ml serum bottles containing 50 ml optimized acetogen medium (pH 5.0-5.3) amended with 30 mM propionate, butyrate, or valerate and refed CO (30 psi) and fresh medium every 48 h. Propionate, butyrate, and valerate were chosen as exemplar fatty acids found in waste streams based on the analysis of fatty acid content in a variety of organic waste streams (Maune 2011). The results of this

experiment are shown in Table 2.7. *C. ragsdalei* consumed 100% of the added propionate and valerate and 92% of the added butyrate, which yielded conversion efficiencies of 88, 92 and 100%, respectively (Table 2.7). Additionally, *C. ragsdalei* produced 236 ± 3.6 mM ethanol, corresponding to an EtOH:Ac ratio of 17 ± 0.1 in propionate amended cultures. Butyrate and valerate amended cultures produced 152 ± 44 and 173 ± 21 mM ethanol, respectively, corresponding to EtOH:Ac ratios of 6.1 ± 3.7 and 29 ± 7.4, respectively. The total solvent concentrations, which included the amount of ethanol produced as well as the amount of alcohol generated from added carboxylate were 265 ± 4.0, 170 ± 45, and 178 ± 22 mM for propionate, butyrate, and valerate-amended *C. ragsdalei* cultures, respectively (Table 2.7). The data indicated that process development was not only successful for increased production of 1-propanol from propionate, but for other VFAs as well.

Initial attempt at the production of alcohols from fatty acids in waste streams by *Clostridium ragsdalei*, growing cell experiment. A screening assay was conducted to determine if *C. ragsdalei* could reduce VFAs in raw feedstocks, beer wastewater and raw sewage, to the corresponding alcohols with CO as the growth substrate and reductant. These waste streams were selected because they contained propionate, butyrate, and valerate (Maune 2011). The *C. ragsdalei* control, which contained MMYE and was fed CO, produced both ethanol and acetate. However, *C. ragsdalei* did not produce alcohols from VFAs in any of the raw feedstock

incubations. The growth limiting factor was not identified in this research, but inhibited growth even in the presence of minimal medium with yeast extract (MMYE). The highest ratio of MMYE to raw feedstock was 1:1. An antibiotic disinfectant was used in the preparation of glassware and could have remained in the brewer's wastewater even after primary fermentation (John Krause, personal communication). Hops, which were used in the brewing process for the wastewater provided in this study, can also produce antibiotic constituents (Simpon and Smith 1992).

Demonstration of carboxylate conversion to alcohols by other carboxidotrophic bacteria. *Clostridium carboxidivorans* and *Alkalibaculum bacchi*, two solventogenic acetogens isolated for their ability to grow and produce ethanol from a syngas analog, were assessed for conversion of propionate to 1-propanol. *C. carboxidivorans* and *A. bacchi* converted 100 percent of the consumed propionate to 1-propanol. The 1-propanol production rate for *C. carboxidivorans* was close to that of *C. ragsdalei*. *C. carboxidivorans* produced 77% of the theoretical maximum 1-propanol from the added propionate (Table 2.8), where as *A. bacchi* had a conversion efficiency of 10% (Table 2.8). This provided evidence that using organisms with defined medium and fermentation parameters (including initial pH, trace element concentrations, preferred gaseous substrate) will allow for higher yield of desired alcohol end-products, especially compared to the recently isolated *A. bacchi* on undefined medium. The comparison of these organisms also indicates

that carboxidotrophic bacteria not only provide a biocatalyst that does not require expensive feedstocks but also, when the process is optimized, outcompetes well-studied sugar-fermenting biocatalysts (Table 2.8). Indeed, the same was noted as the research presented herein was being drafted, in the biocatalyst *C. ljungdahlii* (Perez et al. 2013). *C. ljungdahlii* produced 10.44 ± 1.69 mM 1-propanol from added propionate after 400 h incubation at a conversion efficiency of 67% (Table 2.8). *C. ljungdahlii* was also reported to have converted butyrate, valerate, and caproate to the corresponding alcohols at a conversion efficiency of 68%, 52%, and 46%, respectively (Perez et al. 2013). Gas stripping was purportedly responsible for conversion inefficiency, since incubations were conducted under a constant flow of syngas (Perez et al. 2013). Herein, it is reported that vented and refed cultures can convert propionate, butyrate, and valerate at close to 100% efficiency (86%, 91%, and 92%, respectively) (Table 2.8). While an effect on conversion efficiency may be seen from venting and refeeding cultures of *C. ragsdalei*, the percent theoretical yield of alcohol from added acid is still higher in fed-batch cultures versus batch (Table 2.5).

It might also be beneficial to assess the lower ethanol to acetate ratios previously reported by Perez et al. (2013), which corresponded to 67%, 53%, and 37% of the added propionate, butyrate, and valerate, respectively, being converted to the corresponding alcohols. In this work, EtOH:Ac of 17, 6.1, and 29 were reported, which correspond to 86% of the added propionate being converted to 1-propanol and 92% of the added butyrate and valerate being converted to 1-butanol

and 1-pentanol, respectively. Lower total alcohol production, decreased conversion efficiency with increasing carbon chain length, and lower EtOH:Ac ratios in experiments with *C. ljungdahlii* may also be due to the use of a gas mix containing hydrogen. As the carbon chain length increases for C_2-C_4 linear fatty acids the reactions becomes more energetically favorable when CO is used as a reductant versus hydrogen (Equations 1-6).

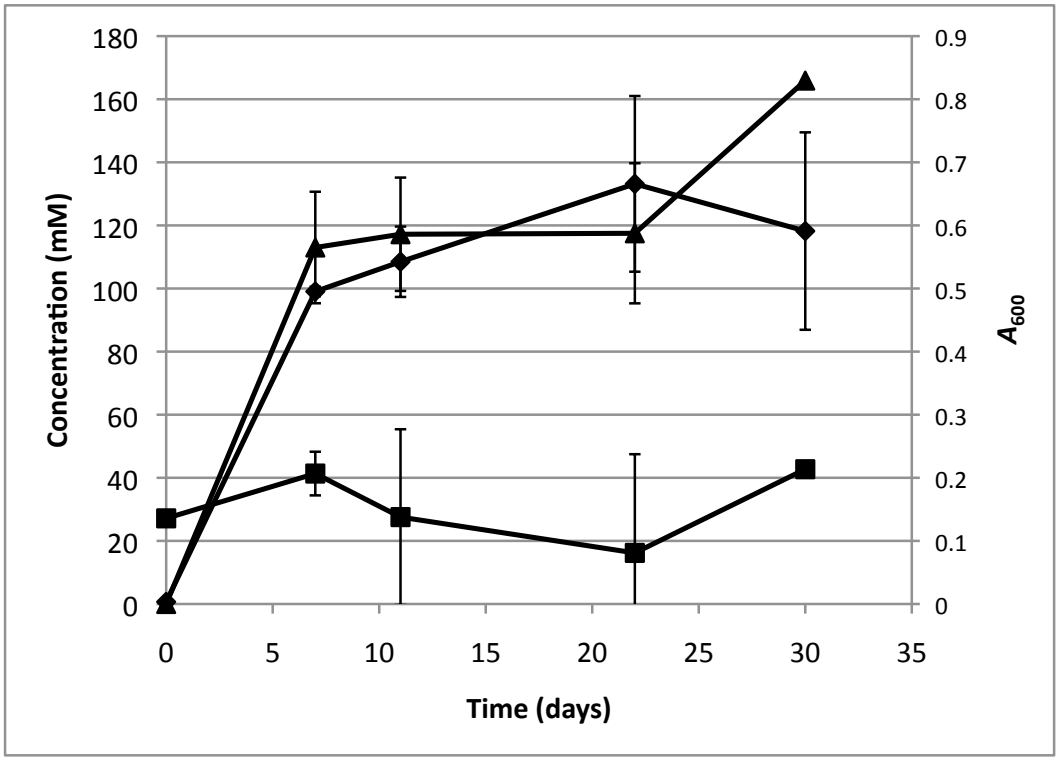


Figure 2.1 Uptake of acetate (■) and production of ethanol (◆) in *Clostridium ragsdalei* during late log phase of growth (A_{600}) (▲).

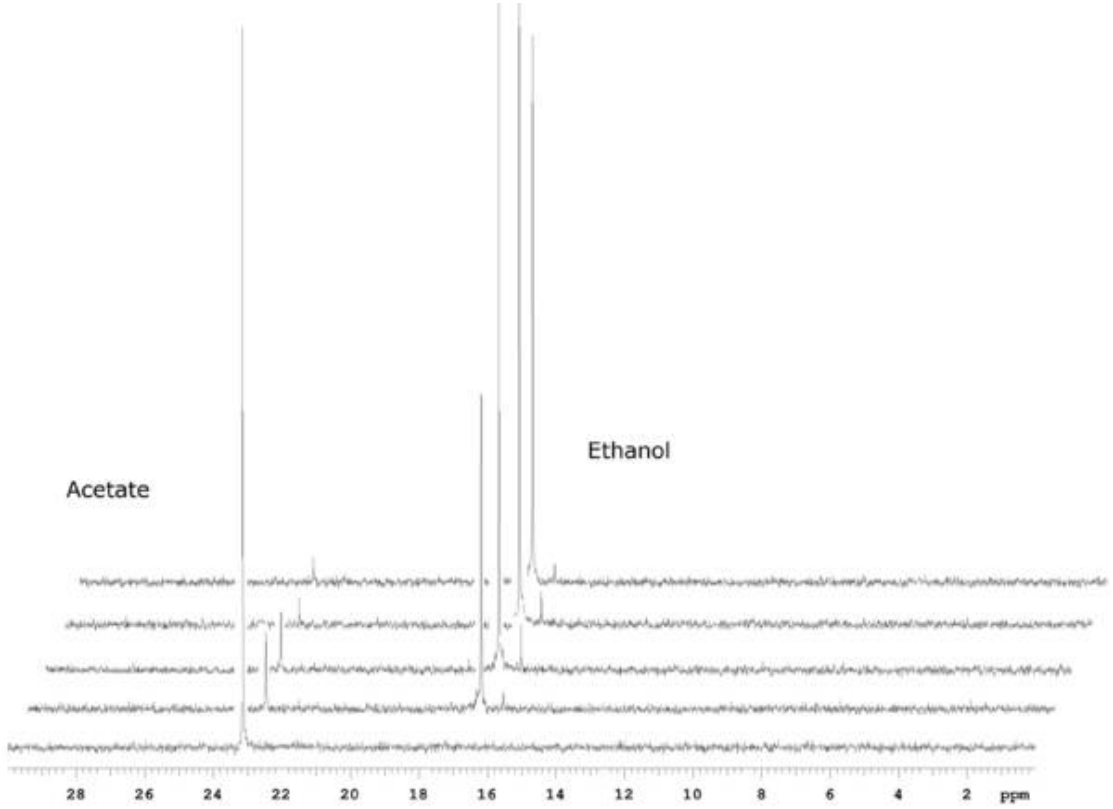


Figure 2.2 The ^{13}C NMR spectrum of the conversion of 2-^{13}C acetate (23.5 ppm) to 2-^{13}C ethanol (16.5 ppm) in *C. ragsdalei* incubations recorded 0, 2, 11, 22, and 30 h after the addition of 2-^{13}C acetate (front to back).

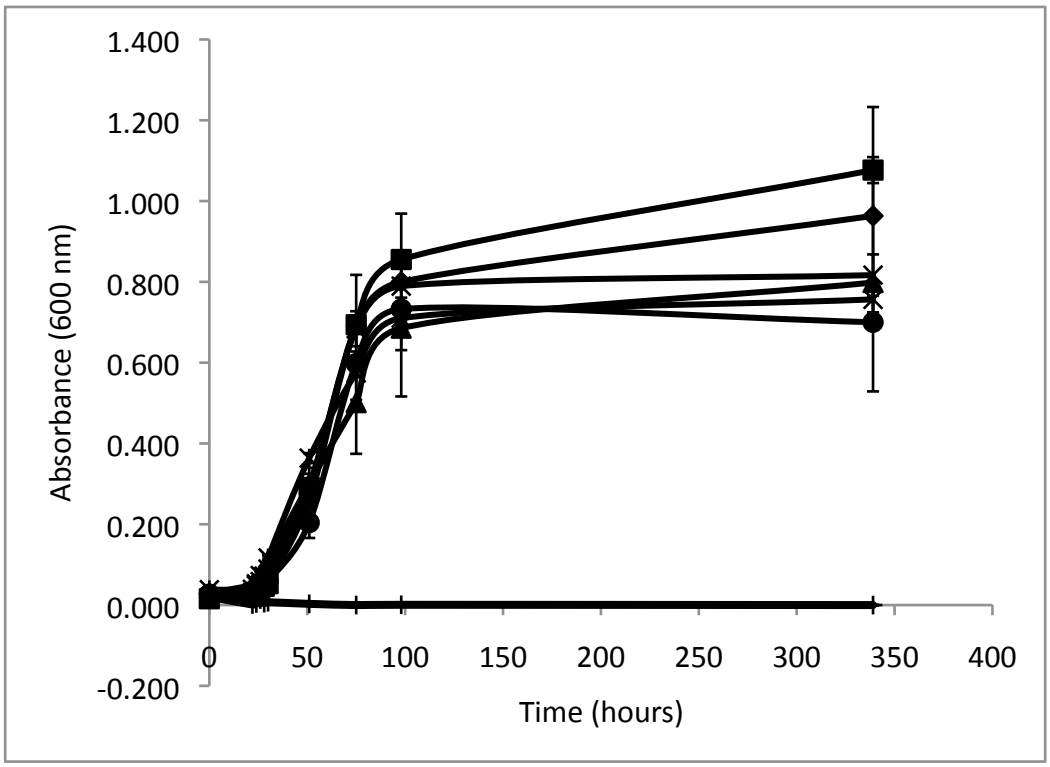


Figure 2.3 Growth analysis of *Clostridium ragsdalei* with acetate (◆), propionate (▲), butyrate (✕), valerate (∗), and hexanoate (●) (1 g L^{-1}). *Clostridium ragsdalei* did not grow with hepatanoate or octanoate (+). Growth was compared to a VFA-free control (■).

Table 2.1 Conversion of propionate to 1-propanol by *Clostridium ragsdalei* grown on general acetogen medium (Tanner 2007) or an acetogen medium optimized to enhance solvent production (Saxena and Tanner 2007).

Condition	EtOH:Ac	Acid consumed (mM)	Alcohol produced (mM)	A_{600}	ΔpH
Acetogen Medium	0.17 ± 0.0	14.9 ± 3.8	4.41 ± 1.3	0.798 ± 0.0	-1.2 ± 0.2
Optimized Medium	0.19 ± 0.0	12.9 ± 1.1	11.9 ± 0.1	0.800 ± 0.1	-1.9 ± 0.2

Table 2.2 Conversion of carboxylates to alcohols by *Clostridium ragsdalei*.

Substrate	EtOH:Ac	A_{600}	ΔpH	% Substrate consumed	% Alcohol produced from consumed substrate	Corresponding alcohol production rate (μM h^{-1})	Final Solvent Concentration[a] (mM)
Propionate	0.22 ± 0.0	0.800 ± 0.1	-1.9 ± 0.2	37.8 ± 3.1	93.1 ± 8.5	35.5 ± 0.3	29.2 ± 1.1
Butyrate	0.17 ± 0.0	0.743 ± 0.1	-1.8 ± 0.1	33.6 ± 9.0	60.6 ± 13	20.6 ± 1.2	14.2 ± 1.7
Valerate	0.04 ± 0.0	0.780 ± 0.0	-1.6 ± 0.0	21.3 ± 2.1	65.4 ± 8.3	8.25 ± 0.2	4.75 ± 0.1
Carboxylate-free Control	0.22 ± 0.1	0.921 ± 0.1	-1.6 ± 0.2				15.9 ± 5.4

[a]Final alcohol produced from added carboxylate combined with final ethanol concentration.

Table 2.3 Conversion of propionate to 1-propanol by *Clostridium ragsdalei* with H_2:CO_2 or CO.

Gas	EtOH:Ac	Acid Consumed (mM)	Alcohol Produced (mM)	A_{600}	ΔpH
H_2:CO_2	0.01 ± 0.0	3.31 ± 1.9	1.08 ± 0.3	0.176 ± 0.0	-1.30 ± 0.0
CO:CO_2	0.18 ± 0.0	10.6 ± 0.9	8.86 ± 0.7	1.010 ± 0.1	-1.50 ± 0.1

Table 2.4 Effect of initial pH on the ethanol to acetate ratio (EtOH:Ac) and growth of *Clostridium ragsdalei* grown on $CO:CO_2$.

Initial pH	EtOH:Ac	A_{600}	ΔpH
4.0	0.00 ± 0.0	0.037 ± 0.0	-0.07 ± 0.0
4.5	0.33 ± 0.2	0.040 ± 0.0	-0.05 ± 0.0
5.0	0.44 ± 0.0	0.820 ± 0.0	-1.14 ± 0.0
5.5	0.28 ± 0.0	0.718 ± 0.1	-1.27 ± 0.2
6.0	0.19 ± 0.0	0.947 ± 0.0	-0.92 ± 1.2
6.5	0.19 ± 0.0	1.072 ± 0.1	-1.99 ± 0.0
7.0	0.34 ± 0.0	1.075 ± 0.1	-2.82 ± 0.0
8.0	0.00 ± 0.0	0.061 ± 0.0	-0.50 ± 0.0

Table 2.5 Effect of continuous addition of CO on the ethanol to acetate ratio (EtOH:Ac) and conversion of propionate to 1-propanol by *Clostridium ragsdalei* grown on CO_2:CO.

Condition	EtOH:Ac	Acid Consumed (mM)	Alcohol Produced (mM)	A_{600}	ΔpH
Batch	0.17 ± 0.0	7.08 ± 0.5	6.6 ± 1.2	0.860 ± 0.1	-1.9 ± 0.0
Fed-batch	0.67 ± 0.1	19.8 ± 1.3	14.1 ± 0.9	0.950 ± 0.0	-1.8 ± 0.0

Table 2.6 Effect of scale of fermentation on the ethanol to acetate ratio (EtOH:Ac) and conversion of propionate to 1-propanol by *Clostridium ragsdalei* grown on $CO:CO_2$.

Ratio of headspace to culture volume	EtOH:Ac	Acid Consumed (mM)	Alcohol Produced (mM)	A_{600}	ΔpH
3.5	0.24 ± 0.0	6.3 ± 0.2	7.9 ± 0.1	0.800 ± 0.4	-1.29 ± 0.24
7	0.33 ± 0.1	8.1 ± 0.8	8.2 ± 0.7	0.910 ± 0.3	-1.62 ± 0.03
9	1.3 ± 0.2	14 ± 2.3	15 ± 1.3	0.880 ± 0.1	-1.53 ± 0.19

Table 2.7 Optimized conversion of propionate, butyrate, and valerate to 1-propanol, 1-butanol, and 1-pentanol, respectively, by *Clostridium ragsdalei*. (Data represent the average of 3 replicates.)

Substrate	EtOH:Ac	A_{600}	ΔpH	% Substrate Consumed	% Alcohol produced from consumed substrate	Corresponding alcohol production rate ($\mu M\ h^{-1}$)
Propionate	17 ± 0.1	1.44 ± 0.1	-1.1 ± 0.1	98 ± 1.2	88 ± 1.5	152 ± 2.3
Butyrate	6.1 ± 3.7	1.32 ± 0.4	-0.85 ± 0.1	92 ± 1.8	100 ± 6.4	110 ± 8.1
Valerate	29 ± 7.4	0.78 ± 0.4	-0.60 ± 0.2	100 ± 0.0	92 ± 17	33 ± 5.9

Table 2.8 Comparison of biocatalysts for the conversion of propionate to 1-propanol.

Biocatalyst	Final Propanol Concentration (mM)	% Theoretical Maximum Propanol Produced	Propanol Production Rate (mM d^{-1})	Source
Clostridium ragsdalei strain P11	29	86	3.6	This study
Clostridium carboxidivorans strain P7^T	23	77	3.3	This study
Clostridium acetobutylicum	19	48	17	Hüsemann and Papoutsakis 1990
Clostridium ljungdahlii	10	67	0.6	Perez et al. 2013
Mixed anaerobic cultures	8.1	33	0.5	Steinbusch et al. 2008
Alkalibaculum bacchi strain CP11^T	2.8	10	0.4	This study
Clostridium formicoaceticum (Crude extracts)	0.1	0.1	1.3	Fraisse and Simon 1988

CONCLUSIONS

The data presented in this study indicated that both ethanol production and conversion of acids to alcohols can be improved by using an acetogen medium optimized by Saxena and Tanner (2010) at an initial pH of 5.0 to 5.3, using CO as the reductant in a fed-batch, shaking incubation with a headspace to culture volume ratio of nine. This is validated by increased ethanol production and decreased acetate production, reflected as an increase in the ethanol to acetate ratio (EtOH:Ac) and higher conversion efficiency of exemplar VFAs to alcohols.

Results of the examination of consumption of VFAs in beer wastewater and raw sewage did not demonstrate that these waste streams could be used by growing cells of *C. ragsdalei* to generate 1-propanol, 1-butanol, or 1-pentanol from propionate, butyrate, and valerate found in these waste streams. The growth-limiting factor was not determined in this research.

ACKNOWLEDGMENTS

Jarrod Warnock and Dr. Mark Nanny for the $^{!3}C$ NMR analysis.

CHAPTER 3

Biochemistry of carboxylate reduction in *Clostridium ragsdalei*

ABSTRACT

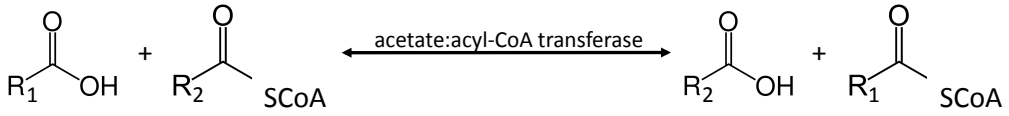
Clostridium ragsdalei is an anaerobic bacterium that produces ethanol, acetate, and biomass from CO and CO_2. *C. ragsdalei* also reduces carboxylates to the corresponding alcohols. The aim of this study was to elucidate the pathway *C. ragsdalei* utilizes for the reduction of carboxylates to alcohols and compare this pathway to known acid reduction pathways in other autotrophic solventogenic bacteria. Enzyme activity was detected in cell-free extracts for an aldehyde oxidoreductase in the acid consuming (0.12 ± 0.03 μmol min^{-1} mg^{-1}) and acid producing (3.28 ± 0.22 μmol min^{-1} mg^{-1}) direction. Additionally, the reduction of ferredoxin was measured with propionaldehyde (592 ± 127 nmol min^{-1} mg^{-1}), CO (37.0 ± 10.6 nmol min^{-1} mg^{-1}), or hydrogen (2.13 ± 0.61 nmol min^{-1} mg^{-1}) as the reductant, which supported the observation that CO-fed *C. ragsdalei* converted more propionate to 1-propanol and generated a 10-fold higher ethanol to acetate ratio than when grown with H_2 as the reductant. NAD(H) was the required electron carrier for alcohol dehydrogenase, which was detected with an activity of 0.12 ± 0.04 μmol min^{-1} mg^{-1}. The AOR-like enzyme reported here differs from the AOR reported for *Moorella thermoacetica* in that it appears to have a narrower substrate range. Comparison of the deduced amino acid sequences of the AOR of *C. ragsdalei* and related Clostridia to *M. thermoacetica* revealed 100% similarity among the conserved N-terminal domain. The other conserved domain of the AOR gene, the C-terminus, was >99% similar among the clostridia, but only 71% similar

to *M. thermoacetica*. This domain is proposed to be involved in substrate binding to AOR and may explain the difference in substrate specificity between carboxidotrophic biocatalysts.

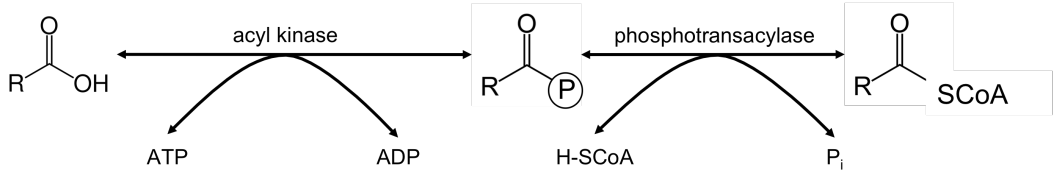
INTRODUCTION

Biochemical pathways for the reduction of carboxylates in acetogenic bacteria. Previous research has demonstrated the conversion of fatty acids to the corresponding alcohols by anaerobic acetogenic bacteria. *Clostridium acetobutylicum* was first reported to convert propionate and propionaldehyde to 1-propanol by Blanchard and MacDonald in 1935. Isopropyl alcohol production from added acetone was later demonstrated in *Clostridium butylicum* (Langlykke et al. 1937). The conversion of propionate to 1-propanol in *C. acetobutylicum* was catalyzed by a CoA transferase, which also activated C_1-C_7 straight chain acids, isobutyrate, DL-2-methylbutyrate, isovalerate, isohexanoate, vinylacetate and crotonate (Hartmanis et al. 1984). *Clostridium kluyveri* was shown to educe hexanoate to 1-hexanol (Kenealy and Waselefsky 1985). Cell-free extracts of *Clostridium formicoaceticum* and resting cells of *Clostridium thermoaceticum*, both grown on fructose, reduced carboxylates to the corresponding alcohols (Fraisse and Simon 1988; Simon et al. 1987).

The different mechanisms for the activation or direct reduction of acids each have unique biochemistry. CoA transferase catalyzes the transfer of a CoA with fatty acid anions producing the different acyl-CoA intermediates (Sramek and Frerman 1975, Wiesenborn et al. 1989).



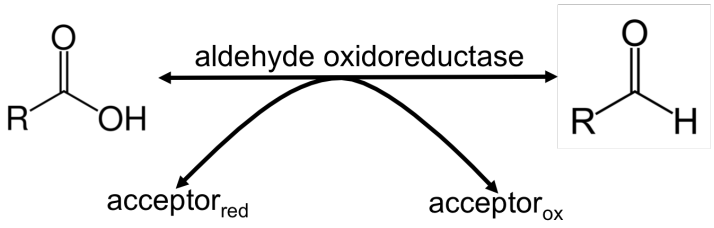
Another mechanism microorganisms use to convert acids to acyl-CoA intermediates is the reversal of the acyl kinase/phosphotransacelyase pathway. Extracts of *C. butyricum* were active for CoA-dependent phosphotransbutyrylase, which reversibly catalyzes the transfer of inorganic phosphate to butyryl-CoA (Valentine and Wolfe 1960). The butyryl-phosphate was then converted to butyrate by butyrate kinase. This process generates one ATP. The reversal of this process will consume one ATP for the phosphorylation of the carboxylate (Lipmann 1944, Valentine and Wolfe 1960).



The phosphotransacetylase/acetate kinase (PTA/AK) pathway has been suggested as the method of acid uptake in *Clostridium carboxidivorans*, a solventogenic, syngas-fermentor (Bruant et al. 2010). *C. carboxidivorans* will reduce propionate to 1-propanol, which was demonstrated in Chapter 2 of this dissertation. The genes for the PTA/AK pathway were identified in the genome of *Clostridium ljungdahlii*, another solventogenic, syngas-fermentor that is 99% similar to *Clostridium*

ragsdalei (Köpke et al. 2010). The enzyme activites of this pathway have not been demonstrated in either bacterium.

As described above, carboxylate reduction typically requires an ATP and CoA to form the thioester intermediate. However, a direct reduction of acids to aldehydes was described (Simon et al. 1987). Cell-free extracts and resting cells of *C. formicoaceticum* and *Moorella thermoacetica* (formerly *C. thermoaceticum*), respectively, converted acids to aldehydes with CO or formate as the reductant (Fraisse et al. 1988). An aldehyde oxidoreductase (AOR) was described for both of these organisms (White et al. 1989, White et al. 1991, White et al. 1993).



An important factor that influences the reduction of acids to alcohols is the energetic constraints of the reaction. Studies on bacteria that use the PTA/AK pathway or CoA transferase to convert acids to acyl-CoA intermediates indicate that the reversal of this pathway is a survival mechanism triggered by the acidic environment generated during acetate and/or butyrate production (Desai et al. 1999). Hence, while the reaction may not be energetically favorable, it appears to be helpful for the survival of these organisms.

Review of the Wood-Ljungdahl Pathway and evidence for direct reduction of carboxylates in *Clostridium ragsdalei*. The direct reduction of acids to aldehydes via an AOR does not require an ATP. However, the reaction requires a redox potential favorable for carboxylate reduction with ferredoxin as an electron donor (E_0' ~550 mV) (Fraisse and Simon 1988, Simon et al. 1987). Simon *et al.* 1987 suggested that the activity of another enzyme, carbon monoxide dehydrogenase (CODH), generates the redox potential necessary for the direct reduction of acids to aldehydes. The oxidation of CO to CO2 generates 2 reducing equivalents and a redox potential of approximately 560 mV. Carbon monoxide dehydrogenase is one of the key enzymes used by acetogens to produce acetyl-CoA from C_1 substrates in the Wood-Ljungdahl pathway (Drake et al. 2006).

The Wood-Ljungdahl Pathway (Figure 3.1) is the accepted mechanism for energy conservation and carbon fixation in acetogenic bacteria (Müller 2003). Formate dehydrogenase (FDH), carbon monoxide dehydrogenase (CODH), and alcohol dehydrogenase (ADH) and/or aldehyde dehydrogenase (ALDH) activities were measured previously in *C. ragsdalei* (Saxena and Tanner 2010). In the methyl-forming branch of the Wood-Ljungdahl Pathway, CO_2 is reduced to formate (Figure 3.1) by FDH. A methylated corrinoid iron sulfur protein is generated via the methyltetrahydrafolate synthetase reaction, which provides the methyl carbon of acetyl-CoA. The carbonyl carbon is generated when CO_2 is reduced to CO by CODH in the carbonyl branch of the Wood-Ljungdahl Pathway (Figure 3.1). As demonstrated in Chapter two of this dissertation, *C. ragsdalei* only produced 2-^{13}C-

ethanol from 2-^{13}C-acetate (Figure 2.2). Therefore, if the labeled acetate were broken down into CO_2 and hydrogen, the labeled CO_2 would then be incorporated into both the methyl and carbonyl carbon of the acetyl-CoA intermediate. As a consequence, the label would be on the methyl and/or carbonyl carbon of acetate and ethanol. The NMR results only showed a methyl labeled ethanol, which suggested direct reduction of acetate to ethanol. This did not, however, clearly indicate whether *C. ragsdalei* converted carboxylates to alcohols via carboxylate activation or direct reduction.

Research objectives. The goal of this research is to test the hypothesis that *C. ragsdalei* directly reduces carboxylates to alcohols at the expense of CO. Additionally, this research will investigate the cofactor, putatively ferredoxin, involved in the reaction. The research objectives are as follows:

1. The direct reduction of propionate to 1-propanol will be tested to further support the reduction of ^{13}C methyl labeled propionate to ^{13}C methyl labeled propanol using ^{13}C Nuclear Magnetic Resonance (NMR) Spectroscopy.
2. Enzyme activities for the conversion of carboxylates to alcohols will be examined. Assays will also be conducted to determine the electron carrier(s) used in this process.

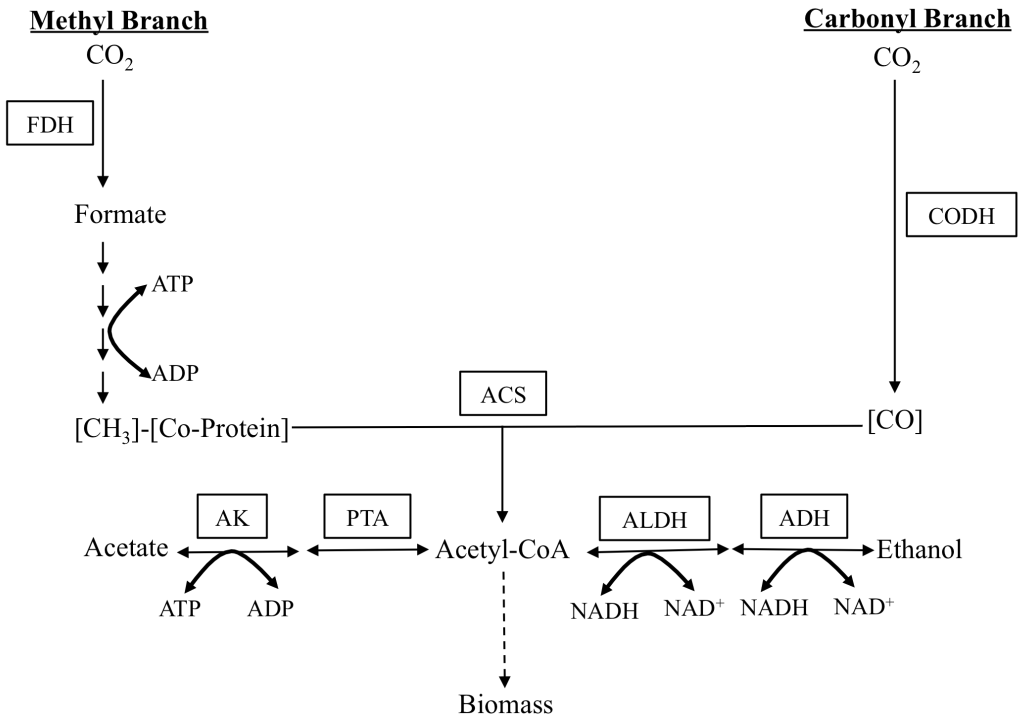


Figure 3.1 Wood-Ljungdahl Pathway with key enzymes highlighted as follows: FDH (formate dehydrogenase), ACS (acetyl-CoA synthetase), CODH (carbon monoxide dehydrogenase), AK (acetate kinase), PTA (phospotransacetylase), ALDH (aldehyde dehydrogenase), and ADH (alcohol dehydrogenase).

MATERIALS AND METHODS

Bacterium, medium, and growth conditions. *Clostridium ragsdalei* strain P11 was maintained on a modified acetogen medium (MAM) prepared using strict anoxic technique (Balch and Wolfe 1976). The medium contained (per L): 20 ml mineral solution (Tanner 2007), modified by using potassium rather than sodium salts of all components; 10 ml vitamin solution (Tanner 2007); 10 ml optimized trace metal solution (Saxena and Tanner 2010); 1 g yeast extract (Difco, Becton Dickinson, Sparks, MD); 10 g 2-(N-morpholino)ethanesulfonic acid (MES), with the pH adjusted to 6.1 using KOH; and 3 ml cysteine sulfide as a reducing agent (Tanner 2007). Inoculation vessels contained a headspace of 1 atm N_2:CO_2 (80:20) and were over-pressurized with CO (207 kPa gauge). For general culture maintenance, *C. ragsdalei* cells were transferred to fresh medium every 8 days using a 2% inoculum into 5 ml of medium in Balch tubes (21 ml headspace). All incubations were done in a shaking incubator at 37°C.

Conversion of propionate to 1-propanol by resting cells of *Clostridium ragsdalei*. *C. ragsdalei* was grown in medium as described above amended with 30 mM propionate. The medium was dispensed in 50 ml aliquotes into 500 ml serum bottles, stoppered, degassed, and sterilized. A 1% inoculum of late log phase *C. ragsdalei* cells was added to sterile medium and the bottles were gassed with CO (207 kPa gauge). Cells were grown to mid-log phase in shaking incubators at 37°C.

Cells were harvested at mid-log phase, when approximately half of the propionate had been consumed and quantitatively converted to 1-propanol. Cells were washed twice using strictly anoxic technique in an anaerobic chamber. A minimal medium (MM), which contained minerals, vitamins, trace metals, MES buffer was amended with 20 mM propionate (MMP). Harvested *C. ragsdalei* cells were resuspended in the prepared MMP in the anaerobic chamber. The slurry was then added to sterile, anoxic, sealed Balch tubes in 3 ml aliquots. Each tube was fed 207 kPa gauge CO. Experiments were conducted in triplicate.

Conversion of 2-^{13}C-propionate to 2-^{13}C-propanol. MAM was amended with 19 mM 2-^{13}C-propionate. Medium was dispensed in 50 ml aliquots into 500 ml bottles (Wheaton) in an anaerobic chamber (Coy) in quadruplicate, crimp sealed with butyl rubber stoppers and aluminum crimp seals, inoculated with mid-log phase *C. ragsdalei* (1% inoculum) and pressurized with CO (207 kPa gauge). Cultures were incubated at 37°C for 9 d. Abiotic and ^{12}C-propionate controls were implemented. Samples (1.2 ml) were treated with 20 µl washed Chelex 100 Resin according to the manufacturers instructions (Bio-Rad Laboratores), vortexed for 30 sec and centrifuged at 6,000 rpm. Supernatant was stored at -80°C until analysis.

Sample volumes were 700 µl and contained 10% D_2O. ^{13}C NMR spectra were obtained on a VNMRS 400 MHz spectrometer at a frequency of 100.5577 MHz using an indirect direction probe. Spectra were collected at 25°C using a single pulse C^{13} experiment with a 45° pulse width (2.95 µs), a delay time of 1 s, an

acquisition time of 1.28 s, a spectral width of 25510.2 Hz and 256 acquisitions. Proton decoupling was achieved using a WALTZ-16 pulse sequence. The number of data points was 65536. The same gain (43) and power (41 db) were used for all samples. Propionate, propanol, acetate, and ethanol were quantified by GC-FID as described in Chapter 2 of this dissertation.

Preparation of cell-free extracts and general enzyme assay protocols. All cell-free extracts were prepared under strictly anaerobic conditions with tightly sealing polypropylene centrifuge bottles and an anaerobic chamber. All plastics materials were kept for at least 24 h in the anaerobic chamber to remove traces of oxygen prior to use. Cells were harvested at mid-log phase when approximately half of the propionate had been consumed and converted to 1-propanol. Cells were harvested in the anaerobic chamber, washed with 100 mM TES (pH 7.0), and either used immediately for enzyme assays or stored at -80°C until needed. Prior to harvesting, samples were taken to measure acid and alcohol concentrations, growth as optical density (A_{600}), and pH measurements.

An approximately 1 g cell pellet (wet weight) was brought to a volume of 3 ml with 100 mM TES (pH 7.0) containing 1 mM dithiothreitol (DTT). The suspension was passed through a French press at 85 MPa under an atmosphere of N_2. The slurry was transferred to centrifuge vials in the anaerobic chamber, sealed and centrifuged at 15,000 x *g* at 4°C for 15 min. The supernatant was used for determining enzyme activities as described in the following sections.

Enzyme assays were carried out in N_2-flushed septum-sealed quartz cuvettes at 37°C. Reaction mixtures were kept anoxic by flushing cuvettes and syringes with N_2 and using strictly anaerobic technique to prepare stock solutions. Controls were run simultaneously. Linearity of the reactions based on protein concentration was established for all preparations. Enzyme activities represent averages for three cell preparations. Protein was determined by the method of Bradford using BSA as standard (Bradford 1976).

Acetate kinase activity. Acetate kinase was assayed in the acyl-phosphate forming direction as previously described with modifications (Bowmann et al. 1976, Lipmann and Tuttle 1945). The assay mixture contained 50 mM Tris-HCl (pH 8.3), 10 mM ATP, 10 mM $MgCl_2$, 0.5 M hydroxylamine (pH 7 with KOH), and 20 mM sodium acetate. Assays were performed using 5 different protein concentrations. No ATP, no fatty acid, and boiled cell-free extract controls were included for each protein concentration. Each reaction mixture was incubated at 37°C for 20 min. The reaction was stopped by the addition of 450 µl ferric reagent (10% $FeCl_3$ and 3% trichloroacetic acid in 0.7 N HCl). Reaction tubes were centrifuged for 5 min at 13,000 *xg* to remove precipitate and the supernatant was read at 535 nm. The molar extinction coeficient for acetyl phosphate under these conditions is 594 M^{-1} cm^{-1}. The activities for controls that did not contain ATP and a separate set of controls that did not contain fatty acid were subtracted from the activities calculated for each sample. The same assay was repeated with the sodium salts of propionate, butyrate,

valerate, hexanoate, and octanoate. *Escherichia coli* cell-free extracts were used as a control. *E. coli* cells were grown in LB medium (Bertani 1951) lysed by sonication at 20% amplitude for a cycle of 20 sec pulse, 60 sec off for a total of 5 min pulse. Debris was pelleted at 10,000 x *g* for 20 min at 4°C.

Detection of CoA-esters. Acetyl-CoA and propionyl-CoA were detected by HPLC equipped with a UV detector set at 260 nm. Each sample was injected into a Kromasil C18 column (250 x 4.6 mm, 10 µm, Supelco) at 0.75 ml min^{-1}. A gradient was initiated with 5% acetonitrile in a buffer that consisted of 50 mM potassium phosphate (pH 6.8) and increased to 20% acetonitrile over 12 min. Samples of cell-free extract, stopped with 1 N HCl and centrifuged at 10,000 rpm for 2 min, were compared against acetyl-CoA and propionyl-CoA (Sigma) standards.

Aldehyde oxidoreductase activity. Aldehyde oxidoreductase (AOR) activity was determined by measuring the initial rate of methyl viologen (MV) reduction at 579 nm and production of propionate from either 1-propanol or propionaldehyde by the addition of propionaldehyde rather than butyralaldehyde as described previously (Simon et al. 1989). The assay mixture contained 100 mM TES buffer (7.0), 0.6 mM oxidized MV (MV_{ox}), 10 µM 1-propanol and 0.68 mM NAD^+, or 10 µM propionaldehyde without NAD^+. The reaction was initiated by the addition of cell-free extract and the activity was determined by measuring changes in the absorbance

at 579 nm caused by reduction of MV. This activity was determined according to the complete reaction:

$$\textbf{1-propanol} + \textbf{NAD}^{+} + \textbf{MV}_{\textbf{ox}} \rightarrow \textbf{propionate} + \textbf{NADH} + \textbf{MV}_{\textbf{red}}$$

AOR activity was determined in the less energetically favorable, aldehyde-forming direction by measuring the initial rate of MV oxidation at 579 nm as described previously (Simon et al. 1989). The assay mixture contained 100 mM TES buffer (pH 7.0) amended with 0.6 mM reduced MV (MV_{red}), reduced drop-wise with 1 mM dithionite, and 10 μM propionate. The reaction was initiated by the addition of cell-free extract and the activity was determined by measuring changes in the absorbance at 579 nm caused by oxidation of MV. This activity was determined according to the reaction:

$$\textbf{Propionate} + \textbf{MV}_{\textbf{red}} \rightarrow \textbf{Propionaldehyde} + \textbf{MV}_{\textbf{ox}}$$

The oxidation of propionaldehyde was measured concomitantly with 1-propanol oxidation by combining alcohol dehydrogenase activity with AOR activity. The assay mixture contained 100 mM TES buffer (pH 7.0) amended with 0.6 mM MV_{ox}, 10 μM 1-propanol, and 0.68 mM NAD^{+}. The reaction was initiated by the addition of CFE and the activity was determined by measuring changes in absorbance at 579 nm caused by the reduction of MV. The production of

propionate and loss of 1-propanol was measured by GC-FID as discussed earlier in this dissertation modified by adjusting the range of the instrument to 1 to detect propionate and 1-propanol at low concentrations (<5 mM). For all AOR assays, substrate free and boiled cell-free extract controls were implemented. Measures were also taken to insure that the order of addition of reaction mixture components did not yield false positive results. The background activity from boiled cell free extract and substrate-free controls were 0.017 ± 0.005 μmol min^{-1} mg^{-1} for the forward and reverse reaction combined. This background activity was removed from the reported AOR activities.

Alcohol dehydrogenase activity. Alcohol dehydrogenase (ADH) activity was determined by measuring the initial rate of NAD^+ reduction at 340 nm according to the reaction:

1-propanol + NAD^+ → propionaldehyde + NADH

The assay mixture contained 100 mM TES buffer (pH 7.0), 10 μM 1-propanol, and 0.68 mM NAD^+. The reaction was initiated by the addition of cell extract and the activity was determined by measuring changes in the A_{340} caused by reduction of NAD^+. The background activity from boiled cell free extract and substrate-free controls were 0.79 ± 0.23 nmol min^{-1} mg^{-1}. This background activity was removed from the reported AOR activities.

Carbon monoxide dehydrogenase and hydrogenase activity. CODH activity was measured as the CO-dependent reduction of MV as measured by spectrophotometry at 579 nm. The reaction mixture contained 100 mM TES (pH 7.0), 1 mM MV_{ox}, and 1 mM DTT. Cell-free extract was added to the reaction mixture and the reaction was initiated by adding 3 ml CO to the headspace of the N_2-flushed cuvette. A N_2 control was implemented.

Hydrogenase was measured in an assay containing 1 mM MV_{ox} in 100 mM TES (pH 7.0) reduced with 1 mM DTT. Cell-free extract was added to the reaction mixture and the reaction was initiated by adding 3 ml H_2 to the headspace of the N_2-flushed cuvette.

Determining the presence of ferredoxin and the role it plays with CODH and hydrogenase. Ferredoxin was determined spectrophotometrically as the reduction of metronidizaole using the method described previously (Chen and Blanchard 1979) modified by adding propionaldehyde instead of the hydrogenase-linked assay described. Additionally, CODH and hydrogenase were linked to ferredoxin oxidation using the same method without propionaldehyde and with the addition of either carbon monoxide or hydrogenase, respectively. The assay mixture contained 100 mM TES buffer (pH 7.0), 0.1 mM metronidazole, and either 10 mM propionaldehyde, 3 ml CO or 3 ml H_2 depending on the activity being measured. The reaction was initiated by the addition of cell extract and the activity was

determined by measuring changes in the A_{360} caused by reduction of metronidazole. The background activity from boiled cell free extract and substrate-free controls for all assays using metronidazole were 0.16 ± 0.08 nmol min^{-1} mg^{-1}. This background activity was removed from the reported AOR activities.

Conversion of propionate to propanol in brewer's wastewater by resting cells of *Clostridium ragsdalei.* Resting cells of *C. ragsdalei* were prepared as described above. Wash buffer (50 mM MES pH 6.1) was used to resuspend harvested cells after the second wash and these cells were dispensed in 3 ml aliquots into anoxic, sterile Balch tubes. Tubes were centrifuged at 10,000 *xg* for 30 min. Supernatant was decanted anoxically and aseptically using a syringe flushed with N_2. Cells were resuspended in the beer wastewater, 1:1 beer wastewater to MMYE, or MMYE described above, fed CO (207 kPa gauge), and incubated at 37°C. Additional incubations were set up, wherein each condition was amended with propionate as an internal standard to determine whether or not the inability of *C. ragsdalei* cells to reduce propionate in beer wastewater to 1-propanol was an issue of substrate availability (ie. substrate bound to solids inaccessible to the bacterial cells). Cultures were monitored for VFA consumption and alcohol production.

RESULTS AND DISCUSSION

Conversion of propionate to 1-propanol by resting cells of *Clostridium ragsdalei.* Conversion of acids to alcohols catalyzed by resting cells or cell-free extracts (Friasse and Simon 1988, Simon et al. 1989) indicates that some solventogenic, acidogenic bacteria can convert acids to alcohols independent of growth. *C. ragsdalei* converts acetate to ethanol during stationary phase of growth and, potentially, does the same with other volatile fatty acids. A resting cell experiment was conducted with cells of *C. ragsdalei* harvested at stationary phase of growth having converted propionate to 1-propanol. These cells were washed twice and amended with minimal medium (MM) containing propionate. CO was used as the reductant. Resting cells of *C. ragsdalei* quantitatively reduced 17.5 ± 0.33 mM of propionate to 18.8 ± 3.5 mM 1-propanol at the expense of CO (Table 3.1). After 5 days, 1.25 ± 0.07 mM of CO was consumed. No change in optical density was noted, nor was a significant change in pH. These results indicated that resting cells could be used to convert VFAs to alcohols without the negative impact of growth limiting factors in the raw waste streams. Additionally, cell-free extracts did not require yeast extract.

Conversion of 2-^{13}C-propionate to 2-^{13}C-propanol. The conversion of acids to alcohols was further supported by the production of 2-^{13}C-propanol from 2-^{13}C-propionate. *C. ragsdalei* amended with 2-^{13}C-propionate consumed 16 mM of the

added propionate and produced 15 mM 1-propanol. The only other end products detected by GC-FID were acetate and ethanol (Table 3.2). In control samples with unlabeled propionate, the day 0 spectrum displayed 5 peaks (Supplemental Figure S1). Four prominent signals between 45 ppm and 65 ppm were due to MES buffer and were observed in all spectra. In 2-^{13}C-propionate samples, the day 0 spectrum prominently displayed only 1 peak at 30.5 ppm, which corresponds to the ^{13}C-label on the number 2 carbon of propionate (Figure 3.1). The day 3 spectrum also contained a signal corresponding to the 2-^{13}C-propionate (30.5 ppm); however, this signal had decreased in intensity when compared to the day 0 spectrum. Additionally, a second signal at 24.6 ppm had appeared. This peak was due to the ^{13}C-label on the number 2 carbon of 1-propanol (Figure 3.1). The day 5 spectra showed a dramatic decrease in 2-^{13}C-propionate (30.5 ppm) and corresponding increase in 2-^{13}C-propanol (24.6 ppm). Additionally, a small peak was seen at 16.8 ppm due to the methyl group on propanol. The day 9 spectrum was similar to day 5 with a slight increase in the signal at 30.5 ppm, which suggests oxidation of the 2-^{13}C-propanol to 2-^{13}C-propionate (Figure 3.2). This evidence not only supported the hypothesis that the carboxylic acid of propionate is being converted to propanol, but also demonstrated that this reaction is reversible *in vivo*. Replicate NMR analysis generated a similar spectrum (Supplemental Figure S2).

Production of alcohols from beer wastewater by resting cells of *Clostridium ragsdalei*. Based on the evidence that resting cells of *C. ragsdalei* converted

propionate to 1-propanol, conversion of propionate in beer wastewater was repeated using resting cells of *C. ragsdalei*. Results were negative for the conversion of propionate in beer wastewater to alcohols. According to one of the beer wastewater suppliers, antibacterial products are used during sterilization (John Krause, personal communication). These antibiotic compounds could remain present on the fermentation equipment through the beer production process. The beer wastewater used in this experiment was collected after primary fermentation. Other compounds could be present that inhibited the reaction from occurring that were not detected in the analyses presented in this work.

Biochemistry for the conversion of acids to alcohols by *Clostridium ragsdalei*.

Cell-free extracts of *C. ragsdalei* harvested during late log phase, when the bacterium is converting propionate to 1-propanol, were assayed for kinase activity toward acetate, propionate, butyrate, valerate, hexanoate, and octanoate (Table 3.3). The acetate kinase (AK) activity for the propionate-amended *C. ragsdalei* cell-free extracts (5.50 ± 0.5 μmol min^{-1} mg^{-1}) was less than half of what was seen for unamended cultures (12.5 ± 2.6 μmol min^{-1} mg^{-1}). AK activity in *C. ragsdalei* during solventogenesis was 10-fold higher than previously described during the solventogenic phase in *Clostridium acetobutylicum* (Andersch et al. 1983). However, *C. ragsdalei* cell-free extracts did not have kinase activity for propionate or for any of the other carboxylates examined. Additionally, a gene annotated as acetate kinase in *C. ragsdalei* was cloned into *Escherichia coli* and the gene product

was partially purified (Appendix A). The partially purified enzyme was active for acetate kinase, but not toward other carboxylates listed in Table 3.3. Though this did not explain the biochemistry of acid reduction in *C. ragsdalei*, the presence of AK activity does add to Saxena and Tanner's (2010) evidence for the use of the Wood-Ljungdahl Pathway for carbon fixation and energy conservation in *C. ragsdalei* (Figure 3.1). Additionally, a propionyl-CoA intermediate was not detected by HPLC (Figure 3.3).

As mentioned previously, it appeared that both acetate (Figure 2.2) and propionate (Figure 3.2) were being directly reduced to the corresponding alcohol based on ^{13}C NMR studies. A mechanism for the direct reduction of acids to alcohols was described previously using an aldehyde oxidoreductase (AOR) to catalyze this reaction (Fraisse et al. 1988, White et al. 1989, White et al. 1991, White et al. 1993). The activity of AOR in the acid-forming direction was demonstrated concomitant with ADH activity in addition to the half reactions (Figure 3.4). ADH activity, alone, was tested as the reduction of propionaldehyde with NADH and the resulting activity was congruent with that reported for ADH in Figure 3.4. Approximately 4 μM 1-propanol was consumed by the reaction. In conjunction with the disappearance of 1-propanol, the activity of ADH was detected (0.12 ± 0.04 μmol min^{-1} mg^{-1}), which is comparable to values reported previously for ADHs active toward 1-butanol in *C. acetobutylicum* (Andersch et al. 1983). The final propionate concentration after the reaction was stopped was 0.31 ± 0.13 mM (Figure 3.4). This does not represent a quantitative conversion of 1-propanol to

propionate as was seen with growing cells, but this result does reflect a conversion that is detectable at very low levels in cell-free extracts of *C. ragsdalei*.

After the AOR activity was demonstrated in the acid-forming direction, efforts were directed at verifying this enzyme's activity in the less energetically favorable, acid-consuming direction. Buffer containing MV was reduced with one drop, anoxic 1 mM dithionite. This provided the best redox envrionment for reproducible AOR activity in the acid-consuming direction as shown in Table 3.4. AOR activity in the propionate-consuming direction was only 3.65% of the activity measured in the propionate-producing direction (0.12 ± 0.03 versus 3.28 ± 0.22 μmol min^{-1} mg^{-1}, respectively). This is comparable to previous observations in *M. thermoacetica* (Strobl et al. 1992), whereby the carboxylic-acid reductase activity of AOR was about 5% of the aldehyde-dehydrogenase acitivty. Unlike acyl kinase, the AOR-like enzyme in *C. ragsdalei* cell-free extracts appeared to be promiscuous (Table 3.4). The AOR enzyme activity when acetate was used as the substrate (0.18 ± 0.02 μmol min^{-1} mg^{-1}) was about the same as when propionate was used as the substrate (0.12 ± 0.03 μmol min^{-1} mg^{-1}). Unexpectedly, however, when octanoate was used as a substrate AOR activity was almost 10-fold higher (0.90 ± 0.35 μmol min^{-1} mg^{-1}) than when propionate was used as the substrate. A 10-fold increase in AOR activity was seen when lactate was used as a substrate (1.26 ± 0.17 μmol min^{-1} mg^{-1}). This was interesting because *C. ragsdalei* did not produce alcohols from octanoate nor lactate. Substrate-free and boiled cell-free extract controls did not oxidize MV.

In an attempt to determine the natural electron mediator of the AOR in *C. ragsdalei* the CODH and hydrogenase activity was measured and these activities were linked to ferredoxin. The CODH activity in *C. ragsdalei* cells, harvested during late log phase while consuming added propionate and producing 1-propanol at the expense of CO, was 1.61 ± 0.8 μmol min^{-1} mg^{-1} (Table 3.5). Hydrogenase activity for these same cell-free extracts (2.15 ± 1.9 μmol min^{-1} mg^{-1}) was higher than expected since these cells were grown with $CO:CO_2$ only, and no H_2.

The activity of ferredoxin in the cell-free extracts was measured as the reduction of metronidazole. When cell-free extract was added to TES buffer (pH 7.0) containing metronidazole and propionaldehyde, the native ferredoxin oxidized propionaldehyde to propionate. Ferredoxin then reduced metronidizole, which was detected spectrophotometrically at A_{360} (Figure 3.5 A). Ferredoxin activity was 592 ± 127 nmol min^{-1} mg^{-1} in cell-free extracts of *C. ragsdalei* (Table 3.6). The ferredoxin activity assay was conducted using H_2 or CO as the substrates of the reaction (Figure 3.5 B and C, respectively). Hydrogenase has a low intrinisic activity toward reducing metronidizole (Chen and Blanchard 1979); however, the reduction of metronidizole in the presence of H_2 and native ferredoxin was only 2.13 ± 0.6 nmol min^{-1} mg^{-1} (Table 3.6). This suggested that there was not an interference of hydrogenase with the assay enough to yield a false positive result. Additionally, this also suggested that another mechanism must be in place for reducing ferredoxin to be used as a cofactor for reducing carboxylates. The reduction of metronidizole in the presence of CO and native ferredoxin was 37.0 ±

10.6 nmol min^{-1} mg^{-1} (Table 3.6), showing strong support that CO is responsible for ferredoxin activation in *C. ragsdalei*.

H_2-fed *C. ragsdalei* only converted 32% of the consumed propionate to 1-propanol and consumed 1/3 of the propionate that CO-fed cultures consumed. The proposed mechanism for acid reduction in *C. ragsdalei* is depicted in Figure 3.6. It is difficult to separate ALDH and ADH activity; thus, ALDH was included in the schematic. The production of ethanol via the reversal of AK cannot be discounted based on the results presented here. However, the absence of kinase activity toward other carboxylates and the absence of propionyl-CoA, suggests that the primary mechanism of alcohol production from carboxylates proceeds via an AOR-like enzyme. The activity of this AOR-like enzyme is comparable to the AOR described in *M. thermoacetica* (Strobl et al. 1992), though the AOR in *M. thermoacetica* shows a broader substrate range in cell-free extracts (Fraisse and Simon 1988).

These findings do not, however, support the process improvement strategies recently suggested, in which Perez et al. (2013) recommended eliminating CO from the gaseous feedstock to essentially inhibit the production of by-products, acetate and ethanol, in *C. ljungdahlii*. This suggestion was developed theoretically and demonstrated the production of one ATP and 2 NADH via hydrogenase-mediated ferredoxin reduction through a set of genes annotated as the Rnf complex (Köpke et al. 2011, Perez et al. 2013). The results presented in Table 3.7 for *C. ragsdalei,* a carboxidotrophic acetogen 99% similar to *C. ljungdahlii*, showed that CO reduced ferredoxin, detected by the reduction of metranidizole by reduced ferredoxin, at

rates over 10-fold those of hydrogen (37.0 ± 10.6 and 2.13 ± 0.61 nmol min^{-1} mg^{-1}, respectively). An Rnf complex was demonstrated in *C. ljungdahlii* and *Acetobacterium woodii* as a proton-translocating ferredoxin:NAD^+ oxidoreductase that appeared to be essential for energy conservation and was similar to the Rnf complex described in *Clostridium kluyveri* (Biegel et al. 2009; Imkamp et a. 2007; Tremblay et al. 2012).

When comparing orthlogous genes for the Rnf complex in *C. kluyveri*, *C. ljungdahlii*, *A. woodii* and *C. ragsdalei* the Rnf complex subunits A, B, D, E, and G were present in all four organisms (Supplemental Figure S3). RnfB is putatively responsible for binding ferredoxin and the subunit RnfC binds NAD(H) (Imkamp et al. 2007). Both subunits have Fe/S binding sites for NAD(H) and Fd with the amino acid (aa) consensus sequence C-XX-C-XX-C-XXX-CP (Imkamp et al. 2007). Two putative RnfB and RnfC genes annotated in the *C. ragsdalei* genome 56% and 31% similar to the *C. kluyveri* RnfB and RnfC aa sequence contained putative Fe/S binding sites with the aforementioned aa sequence CIQCNQCSYVCP and CIQCNQCSFVCP, respectively. These genes were annotated as putative pyruvate:ferredoxin oxidoreductases. *C. ragsdalei* did not have an ortholog neighbor match to the *C. kluyveri* gene encoding RnfC, even though Blast analysis revealed the conserved putative Fe/S binding site aa sequence (Supplemental Figure S3). The presence of Rnf subunits A, B, D, E, G and the consensus sequences for Fe/S binding sites of RnfB and RnfC suggested that *C. ragsdalei* may link energy conservation to fatty acid oxidation/reduction as described for *C. ljungdahlii*, *A.*

woodii, and *C. kluyveri* (Biegel et al. 2009; Imkamp et a. 2007; Tremblay et al. 2012).

AOR in solventogenic Clostridia and other carboxidotrophic bacteria. A noted difference between *C. ragsdalei* and *M. thermoacetica* when reviewing the activities of enzymes involved in the conversion of acids to alcohols is the use of NAD(H) by *C. ragsdalei* versus NADP(H) in *M. thermoacetica.* Though this does not necessarily explain the difference in the carboxylate range that can be reduced in these carboxidotrophic acetogens. The gene sequence of the proposed AOR in *C. ragsdalei* was compared, *in silica*, to AORs of related organisms that have been shown to reduce acids to alcohols in the National Center for Biotechnology Information (NCBI) database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). These sequences were translated using the ExPASy Bioinformatics Resource (http://web.expasy.org/translate/). The resulting amino acid sequences were aligned using the European Bioinformatics Institute's online sequence alighment resource, ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The aligned sequences are shown in Figure 3.7. *C. ragsdalei* is 99% similar to *C. ljungdahlii*, another acetogen that quantitatively reduced 2-^{13}C-acetate to 2-^{13}C-ethanol (Figure 3.8) and was shown to reduce propionate, butyrate, valerate, hexanoate, and isobutyrate to the corresponding alcohols with syngas (Perez et al. 2013). *C. carboxidivorans* reduced propionate to 1-propanol, as described in Chapter 2 of this dissertation (Table 2.8). The *C. ragsdalei* AOR was 99% and 92% similar to the AOR of *C. ljungdahlii* and

C. carboxidivorans, respectively, where as the max identity of the *C. ragsdalei* AOR with *M. thermoacetica* AOR was only 50%. Two conserved domains are described for AOR, an N-terminus and a C-terminus domain. The N-terminus is described as interacting with the tungsten cofactor. This domain is 100% conserved across all of the aligned sequences (Table 3.7), which may explain why the general function of AOR is possible in all of these bacteria. The C-terminus, while 100% similar in the aligned Clostridial AOR amino acid sequences, was only 71% similar to the *M. thermoacetica* C-terminus amino acid sequence (Table 3.7). The C-terminus is composed of 2 structural domains that bind the tungsten cofactor. Another proposed role of this domain is regulation of substrate binding. This could explain the difference in carboxylate substrates available for reduction to the corresonding alcohols in *C. ragsdalei* versus *M. thermoacetica.* Cell-free extracts reduced exemplar substrates, lactate and octanoate, that growing cells did not.

Table 3.1 Conversion of propionate to 1-propanol by resting cells of *Clostridium ragsdalei* with CO.

	Time (Days)	
	0	5
CO (mM)	1.39 ± 0.01	0.14 ± 0.07
Propionate (mM)	22.2 ± 0.07	2.47 ± 0.33
1-propanol (mM)	0.00 ± 0.00	18.8 ± 3.49
pH	5.31	5.47 ± 0.16

Table 3.2 Propionate, 1-propanol, acetate, and ethanol produced by *Clostridium ragsdalei* amended with 2-^{13}C-propionate as determined by GC-FID.

	Concentration (mM)			
Day	Propionate	1-propanol	Acetate	Ethanol
0	20.0 ± 0.6	0.00 ± 0.0	2.31 ± 0.2	0.00 ± 0.0
3	10.5 ± 3.8	6.43 ± 2.6	8.54 ± 1.2	6.25 ± 2.6
5	3.62 ± 0.9	15.2 ± 1.8	76.9 ± 13	134 ± 21
9	4.11 ± 1.2	14.8 ± 2.4	81.8 ± 17	125 ± 25

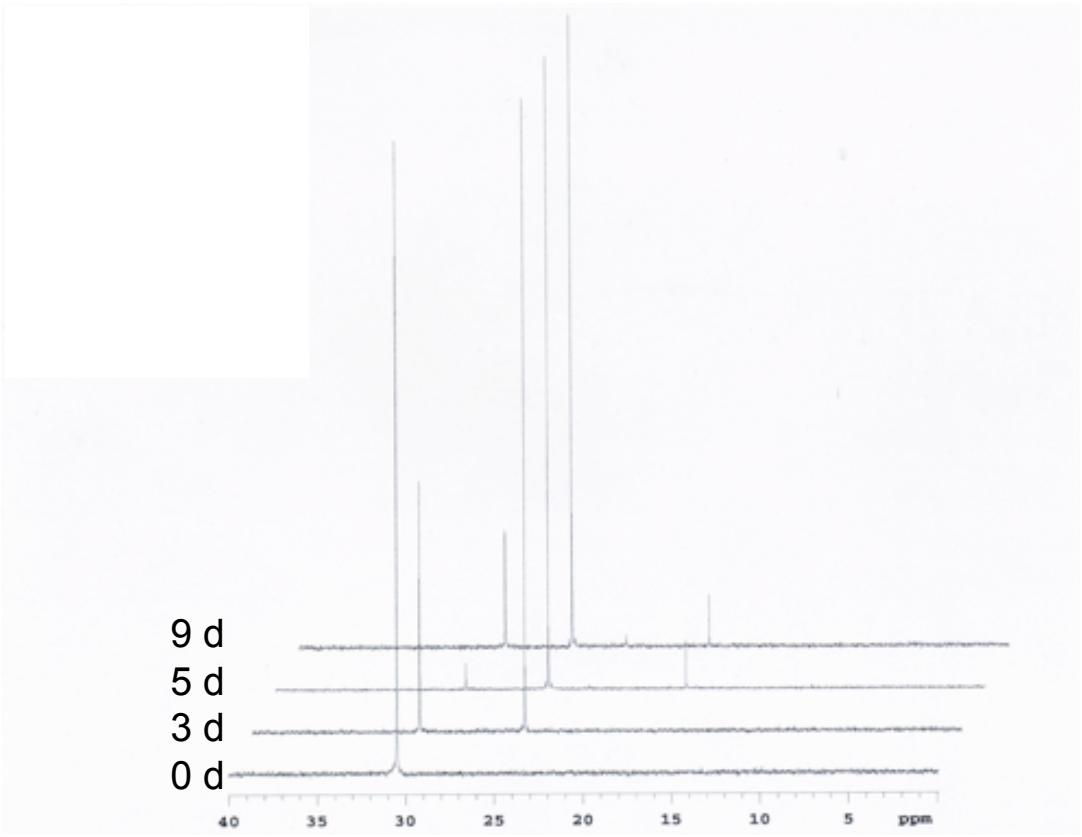


Figure 3.2 ^{13}C NMR spectra for *C. ragsdalei* reducing 2-^{13}C-propionate (30.5 ppm) to 2-^{13}C-propanol (24.6 ppm) for days 0, 3, 5, and 9.

Table 3.3 Catalytic activities of *Clostridium ragsdalei* acetate kinase and other acyl kinases.

	Clotridium ragsdalei (propionate amended)	*Clostridium ragsdalei* (unamended)	*Escherichia coli* (positive control)
Substrate	Specific activity (μmol min^{-1} mg^{-1})		
Acetate	5.50 ± 0.5	12.5 ± 2.6	2.05
Propionate	ND[a]	ND	
Butyrate	ND	ND	
Valerate	ND	ND	
Hexanoate	ND	ND	
Octanoate	ND	ND	

[a]Colorimetric change was not detected by the spectrophotometer.

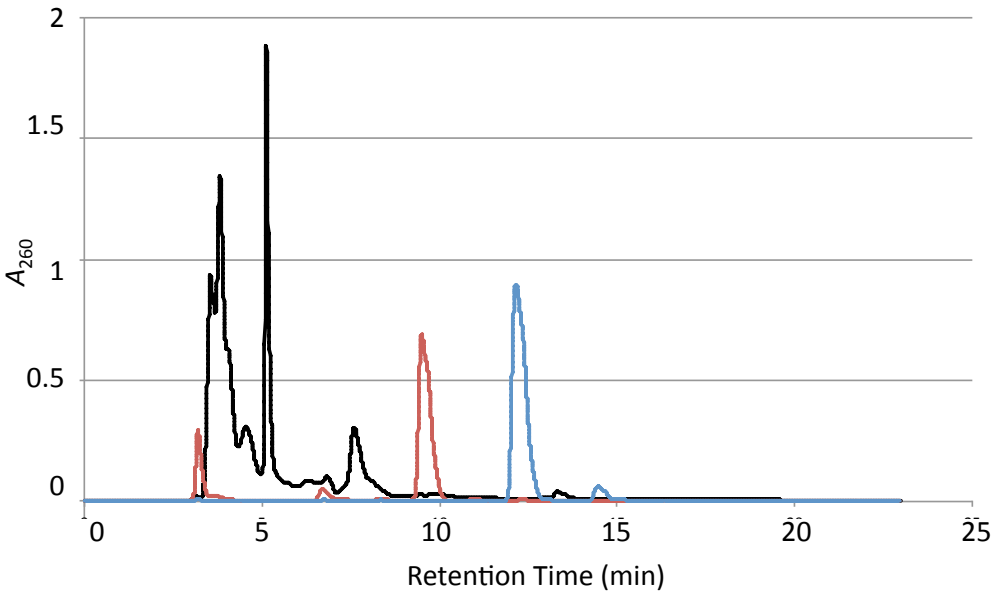


Figure 3.3 Chromatographic analysis for the presence of CoA thioesters in *Clostridium ragsdalei* amended with propionate and grown on CO:CO_2 as detected by HPLC/UV (Black line). Retention times for the CoA thioesters are as follows: acetyl-CoA (9.4 min, red line), and propionyl-CoA (12.5 min, blue line).

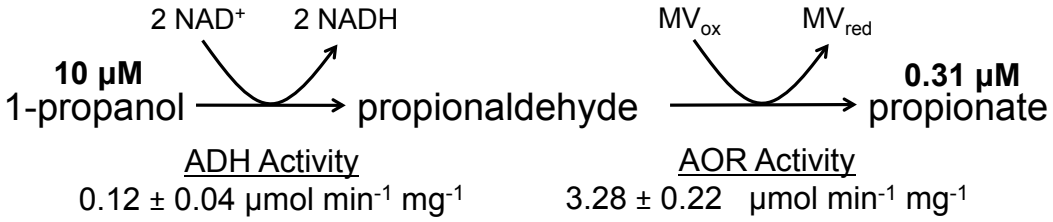


Figure 3.4 Conversion of 1-propanol to propionate by desalted cell-free extracts of *C. ragsdalei* with corresponding alcohol dehydrogenase (ADH) and aldehyde oxidoreductase (AOR) activity.

Table 3.4 Catalytic activities of *Clostridium ragsdalei* aldehyde oxidoreductase

Substrate	Electron Carrier	Specific activity (μmol min^{-1} mg^{-1})
Propionaldehyde	MV_{ox}	3.28 ± 0.22
Propionate	MV_{red}	0.12 ± 0.03
Acetate	MV_{red}	0.18 ± 0.02
Octanoate	MV_{red}	0.90 ± 0.35
Lactate	MV_{red}	1.26 ± 0.17

Table 3.5 Catalytic activities of *Clostridium ragsdalei* carbon monoxide dehydrogenase (CODH) and hydrogenase.

	Substrate	Electron carrier	Specific activity (μmol min^{-1} mg^{-1})
CODH	CO	MV_{ox}	1.61 ± 0.8
Hydrogenase	H_2	MV_{ox}	2.15 ± 1.9

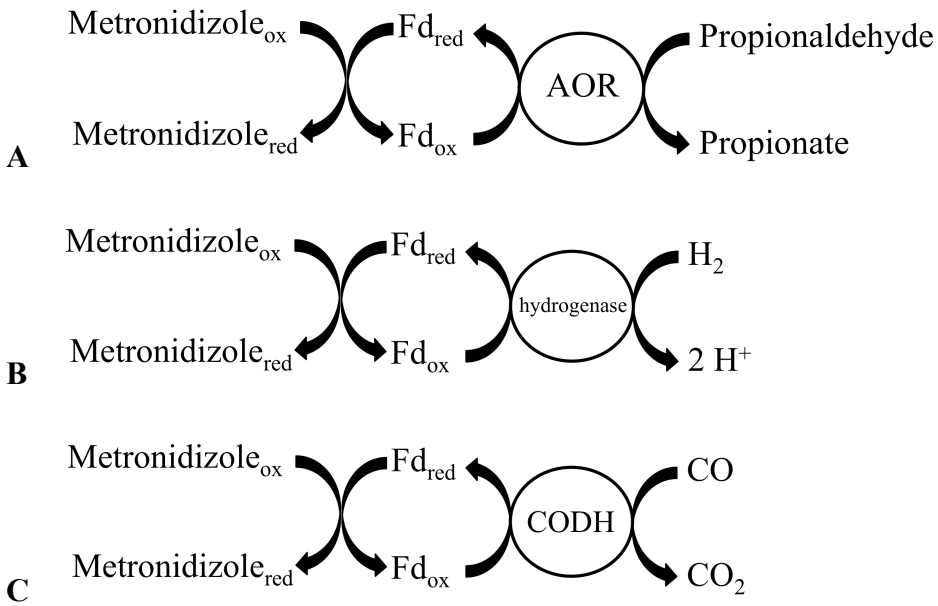


Figure 3.5 The reduction of metronidizole by native *Clostridium ragsdalei* ferredoxin (Fd) that oxidized propionaldehyde (A). The reduction of ferredoxin (Fd) coupled to hydrogenase activity in *Clostridium ragsdalei* cell-free extracts (B). The reduction of ferredoxin (Fd) coupled to CODH activity in *Clostridium ragsdalei* cell-free extracts (C).

Table 3.6 CODH and hydrogenase activity with *Clostridium ragsdalei* ferredoxin.

Substrate	Electron carrier	Specific activity (1 nmol min^{-1} mg^{-1})
Propionaldehyde	Fd	592 ± 127
CO	Fd	37.0 ± 10.6
H_2	Fd	2.13 ± 0.61

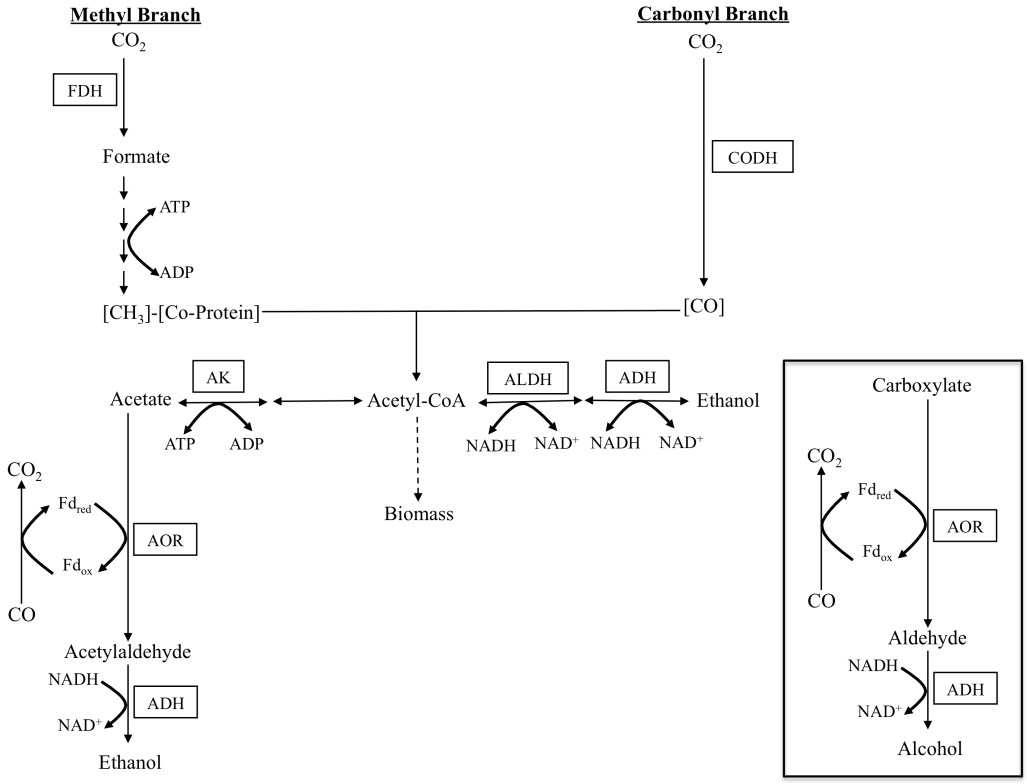


Figure 3.6 Proposed pathway for the reduction of acids to alcohols coupled to the Wood-Ljungdahl Pathway with key, empirically supported enzymes highlighted as follows: FDH (formate dehydrogenase), CODH (carbon monoxide dehydrogenase), AK (acetate kinase), ALDH (aldehyde dehydrogenase), ADH (alcohol dehydrogenase), and aldehyde oxidoreductase (AOR).

```
ragsdalei           MYGYNGKVLRINLSSKTYIVEELKIDKAKKFIGARGLGVKTLFDEVDPKVDPLSPDNKFI 60
ljungdahlii         MYGYDGKVLRINLKERTCKSENLDLDKAKKFIGCRGLGVKTLFDEIDPKIDALSPENKFI 60
carboxidivorans     MYGYNGKVLRINLSNKTYVVEELKIDQAKKFIGSRGLGVKTLFDEVDPKIDPLSPDNKFI 60
thermoacetica       MYGWTGQLLRVNLSNGKCRTERLDPILARDYVGARGLASKILWNEIDPQVDPLAPENKLI 60
                    ***: *::**:**.. .   *.*.   *:.::*.***. * *::*:**::*.*:*:**:*

ragsdalei           IAAGPLTGAPVPTSGRFMVVTKSPLTGTIAIANSGGKWGAEFKAAGYDMIIVEGKSDKEV 120
ljungdahlii         IVTGPLTGAPVPTSGRFMVVTKAPLTGTIGISNSGGKWGVDLKKAGWDMIIVEDKADSPV 120
carboxidivorans     IAAGPLTGAPVPTSGRFMVVTKSPLTGAIAISNSGGKWGVELKMAGYDMIIVEGKSDKEV 120
thermoacetica       FMTGPLTGTTAISGNRYNVVTKSPLTGAIAASSSGGYFGSELKYAGFDGIIFEGRAPEPV 120
                    : :*****:.. :..*: ****:****:*. :.*** :* ::* **:* **.*.:: . *

ragsdalei           YVNIVDDKVEFRDASHVWGKLTEETTKMLQQETDSRAKVLCIGPAGEKLSLMAAVMNDVD 180
ljungdahlii         YIEIVDDKVEIKDASQLWGKVTSETTKELEKITENKSKVLCIGPAGERLSLMAAVMNDVD 180
carboxidivorans     YVNIVDDKVEFKDASHVWGKLTEETTKMLQNENDAKAKVLCIGPAGEKLSLMAAVMNEVD 180
thermoacetica       YLWIEDGSFELRPAGELWGKNVHETEDAIKAVTCPHAKVACIGPAGEKLVRFACIMNDKN 180
                    *: * *...*:: *..:*** . ** . ::  .  ::** *******:*  :*.:**: :

ragsdalei           RTAGRGGVGAVMGSKNLKAIVVKGSGKVKLFDEQKVKEVALEKTNILRKDPVAGGGLPTY 240
ljungdahlii         RTAARGGVGAVMGSKNLKAITVKGTGKIALADKEKVKKVSVEKITTLKNDPVAGQGMPTY 240
carboxidivorans     RTAGRGGVGAVMGSKNLKAIVVKGSGKVKLFDEEKVKAVSLEKSNILRKDPVAGSGLPTY 240
thermoacetica       RAAGRSGVGAVMGSKNLKAIAVRGHGGVKVADGPAFREAVLASLAKIKANDVTHGGLPAY 240
                    *:*.*.**************.*:* * : : *   .: . : .   :: : *:  *:*:*

ragsdalei           GTAVLVNIINENGVHPVKNFQKSYTDQADKISGETLTKDCLVRKNPCYRCPIACGRWVKL 300
ljungdahlii         GTAILVNIINENGVHPVKNFQESYTNQADKISGETLTANQLVRKNPCYSCPIGCGRWVRL 300
carboxidivorans     GTAVLVNIINENGIHPVKNFQESYTPEADKISGETMTKDCLVRKNPCYRCPIACGRWVKL 300
thermoacetica       GTGVLVNVINAHGGLPTRNFQTGIFPGAEKISGEALAATYLVRKKACLACPMACGRATMV 300
                    **.:***:** :*  *.:*** .    *:*****:::   ****:.*  **:.*** . :

ragsdalei           DDGTECG---GPEYETLWSFGSDCDVYDINAVNTANMLCNEYGLDTITAGCTIAAAMELY 357
ljungdahlii         KDGTECG---GPEYETLWCFGSDCGSYDLDAINEANMLCNEYGIDTITCGATIAAAMELY 357
carboxidivorans     DDGTECG---GPEYETLWSFGSDCDVYDINAANTANMLCNEYGLDTISAGATIAAAMELY 357
thermoacetica       PSGPYAGHGEGPEYEAQWSLGADCGIDDLAAILKANFLANELGYDPISFGSTLACAMELY 360
                     .*. .*   *****: *.:*:**.  *: *   **:*.** * *.*: *.*:*.*****

ragsdalei           QRGYIKDEEIAADGLSLNWGDAKSMVEWVKKMGLREGFGDKMADGSYRLCDSYGVPEYSM 417
ljungdahlii         QRGYIKDEEIAGDNLSLKWGDTESMIGWIKRMVYSEGFGAKMTNGSYRLCEGYGAPEYSM 417
carboxidivorans     QRGYIKDEEIAADGLSLKWGDAKSMVEWVKKMGRREGFGDKMADGSYRLCESYGAPEYAM 417
thermoacetica       EKGYLP---AGDTEVPLEFGNAAVMVETARKVGYREGIGDLLAEGSYRLASRYGHPELSM 417
                    ::**:     .   :.*::*::  *:   :::   **:*  :::*****.. ** ** :*

ragsdalei           TVKKQEIPAYDPRGIQGHGITYAVNNRGGCHIKGYMVSPEILGYPEKLDRLAVEGKAGYA 477
ljungdahlii         TVKKQEIPAYDPRGIQGHGITYAVNNRGGCHIKGYMINPEILGYPEKLDRFALDGKAAYA 477
carboxidivorans     TVKKQELPAYDPRGAQGHGITYAVNNRGGCHIKGYMISPEILGYPEKLDRFALEGKPAYA 477
thermoacetica       TSKKQEYPAYDPRAFQGIGLNYATSNRGGCHVRGYTIAAEALGTPVQADPLSSEGKAALD 477
                    * **** ******. ** *:.**..******::** : .* ** * : * :: :**..

ragsdalei           RVFHDLTAVIDSLGLCIFTTFGLGAQDYVDLYNAVVGGELHDVDSLMLAGDRIWTLEKIF 537
ljungdahlii         KLFHDLTAVIDSLGLCIFTTFGLGIQDYVDMYNAVVGESTYDADSLLEAGDRIWTLEKLF 537
carboxidivorans     RVFHDLTAVIDSLGLCIFTTFGLGAQDYVDMYNAVVGGELHDVNSIMMAGDRIWTLEKIF 537
thermoacetica       KAFQDLTALVDASGICLFTTFALGAPDVASMLATATG-VPYTEESGLLAGERIYNLERLF 536
                    : *:****::*: *:*:****.**  * ..:  :..*   :  :* : **:**:.**::*

ragsdalei           NLKAGIDSSQDTLPKRLLEEPVPEGPSKGEIHRLDVLLPEYYSVRGWDKNGIPTEETLKK 597
ljungdahlii         NLAAGIDSSQDTLPKRLLEEPIPDGPSKGEVHRLDVLLPEYYSVRGWSKEGIPTEETLKK 597
carboxidivorans     NLKAGIDSSQDTLPKRLLEEPVPEGPSKGEVHRLDVLLPEYYSVRGWDSNGIPTEETLRK 597
thermoacetica       NFAAGLTKADDTLAPRLLNEPMPEGPAKGKTSALTKMLAEYYQLRGWDEEGRVTAATRER 596
                    *: **: .::***. ***:**:*:**:**:   *  :*.***.:***..:*  *  * .:

ragsdalei           LGLDEYVGKF 607
ljungdahlii         LGLDEYIGKF 607
carboxidivorans     LGLEEYIGKI 607
thermoacetica       LGL------- 599
                    ***
```

Figure 3.7 Alignment of *Clostridium ragsdalei* AOR to that of *C. ljungdahlii* (max identity 99%), *C. carboxidivorans* (92%), and *Moorella thermoacetica* (50%). Sequences were aligned using the online alignment tool, ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Identical residues, observed substituations, and semi-conserved substiutations are demarkated with an asterisk (*), semi-colon (:), and period (.), respectively.

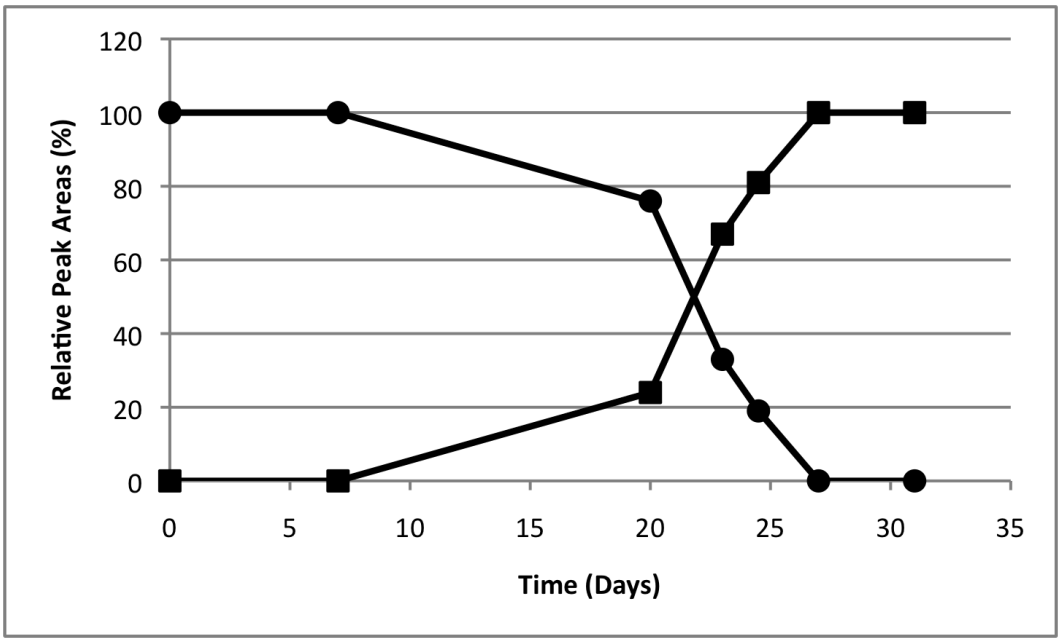


Figure 3.8 Quantitative conversion of acetate (●) to ethanol (■) by *Clostridium ljungdahlii* as detected by ^{13}C NMR.

Table 3.7 Analysis of conserved domains of AOR in *Clostridium ragsdalei*.

Domain	Sequence	Amino Acids	% ID[a]	Proposed Function[b]
N-terminus	GVGAVMGSKNLKAI	187-200	100	Interacts with W cofactor.
C-terminus	RLLNEPMPEGPAKG	553-566	71	Binds W cofactor. Involved in regulating substrate binding to AOR.

[a]Percent identity of the clostridial AOR conserved domains with the sequence for the conserved domain in *M. thermoacetica* AOR.
[b]Marchler-Bauer et al. 2013

CONCLUSIONS

The ^{13}C NMR studies, enzyme data, and absence of CoA intermediates support the conclusion that *Clostridium ragsdalei* uses an AOR-like enzyme to reduce carboxylates of varying chain lengths to the corresponding alcohols. The use of this pathway has been only been demonstrated in *Moorella thermoacetica* and *Clostridium formicoaceticum* previously. Reduction of carboxylates via an AOR is the suggested mechanism in other carboxidotrophic microorganisms, including *Clostridium ljungdahlii*. The lack of activity for the acyl kinase/phosphotransacylase pathway and the absence of an acyl-CoA intermediate further support these claims.

The AOR activity in cell-free extracts of *C. ragsdalei* was linked to ferredoxin as an electron carrier. Furthermore, the ferredoxin in *C. ragsdalei* appeared to favor CO as a reductant over hydrogen as was demonstrated by a more than 10-fold increase in reduction of ferredoxin in the presence of CO versus hydrogen. The reduction of a carboxylate to the aldehyde intermediate appeared to rely strictly on reductant, either methyl viologen or ferredoxin. Only the further conversion of the aldehyde to alcohol required pyridine nucleotides, in the form of NADH.

AOR appeared to be a promiscuous enzyme with activity toward lactate and octanoate, in addition to acetate and propionate even though growing cells of *C. ragsdalei* did not reduce lactate or octanoate to the respective alcohols. Kinase

activity was negative for all carboxylates examined except acetate. This was also true for a partially purified recombinant protein annotated as acetate kinase in *C. ragsdalei*.

The use of this system explains how *C. ragsdalei* and similar carboxidotrophic acetogens can produce alcohols from carboxylates at the expense of CO. This provides *C. ragsdalei* with another mechanism for generating electron acceptors (oxidation of ferredoxin and NADH) and, potentially, ATP (via chemiosmotic processes). The AOR-mediated carboxylate reduction pathway is annotated in the genomes of *C. ljungdahlii* and *C. carboxidivorans* at 100% similarity and *M. thermoacetica* at 50% similarity. It has yet to be tested if *C. ragsdalei* or these related bacteria posess a functional Rnf complex for the regeneration of electron carriers as has been described for *Acetobacterium woodii*.

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SUPPLEMENTAL DATA

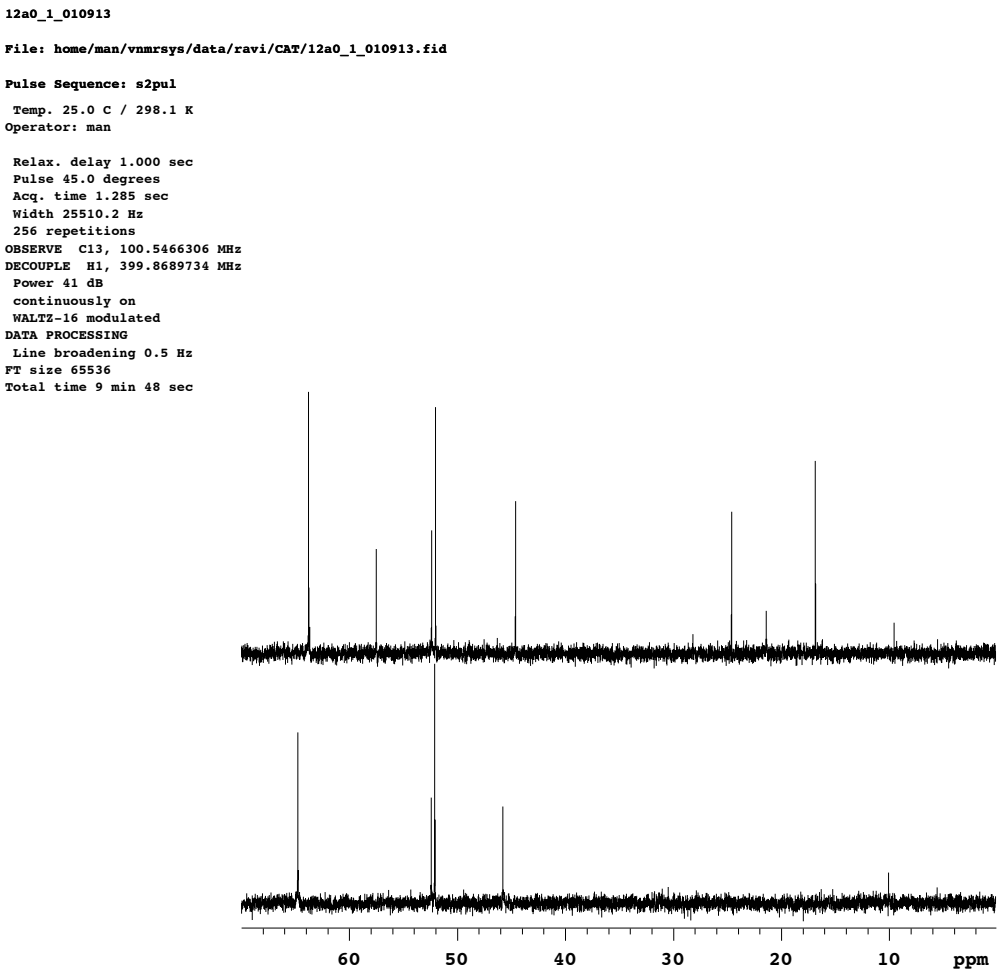

Figure S1. Day 0 unlabeled control ^{13}C NMR spectrum (bottom) for propionate to propanol experiment. Four prominent signals between 45 ppm and 65 ppm due to MES and 1 peak at 30.5 ppm corresponds to the methyl carbon of propionate. Day 9 unlabeled control ^{13}C NMR spectrum (top) for propionate to propanol experiment also showed prominent signals for MES in addition to 3 prominent signals corresponding to the 3 carbons of 1-propanol and a peak a 22.5 ppm that could be due to the methyl carbon of acetate.

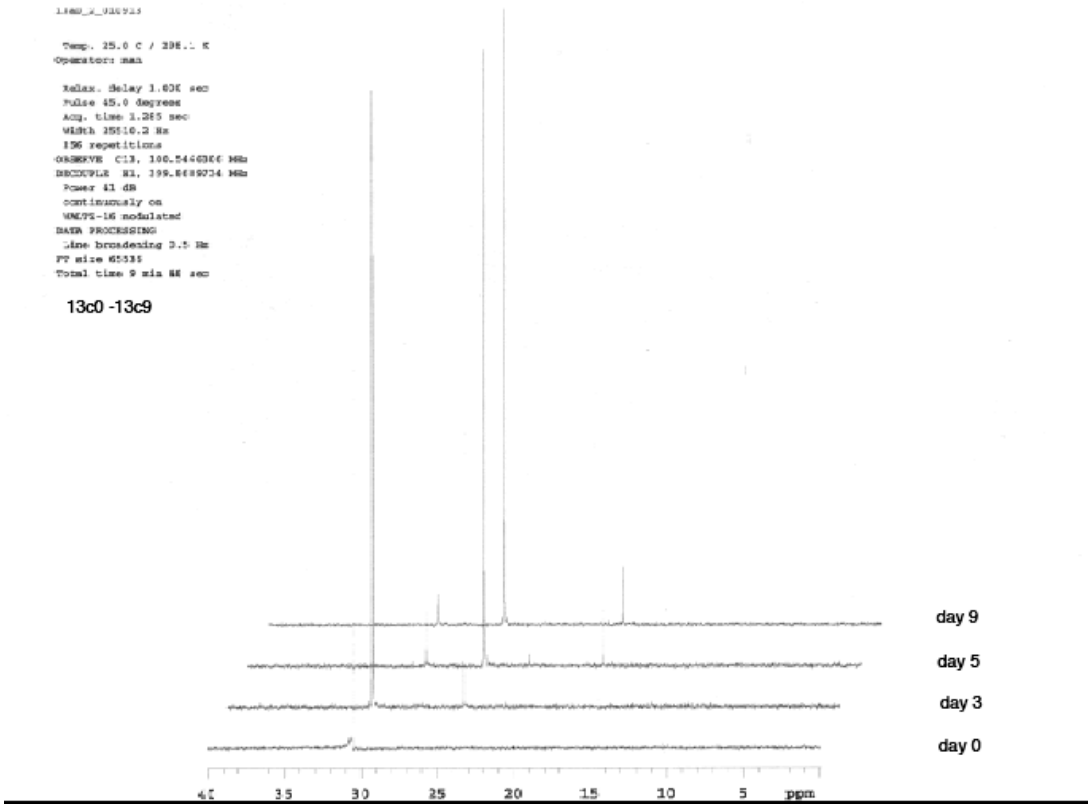

Figure S2. Replicate ^{13}C NMR spectra for *Clostridium ragsdalei* reducing 2-^{13}C-propionate (30.5 ppm) to 2-^{13}C-propanol (24.6 ppm) for days 0, 3, 5, and 9 (front to back).

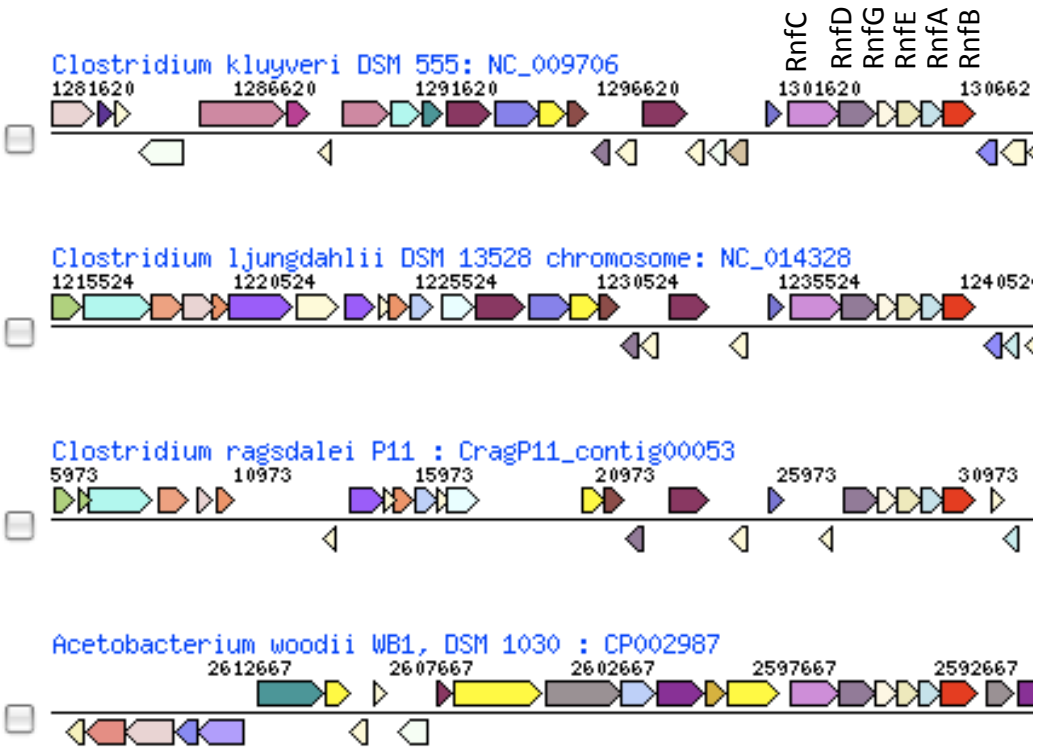

Figure S3. Neighbor orthologs for putative Rnf complex in *Clostridium kluyveri*, *Clostridium ljungdahlii*, *Clostridium ragsdalei* and *Acetobacterium woodii* with the Rnf subunits labeled.

APPENDIX A

Partial purification of recombinant *Clostridium ragsdalei* acetate kinase (AK) in *Escherichia coli*

ABSTRACT

The enzyme acetate kinase (AK) catalyzes the reversible dephosphorylation of acetyl-phosphate in the fermention of acetogens. The production of acetate in acidogenic fermentation is important in that it generates one ATP via substrate level phosphorylation. Though not necessarily energetically favorable, the reversal of AK might be responsible for activation of acetate for the subsequent reduction to ethanol. Enzyme activities for the partially purified recombinant *Clostridium ragsdalei* acetate kinase AK were 35-fold higher than those of cell-free extracts of *C. ragsdalei* strain P11. Neither cell-free extracts of *C. ragsdalei* nor the partially purified recombinant *C. ragsdalei* AK were active towards the other carboxylates examined. This provides further evidence that acetate kinase does not catalyze the reduction of acetate to ethanol in *C. ragsdalei*.

INTRODUCTION

Carboxidotrophic acetogens are a group of bacteria that are biochemically distinct from other prokaryotes in that they are capable of metabolizing C_1 substrates to acetate and cell biomass (Müller 2003). The method by which these bacteria generate an ATP via substrate level phosphorylation (SLP) is through the Wood-Ljungdahl Pathway, also known as the acetyl-CoA pathway (Müller 2003). One ATP is consumed in the methyl branch of the Wood-Ljungdahl Pathway (Figure A.1); thus, there is no net energy conservation via SLP. *Clostridium ragsdalei* strain P11 is a mesophilic, carboxidotrophic acetogen that was isolated from anoxic, freshwater sediement and which grows on $CO:CO_2$ (Huhnke et al. 2010). The proposed carbon fixation pathway in *C. ragsdalei* is the Wood-Ljungdahl Pathway. Additionally, *C. ragsdalei* appears to consume acetate generated earlier in the fermentation and produce ethanol (Figure 2.1). It was initially proposed that acetate activation was accomplished via the reversal of the acetate kinase (AK) (Desai et al. 1999). According to the proposed mechanism, the phosphorylation of acetate via AK would consume one ATP. This was not entirely impossible since the majority of ethanol production occurred during late log/early stationary phase of growth (Figure 2.1). In the attached dissertation, the previous research that carboxidotrophic acetogens directly reduce carboxylates to the corresponding alcohols (ie. acetate to ethanol) via an aldehyde oxidoreductase (AOR) without activation of the carboxylate via phosphorylation and then dephosphorylation with

CoA transfer were supported (White et al. 1993). At the time of the work presented herein, however, it was still unclear as to whether or not the production of alcohols from carboxylates proceeded via a CoA intermediate. Thus, a gene annotated as AK in *C. ragsdalei* was recombinantly expressed and partially purified. This report summarizes this partial purification, describes some of the properties of the enzyme, and tests the substrate specificity of the enzyme.

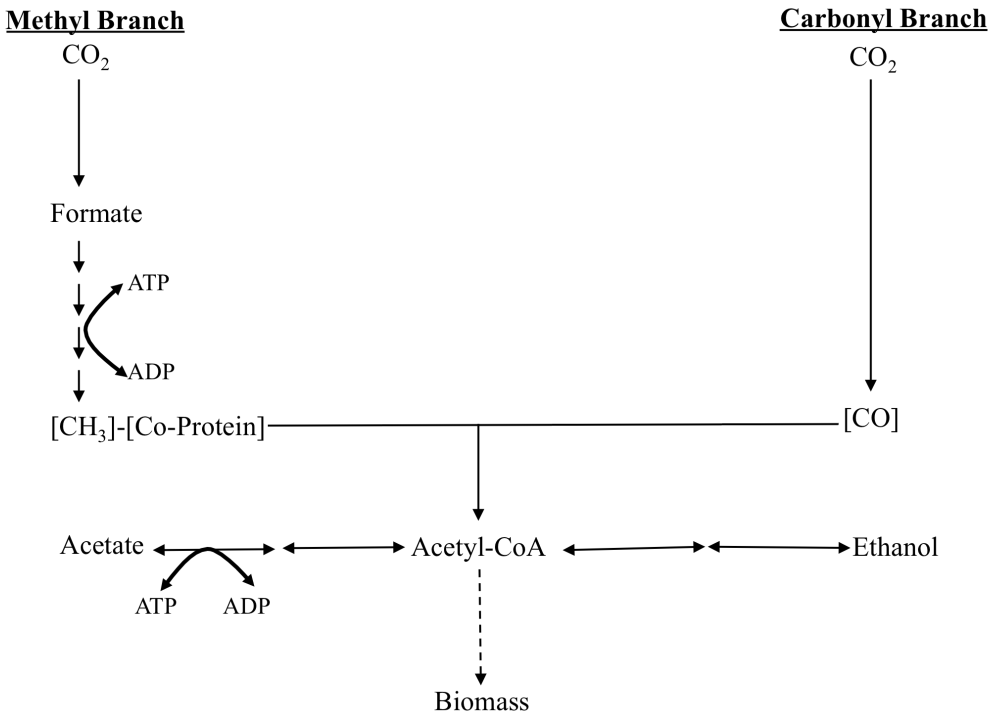

Figure A.1 Wood-Ljungdahl Pathway including the initially proposed mechanism for the production of ethanol from acetyl-CoA.

MATERIALS AND METHODS

Growth of organisms, isolation of genomic DNA, and preparation of cell-free extracts. *Clostridium ragsdalei* strain P11 (ATCC BAA-622) was maintained on the modified acetogen medium (Saxena and Tanner 2010). Media were prepared using strictly anoxic technique (Balch and Wolfe 1976). *C. ragsdalei* medium contained (L^{-1}): 20 ml mineral solution (Tanner 2007); 10 ml vitamin solution (Tanner 2007); 10 ml optimized trace metal solution (Saxena and Tanner 2010); 1 g yeast extract (Difco, Becton Dickinson, Sparks, MD); 10 g MES, with the pH adjusted to 6.1 using KOH; and 3 ml cysteine sulfide as a reducing agent (Tanner 2007). Genomic DNA was isolated from *C. ragsdalei* using the ZR Fungal/Bacterial Miniprep kit (Zymo Research). All DNA quantitation was performed with the QuantIT® Broad Range (BR) DNA assay (Invitrogen). Approximately 1 g cell pellet was brought to a volume of 3 ml with 100 mM TES (pH 7.0). The solution was lysed by sonication at 20% amplitude for a cycle of 20 sec pulse, 60 sec off for a total of 5 min pulse. Debris was pelleted at 10,000 x *g* for 20 min at 4°C.

Cloning and expression of recombinant AK. The AK open reading frame was PCR amplified from geonomic DNA of *C. ragsdalei* using the forward primer (1) and reverse primer (2) with restriction enzyme cut sites as follows:

TTCAG BAMHI ATGAAAATATTAGTAGTAAAC (1)

TTCAG HINDIII TTATTTATTTTCAACTATTTC (2)

The PCR products were cloned into a vector containing an N-terminal His-tag. The resulting plasmid was cloned into *Escherichia coli* Rosetta (Novagen). The His-tagged plasmid was expressed by induction at an optical density (A_{600}) of 0.450 using 0.1 mM isopropyl-ß-D-thiogalactopyranosid (IPTG). Cultures were incubated at 18°C and harvested after 18 h by centrifugation (10,000 *xg*, 20 min, 4°C). Cells were resuspended in lysis buffer (per L): 50 mM NaH_2PO_4, 0.5 M NaCl, 10 mM imidazole, 5% glycerol, pH 8.0) and lysed by sonication. Protein was purified using Ni-NTA purification system (Invitrogen) according to the manufacture's guidelines by increasing the amount of imidizole in the buffer step-wise up to 250 mM across a gradient (75, 125, 250 mM). Protein was quantitated using the methods of Bradford using BSA as a standard (Bradford 1976). Fraction were check for purity by SDS PAGE. Samples were diluted 1:1 with 2X SDS PAGE sample buffer and boiled for 5 min. 5 µl sample was loaded onto a 5/15% stacked polyacrylamide gel and ran at 200 V, 350 mA for 40 min in 1X SDS PAGE running buffer. Gels were stained with GelCode Blue Stain Reagent (Pierce). Recombinant plasmid DNA was prepared for sequencing using a kit (Qiagen). DNA was sequenced at the Oklahoma Medical Research Foundation (OMRF, Oklahoma City, OK).

Enzyme activity of acetate kinase. Acetate kinase was assayed in the acetyl-phosphate forming direction as previously described (Bowmann et al. 1976, Lipmann and Tuttle 1945). The assay mixture contained 50 mM Tris-HCl (pH 8.3), 10 mM ATP, 10 mM $MgCl_2$, 0.5 M hydroxylamine (pH 7 with KOH), and 20 mM sodium acetate. Assays were performed using 5 different protein concentrations. No ATP, no fatty acid, and boiled cell-free extract controls were included for each protein concentration. The reaction mixture was incubated at 37°C for 20 min. The reaction was stopped by the addition of 450 µl ferric reagent (10% $FeCl_3$ and 3% trichloroacetic acid in 0.7 N HCl). Reaction tubes were centrifuged for 5 min at 13,000 rpm to remove precipitate and the supernatant was read at 535 nm. The molar extinction coeficient for acetyl phosphate under these conditions is 594 M^{-1} cm^{-1}. The background absorbance (no ATP and no fatty acid controls) was subtracted from the activities calculated for each sample. The same assay was repeated with the sodium salts of propionate, butyrate, valerate, hexanoate and octanoate. *Escherichia coli* Rosetta cell-free extracts were used as a positive control. *E. coli* cells were lysed by sonication at 20% amplitude for a cycle of 20 sec pulse, 60 sec off for a total of 5 min pulse. Debris was pelleted at 10,000 x *g* for 20 min at 4°C. Since *E. coli* has an acetate kinase activity, the purified fractions corresponding to recombinant *C. ragsdalei* AK were also collected for a analysis by SDS PAGE.

RESULTS AND DISCUSSION

A His-tagged *Clostridium ragsdalei* acetate kinase (AK) was successfully cloned, overexpressed and partially purified (Figure A.2). The recombinant plasmid containing *C. ragsdalei* AK was verified by sequence analysis. The resulting AK activity following partial purification is presented in Table A.1. AK activity in fraction following elution with 75 mM imidizole was 35-fold that of the *C. ragsdalei* cell-free extract control and contained the brightest band in the molecular weight range for AK of 25 kDa (Table A.1 and Figure A.2). Extract has AK activity of 22.5 and 7.42 μmol min^{-1} mg^{-1} in cell-free extracts of propionate-unamended and propionate-amended *C. ragsdalei*, respectively (Table A.1). The recombinant *C. ragsdalei* AK phosphorylated acetate at a rate of 789 μmol min^{-1} mg^{-1}, which shows a partial His-tag purification with approximately 35-fold AK activity as compared to the native AK in *C. ragsdalei* (Table A.1). Recombinant *C. ragsdalei* AK did not phosphorylate propionate, butyrate, valerate, hexanoate or octanoate. This aligns with the results presented in Table 3.3 for cell-free extracts of propionate-amended *C. ragsdalei*. AK activity was not observed in no ATP, no substrate, and boiled cell-free extract or boiled eluate controls.

AK activity was three times that for extracts of *C. ragsdalei* grown without propionate as compared to those grown with propionate in the medium. This is important when attempting to identify the mechanism for carboxylate reduction in *C. ragsdalei* since cells could be easily monitored by GC-FID for the reduction of

propionate to 1-propanol as opposed to needing ^{13}C NMR analysis for reduction of acetate to ethanol, as described previously (See Chapter 2 of this dissertation). Additionally, neither extracts of *C. ragsdalei* nor a partially purified recombinant *C. ragsdalei* AK showed acyl kinase activity toward any carobxylate other than acetate. This work further supports evidence for an AOR-like enzyme that reduced acetate, propionate, octanoate, and lactate in cell-free extracts of *C. ragsdalei* (See Chapter 3 of this dissertation).

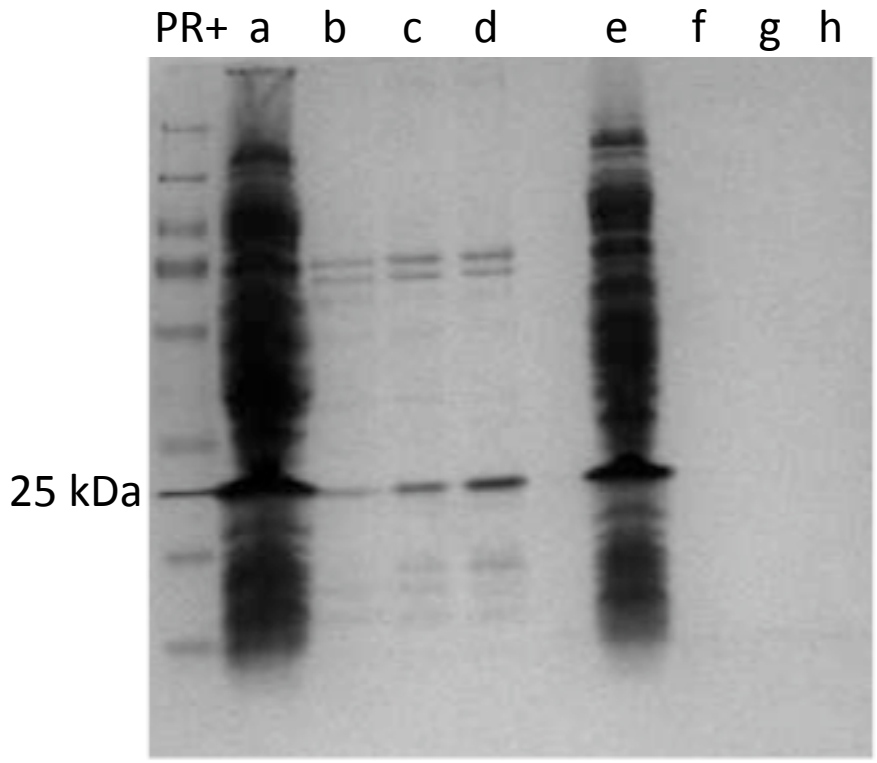

Figure A.2 Image of partial purification of recombinant *Clostridium ragsdalei* acetate kinase (AK) (His-tag® AK is approximately 25 kDa). Lane 1 shows the Fermentas PageRuler™ Plus Prestained Protein Ladder (PR+). Lane 2 shows induced cell lysate (a). Lanes 3-5 show column eluate after the addition of 250 (b), 125 (c) and 75 mM (d) imidizole, respectively. Lane 7 shows the *Escherichia coli* Rosetta control lysate (e). Lanes 8-10 contain the eluates of *E. coli* Rosetta purification as described for the recombinant *C. ragsdalei* AK (f-h).

Table A.1 Acetate kinase (AK) enzyme activity for *Clostridium ragsdalei* cell-free extracts of cells grown with or without propionate and AK activities for partially purified recombinant *C. ragsdalei* AK.

	Activity (μmol min^{-1})	Protein (mg)	Specific activity (μmol min^{-1} mg^{-1})
Clostridium ragsdalei			
lysate (0X propionate)	1.42	0.06	22.5
lysate (+propionate)	0.74	0.10	7.42
Escherichia coli Rosetta with AK			
Lysate	4420	6.58	672
Eluate (75 mM imidizole)	545	0.69	789
Eluate (125 mM imidizole)	42	0.23	183
Eluate (250 mM imidizole)	7	0.09	77.8
E. coli Rosetta			
Lysate	31.7	15.5	2.05

CONCLUSIONS

A partially purified recombinant *Clostridium ragsdalei* AK generated 35-fold the AK activity of the *C. ragsdalei* cell-free extract control. Recombinant *C. ragsdalei* AK did not phosphorylate propionate, butyrate, valerate, hexanoate or octanoate. This work further supports evidence for an AOR-like enzyme that reduced acetate, propionate, octanoate, and lactate in cell-free extracts of *C. ragsdalei.*

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