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Improved conversion efficiencies for *n*-fatty acid reduction to primary alcohols by the solventogenic acetogen "*Clostridium ragsdalei*"

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Abstract "*Clostridium ragsdalei*" is an acetogen that ferments synthesis gas (syngas, predominantly H_2:CO_2:CO) to ethanol, acetate, and cell mass. Previous research showed that *C. ragsdalei* could also convert propionic acid to 1-propanol and butyric acid to 1-butanol at conversion efficiencies of 72.3 and 21.0 percent, respectively. Our research showed that *C. ragsdalei* can also reduce pentanoic and hexanoic acid to the corresponding primary alcohols. This reduction occurred independently of growth in an optimized medium with headspace gas exchange (vented and gassed with CO) every 48 h. Under these conditions, conversion efficiencies increased to 97 and 100 % for propionic and butyric acid, respectively. The conversion efficiencies for pentanoic and hexanoic acid to 1-pentanol and 1-hexanol, respectively, were 82 and 62 %. *C. ragsdalei* also reduced acetone to 2-propanol at a conversion efficiency of 100 %. Further, we showed that *C. ragsdalei* uses an aldehyde oxidoreductase-like enzyme to reduce *n*-fatty acids to the aldehyde intermediates in a reaction that requires ferredoxin and exogenous CO.

Keywords Biofuels · Syngas · Acetogenesis · Aldehyde oxidoreductase · Propanol

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Introduction

Depletion of petroleum-based energy reserves and effects of petroleum combustion on the global climate have necessitated the development of renewable energy sources to supplement petroleum. Transportation accounts for approximately 74 % of refined crude oil consumption and the demand will only grow with the population at an estimated 0.6 million more barrels of refined crude oil per day from 2010 to 2035 [9]. The chemical products industry also represents a significant fraction of the petroleum demand (16.8 %, [14]) and, like transportation, this demand will also increase with growing populations. Several potential transportation fuel additives and replacements have been investigated, including biologically produced alcohols and biodiesel; however, 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 include perennials grown on agriculturally abandoned land, crop residues, sustainably harvested woody crops, crop rotation/mixed crop systems, and municipal/industrial waste rather than sugar-rich food crops [35]. Synthesis gas (syngas, predominantly H_2:CO_2:CO), produced via the gasification of organic waste materials, is another sustainable feedstock that can be used, primarily, by mesophilic microorganisms to generate multicarbon compounds [12]. Some acetogenic

bacteria (acetogens) produce acetate, ethanol, and cell mass from syngas [8].

While ethanol remains a useful renewable fuel source, there exists a need for products with higher energy yields that are less corrosive than ethanol. The energy density of these products must be comparable with the current transportation fuels and petroleum infrastructures. Fatty acids, such as propionic and butyric acids, are energy-rich, but the high oxygen to carbon ratio makes fatty acids less suitable for fuel applications; converting energy-dense fatty acids to alcohols, however, yields a product more applicable to the fuel industry [7]. Biologically produced C_2–C_6 alcohols can be blended with gasoline as fuel oxygenates [7]. These solvents are also used in a variety of industrial processes. For example, 1-propanol can be used as a solubility agent in the pharmaceuticals industry [11]. The butanol industry was estimated to be an approximately 7 billion dollar expanding market [16]. Like propanol and other lower molecular weight solvents, 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. These are just a few examples of the use of alcohols in industrial processes.

Recently, application of the microbial conversion of acetic, propionic, and butyric acids to alcohols as a waste treatment practice was described for mixed bacterial consortia (without the requirement of fermentable sugars or starches) with hydrogen as the electron donor [30]. Hydrogen is produced during anaerobic digestion, which reinforces the waste-based feedstock model of this work; however, the majority of this is consumed by hydrogenotrophic methanogens in the mixed cultures [31]. Alternatively, some solventogenic acetogens can produce alcohols and cell mass from syngas and *n*-fatty acids. This was demonstrated in the acetogens *Clostridium kluyveri* [15], *Clostridium formicoaceticum* [10], *Moorella thermoacetica* (f. *Clostridium thermoaceticum*) [28], *Clostridium acetobutylicum* [13], *Clostridium ljungdahlii* [22], and *Alkalibaculum bacchi* strains CP11^T, CP13 and CP15 [19].

Both *C. ljungdahlii* and "*Clostridium ragsdalei*" (ATCC BAA-622, DSM 15248) were shown to produce 1-propanol and 1-butanol from propionic and butyric acid, respectively, and it was suggested that optimizing fermentation would improve conversion efficiencies for this process [22]. Previously, our lab has shown a four-fold increase in ethanol production by *C. ragasdalei* in optimized medium [26]. The research presented herein was focused on achieving higher conversion efficiencies of acids to alcohols by *C. ragsdalei*. This was achieved by developing a better understanding of the enzymes and reductants used by *C. ragsdalei* to convert acids to aldehyde intermediates and using optimized medium components and incubation conditions for alcohol production described previously [26].

Materials and methods

Cultures, media, and syngas analog

"*Clostridium ragsdalei*" (ATCC BAA-622, DSM 15248) medium contained (L^{-1}): 20 ml mineral solution [33], modified by the elimination of sodium; 10 ml vitamin solution [33]; 10 ml optimized trace metal solution [26]; 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 potassium hydroxide; and 3 ml cysteine sulfide solution (0.4 g L^{-1} stock) as a reducing agent [33]. *Clostridium carboxidivorans* strain P7^T (DSM 15243) medium was prepared in the same manner as *C. ragsdalei* medium using unmodified trace metal solution [33]. *Alkalibaculum bacchi* strain CP11^T (ATCC BAA-1772) medium was prepared according to Allen et al. [1]. Media were prepared using strict anoxic technique [2]. Incubation vessels contained a headspace of 101.3 kPa N_2:CO_2 (80:20) and were over-pressurized with carbon monoxide (CO) to a total gas final pressure of 207 kPa. Shaking incubations (60 rpm) were conducted at 37 °C in stoppered and crimp-sealed Balch tubes or bottles depending on the experimental set-up (see below). All experiments were prepared in this manner unless otherwise noted. All chemicals used in this research were obtained from Sigma–Aldrich unless otherwise stated (Sigma–Aldrich Corp.). Additionally, for all studies, the growth phases were detected by measuring the absorbance at 600 nm of cultures used for inoculum. Growth curves for *C. ragsdalei* in the presence of the different *n*-fatty acids were determined prior to conducting the experiments described herein (Supplemental Fig. 2).

Nuclear magnetic resonance (NMR) study

C. ragsdalei medium was amended with [2-^{13}C]-acetate or [2-^{13}C]-propionic acid, dispensed as 20 ml aliquots into 160 ml glass bottles (Wheaton), inoculated with mid-log phase *C. ragsdalei*, and pressurized with CO (207 kPa gauge) in triplicate. Cultures were incubated at 37 °C for 30 days as static incubations (acetate amended) and 9 days at 60 rpm (propionic acid amended), corresponding to consumption of the *n*-fatty acid. Sterile (autoclaved post-inoculation) and unlabeled controls were implemented. Samples (1.2 ml) were treated with 20 µl washed Chelex 100 Resin to remove paramagnetic ions from the solution that interfere with NMR analysis. Samples were treated with Chelex 100 according to the manufacturer's instructions (Bio-Rad Laboratories), vortexed for 30 s and centrifuged at 6,000 rpm. The supernatant was stored at −80 °C until analysis. Samples were diluted 1:1 with ^2H_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 ^{13}C experiment with a 45° pulse width (2.95 µs), a delay time of 2 s (acetate-amended samples) and 1 s (propionate-amended samples), an acquisition time of 1.28 s, and a spectral width of 25,510.2 Hz and 256 acquisitions. Proton decoupling was achieved using a WALTZ-16 pulse sequence. Propionate, propanol, acetate, and ethanol were quantified by GC-FID as described in analytical methods below.

Conversion of propionic acid to 1-propanol by resting cells of *C. ragsdalei*

In an anaerobic chamber, 50 ml aliquots of medium were dispensed into 500 ml serum bottles that were sealed with butyl-rubber stoppers and aluminum crimp seals, degassed, and then sterilized by autoclaving. Propionic acid was added as a sterile, anoxic stock to a final concentration of 30 mM. A 1 % (v/v) inoculum of log phase *C. ragsdalei* cells was added to the medium and the bottles were pressurized with CO to a final pressure of 207 kPa. Cells were grown in shaking incubators (60 rpm) at 37 °C and harvested at mid-log phase, corresponding to approximately, 50 % propionic acid consumption. Cells were washed twice in a MES buffered solution (pH 5.3) using strictly anoxic techniques in an anaerobic chamber. A minimal medium (MM), which contained all medium components excluding yeast extract, was amended with 20 mM propionic acid (MMP). Washed *C. ragsdalei* cells were resuspended in the prepared MMP in the anaerobic chamber, and subsequently added to sterile, anoxic, sealed Balch tubes in 3 ml aliquots. Each tube was pressurized with 207 kPa gauge CO. All resting cell experiments were conducted in triplicate. Along with CO, reduced benzyl viologen (BV), hydrogen, and formate were also tested as potential reductants. N_2 headspace controls were also conducted.

In silico comparison of aldehyde oxidoreductases (AORs)

Using the Joint Genome Institute's (JGI) Integrated Genomes and Metagenomes/Expert Review (IMG/ER) comparative analysis software [21], the gene sequence of the proposed AOR in *C. ragsdalei* was compared to AORs of related organisms that have been shown to reduce carboxylates (*n*-fatty acids and branched chain fatty acids) and ketones to alcohols. These sequences were translated using the ExPASy Bioinformatics Resource (http://web.expasy.org/translate/) and aligned in ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) to compare the conserved domains as predicted in IMG/ER.

Enzyme activity assays for AOR, carbon monoxide dehydrogenase (CODH), and alcohol dehydrogenase (ADH)

All cell-free extracts were prepared under strictly anaerobic conditions with tightly sealed polypropylene centrifuge bottles in an anaerobic chamber. All plastic materials were kept for at least 48 h in the anaerobic chamber to remove traces of oxygen prior to use. Cells were harvested in the anaerobic chamber when the propionic acid concentration had decreased by approximately, 50 %. Cells were washed with anoxic, sterile 2-[(2-hydroxy-1,1-bis(hydroxymethyl) ethyl)amino]ethanesulfonic acid (TES, pH 7.0) buffered solution. Prior to harvesting, samples were taken to measure acid and alcohol concentrations, growth, and pH as described below. 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 crude lysate was transferred to centrifuge vials in the anaerobic chamber, sealed and centrifuged at 15,000×*g* and 4 °C for 15 min. The supernatant was passed through a desalting column (PD-10, GE Health Care Life Sciences) and used for determining enzyme activities. 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. Substrate-free and boiled cell-free extract controls were implemented and activities presented here were reported relative to these background activities. Linearity of the reactions based on protein concentration was established for all preparations. Enzyme activities represent averages for three replicate cell-extract preparations. Protein concentration was determined by Bradford protein assays using bovine serum albumin (BSA) as the standard [4]. AOR activity was determined by measuring the initial rate of methyl viologen (MV) reduction at 579 nm and production of propionic acid from propionaldehyde as described previously for butyrate production [29]. The assay mixture contained 100 mM TES buffer (pH 7.0), 0.6 mM oxidized MV (MV_{ox}), and 10 µM propionaldehyde. 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 (A_{579}) caused by accumulation of reduced MV (MV_{red}).

AOR activity was also determined in the less energetically favorable, aldehyde-forming direction by measuring the initial rate of MV_{red} 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 MV, reduced drop-wise with 1 mM dithionite, and 10 μM propionic acid. The reaction was initiated by the addition of cell-free extract and the activity was determined by measuring changes in A_{579} caused by oxidation of MV_{red}.

ADH activity was determined by measuring the initial rate of NAD^+ reduction as a change in A_{340}. 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-free extract.

CODH activity was determined as described previously, modified by measuring MV reduction at A_{579} rather than A_{603} [27].

C. ragsdalei ferredoxin was determined spectrophotometrically as the reduction of metronidazole using the method described previously [6] modified by adding propionaldehyde or CO instead of hydrogen. The assay mixture contained 100 mM TES buffer (pH 7.0), 0.1 mM metronidazole, and 10 mM propionaldehyde or CO. The reaction was initiated by the addition of cell-free extract and the concentration was determined by measuring changes in A_{360} caused by reduction of metronidazole.

Reduction of carboxylates to alcohols

Anoxic, sterile stocks of *n*-fatty acids, lactate, and isobutyrate were individually added to prepared medium to a concentration of 30 mM, a 60 mM addition was used for acetone only, prior to inoculation with *C. ragsdalei*. *C. carboxidivorans* and *A. bacchi* were analyzed for the conversion of propionic acid to 1-propanol only. Solventogenic cells (mid-log phase) were added to media and pressurized with CO to a final pressure of 207 kPa. 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- and ketone-free and sterile controls were implemented.

Analytical methods

Branched chain fatty acids, *n*-fatty acids, ketone, *n*-alcohol, and branched chain 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 × 5 mm × 2.6 mm) packed with 4 % Carbowax 20 M 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). Sample pH was determined using the Fisher Accumet Basic pH Meter (Fisher Scientific, Pittsburgh, PA).

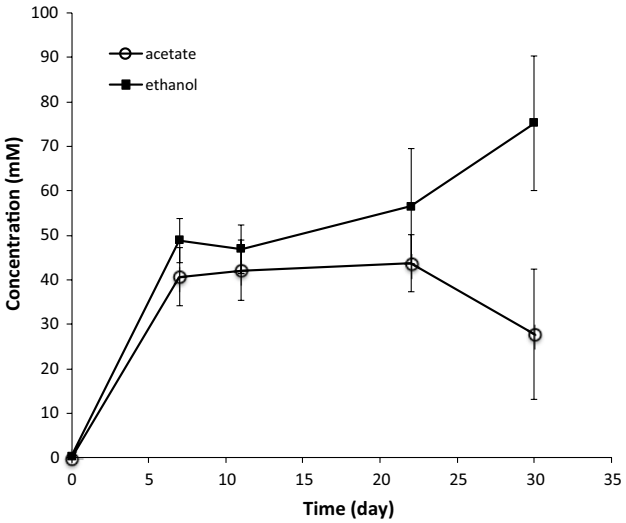

Fig. 1 The production of acetate (*open circles*) and ethanol (*solid squares*) by "*Clostridium ragsdalei*" during 30 days incubation as detected by GC-FID

Results and discussion

Evidence for the reduction of *n*-fatty acids to alcohols

The concentration of acetate and ethanol in "*Clostridium ragsdalei*" incubations with syngas was monitored over 30 days (Fig. 1). Decreases in the acetate concentration suggested that *C. ragsdalei* consumed some of the acetate produced earlier in the fermentation. In conjunction with this acetate consumption, was the production of ethanol. Ethanol production did not appear to be growth-dependent and may proceed via the reduction of acetate, though this conclusion cannot be drawn from GC-FID analysis alone given that *C. ragsdalei* produces both acetate and ethanol from C_1 substrates intrinsically. Thus, labeled [2-^{13}C]-acetate was added to fresh medium to evaluate the hypothesis that some of the ethanol produced by *C. ragsdalei* was derived from the reduction of acetate. The results of this experiment are shown in Fig. 2. The collection of NMR spectra obtained over a 30-day period shows a peak at 23.5 ppm for [2-^{13}C]-acetate and an increasing peak at 16.5 ppm for [2-^{13}C]-ethanol (Fig. 2). The ^{13}C NMR analysis shows that [2-^{13}C]-ethanol is the only labeled end product (Fig. 2). $^{13}CO_2$, [2-^{13}C]-acetyl-CoA, and [2-^{13}C]-acetyl phosphate were not detected.

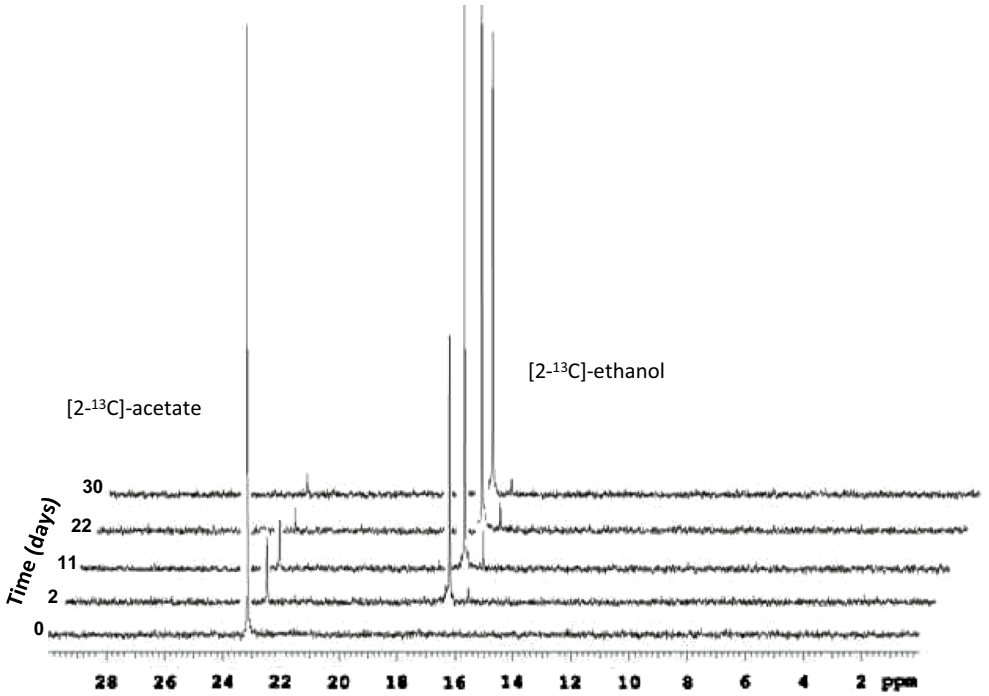

Fig. 2 ^{13}C NMR spectrum obtained from "*Clostridium ragsdalei*" incubations showing the conversion of [2-^{13}C]-acetate (23.5 ppm) to [2-^{13}C]-ethanol (16.5 ppm) at 0, 2, 11, 22, and 30 d after the addition of 2-^{13}C acetate (*front* to *back*, respectively)

Table 1 Conversion of propionic acid to 1-propanol by resting cells of "*Clostridium ragsdalei*" with carbon monoxide (CO)

	Time (days)	
	0	5
CO (mM)	9.64 ± 0.63	0.93 ± 0.47
Propionic acid (mM)	21.9 ± 0.24	2.47 ± 0.33
1-propanol (mM)	0.00 ± 0.00	18.8 ± 3.49
pH	5.30 ± 0.01	5.47 ± 0.16

The conversion of carboxylates to alcohols catalyzed by resting cells or cell-free extracts of solventogenic *Clostridia* [10, 28] indicates that these bacteria can convert carboxylates to alcohols independent of growth. A resting cell experiment was conducted with cells of *C. ragsdalei* harvested at stationary phase of growth. These cells were washed twice and amended with minimal medium containing propionic acid (21.9 ± 0.24 mM). After 5 days, *C. ragsdalei* consumed 8.71 ± 0.63 mM CO and produced 18.8 ± 3.5 mM 1-propanol (Table 1). This suggested that the oxidation of 1 CO provided 2 of the reducing equivalents required to produce 1-propanol from propionic acid. No change in pH was observed.

The reduction of propionic acid to 1-propanol was further supported by the production of [2-^{13}C]-propanol from [2-^{13}C]-propionic acid (Fig. 3). *C. ragsdalei* cultures amended with [2-^{13}C]-propionic acid consumed 16 mM

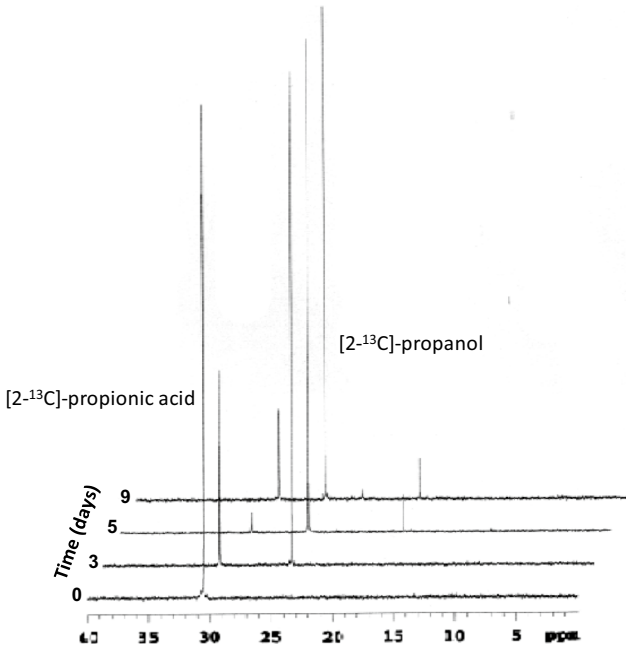

Fig. 3 ^{13}C NMR spectra obtained from samples of "*Clostridium ragsdalei*" incubations showing the reduction of [2-^{13}C]-propionic acid (30.5 ppm) to [2-^{13}C]-propanol (24.6 ppm) at 0, 3, 5, and 9 d (*front to back*, respectively)

of the added propionic acid and produced 15 mM [2-^{13}C]-propanol after 9 d incubation. The only other end products detected by GC-FID were acetate (81.8 ± 17 mM) and

ethanol (125 ± 25 mM). In [2-^{13}C]-propionic acid samples, the day 0 spectrum prominently displayed only one peak at 30.5 ppm, corresponding to the ^{13}C-label on the number 2 carbon of propionic acid (Fig. 3). The day 3 spectrum also contained a signal corresponding to the [2-^{13}C]-propionic acid (30.5 ppm); however, this signal had decreased in intensity when compared to the day 0 spectrum. Additionally, a second signal had appeared at 24.6 ppm, which was identified as the ^{13}C-label on the number 2 carbon of 1-propanol. The day 5 spectrum showed a dramatic decrease in [2-^{13}C]-propionic acid (30.5 ppm) and corresponding increase in [2-^{13}C]-propanol (24.6 ppm) signal intensities. The day 9 spectrum was similar to day 5 with a slight increase in the signal intensity at 30.5 ppm, suggesting the oxidation of the [2-^{13}C]-propanol back to [2-^{13}C]-propionic acid. This evidence not only supported the hypothesis that the number 2 carbon of propionic acid is being converted to the number 2 carbon of 1-propanol, but also demonstrated that this reaction is reversible in vivo. Replicate NMR analyses generated similar spectra.

CO as the preferred reductant for the reduction of acids to alcohols

CODH activity was measured in *C. ragsdalei* cell-free extracts that reduced propionic acid to 1-propanol at the expense of CO. The CODH activity was 1.61 ± 0.8 μmol min^{-1} mg^{-1}. Even though CODH activity was lower in this study than the activities demonstrated previously [26], it was evident that CO was a preferred reductant based on the activities observed in the presence of alternative reductants (no activity observed with BV and formate). Additionally, *C. ragsdalei* did not reduce propionic acid to 1-propanol in the presence of hydrogen (data not shown). CO was also the only reductant examined that supported the conversion of propionic acid to 1-propanol by resting cells. The free energy change for the biological conversion of acids to alcohols, though exothermic, is much less than that for the fermentation of glucose to acetate (−225.5 kJ mol^{-1}) [30]. The energetics for acetate reduction to ethanol (Eq. 1), propionic acid to 1-propanol (Eq. 2), and butyric acid to 1-butanol (Eq. 3) with hydrogen as the electron donor are shown below at [34]:

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

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

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

The thermodynamics of acetate reduction (Eq. 4) do not change when CO replaces hydrogen as the reductant. However, the reaction becomes more favorable for the reduction of propionic acid (Eq. 5) and butyric acid (Eq. 6) when CO is used in place of hydrogen [34].

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

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

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

Thermodynamics indicate that the reduction of propionate and butyrate to the corresponding alcohols would be more exothermic with CO as the reductant rather than hydrogen. This analysis was ultimately supported by the use of CO as the preferred electron donor for whole resting cells of *C. ragsdalei* and measured CODH activity in *C. ragsdali* cell-free extracts.

Sequence similarity for AOR-mediated *n*-fatty acid reduction

The reduction of carboxylates to corresponding aldehydes was previously described as being mediated by AOR [37]. The amino acid sequence of AOR in *C. ragsdalei* DSM 15248 (CragP11_contig00091) was compared to the amino acid sequences of AORs in *C. ljungdahlii* DSM13528 (NC_014328) [17], *C. carboxidivorans* strain P7^T DSM 15243 (NZ_ACV101000065) [5], and *M. thermoacetica* DSM 521 [23] using JGI's IMG comparative analysis software [21] (Fig. 4). All of these acetogens have been shown to reduce *n*-fatty acids to alcohols at the expense of CO and/or H_2. C. *ragsdalei* AOR exhibited 99 % amino acid sequence identity with *C. ljungdahlii* AOR. Comparison of the AOR of *C. ragsdalei* to the AOR of *C. carboxidivorans* revealed an amino acid sequence similarity of 92 %. By contrast, the amino acid sequence homology of *C. ragsdalei* AOR to the previously described *M. thermoacetica* AOR was only 50 % (Fig. 4). Comparatively, *M. thermoacetica* has a broader substrate range than other acetogens, having reduced acetate, propionic butyric, pentanoic and hexanoic acids, (R)-lactate, (S)-lactate, succinate, glutarate, adipate, suberate, benzoic acid, (2R,S)-2-phenylbutyrate, (E)-2-methyl-2-butenoate, (E)-2-methylcinnamate, vinyl acetate, and sorbate to alcohols [29]. The reduction of acetone, lactate, isobutyrate and propionic, butyric, pentanoic and hexonic acids to alcohols by *C. ragsdalei* was examined (Table 2). Using CO as a substrate, *C. ragsdalei* reduced *n*-fatty acids up to 6 carbons in length with production of the corresponding alcohols. However, when *n*-fatty acids >6 carbons were initially added to the growth medium, growth was inhibited (Supplemental Fig. 2). *C. ragsdalei* converted 100 % of the added acetone to 2-propanol with a final isopropanol concentration of 60 mM (Table 2).

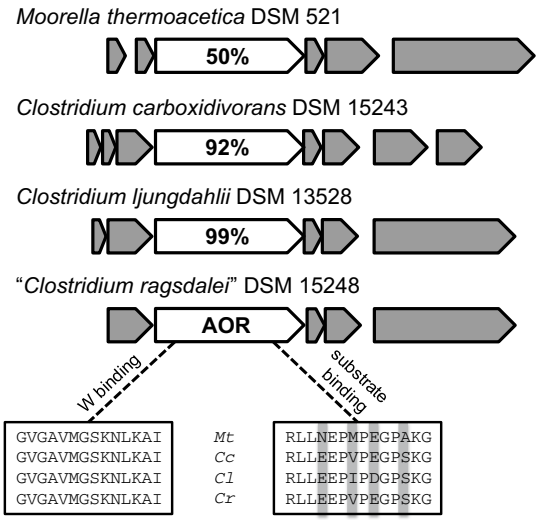

Fig. 4 Comparison of the gene sequence for the aldehyde oxidoreductase (AOR) of "*Clostridium ragsdalei*" DSM 15248(CragP11_contig00091) versus the annotated AORs of *Clostridium ljungdahlii* DSM 13528 (NC_014328), *Clostridium carboxidivorans* strain DSM 15243 (NZ_ACV101000065), and *Moorella thermoacetica* DSM 521. The N-terminal tungsten (W) binding domain was 100 % similar across all species (*top* to *bottom*: *M. thermoacetica*, Mt; *C. carboxidivorans*, Cc; *C. ljungdahlii,* Cl; and *C. ragsdalei*, Cr); whereas the C-terminal substrate binding domain was 71 % similar between *C. ragsdalei* and *M. thermoacetica* and 80 % similar among the compared *Clostridia*

Acetone conversion was examined due to the application of this ketone as a syngas scrubbing agent prior to fermentation [24]. Although not the first to show the conversion of acetone to 2-propanol by a biocatalyst [24], our research showed complete conversion of twice the amount of acetone to 2-propanol (Table 2). Lactate and isobutyrate did not inhibit growth when added to growth medium, however, 1,2-propanediol and 2-butanol, respectively, were not produced from these substrates (Table 2).

Two conserved domains are described for AOR: an N-terminus described as interacting with the tungsten (W) cofactor and a C-terminus that putatively regulates substrate binding [20]. The N-terminal domain amino acid sequence is 100 % conserved across all of the aligned sequences (Fig. 4). The C-terminal domain, while 80 % similar in the aligned clostridial AOR amino acid sequences, was only 71 % similar to the *M. thermoacetica* C-terminal amino acid sequence (Fig. 4). This could explain the difference in substrate specificity between *C. ragsdalei* and *M. thermoacetica*, since a change in predicted secondary structure of the substrate binding domain is observed *in silica* due to the amino acid differences. This, however, is speculative and requires further research.

AOR activity was measured in *C. ragsdalei* cell-free extracts. AOR activity in the propionic acid-reducing direction was 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) for cell-free extracts of *C. ragsdalei* (Fig. 5). This is comparable to previous observations in *M. thermoacetica* cell-free extracts [32], whereby the carboxylic-acid reductase activity of AOR was about 5 % of the aldehyde-dehydrogenase activity. *C. ragsdalei* produced approximately, 0.31 μM propionic acid when 10 μM propionaldehyde and MV_{ox} were amended with cell-free extracts. Unlike acyl kinase, the AOR-like enzyme in *C. ragsdalei* cell-free extracts appeared to be promiscuous with respect to substrate, as indicated by the reduction of lactate and octanoic acid by cell-free extracts but not whole cells. The absence of kinase activity (Supplemental Table 1) and CoA intermediates (Supplemental Fig. 1) further supports the finding that the primary mechanism of alcohol production from carboxylates occurs via an AOR-like enzyme. In addition to AOR activity, ferredoxin activity was measured as the reduction of metronidazole. The measured ferredoxin activity in *C. ragsdalei* cell-free extracts using propionaldehyde as the substrate was 0.59 ± 0.13 μmol min^{-1} mg^{-1}. The proposed mechanism for propionic acid reduction in *C. ragsdalei* is depicted in Fig. 5.

Enhanced 1-propanol production from propionic acid

Using *C. ragsdalei* as a biocatalyst, the conditions for the enhanced conversion of propionic acid to 1-propanol were determined to be fed-batch incubations (CO pressurized

Table 2 Highest measured alcohol product concentration (mM) from listed substrates by "*Clostridium ragsdalei*"

Substrate	Product	Highest measured final alcohol concentration (mM)	Conversion efficiency (%)[a]
Acetone	2-propanol	60	100
Propionic acid	1-propanol	29	97
Butyric acid	1-butanol	28	100
Pentanoic acid	1-pentanol	2.8	82
Hexanoic acid	1-hexanol	11	62
Lactate	1,2-propanediol	ND[b]	–
Isobutyrate	2-butanol	ND	–

[a] Calculated as the percent of consumed acid converted to alcohol. Standard deviations <15 %

[b] Not detected

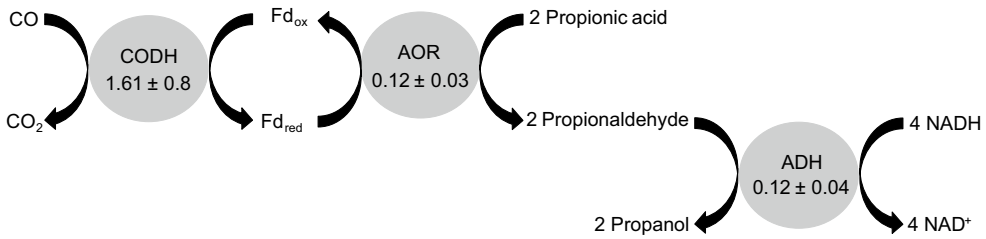

Fig. 5 The proposed mechanism for "*Clostridium ragsdalei*"-mediated reduction of propionic acid to 1-propanol using aldehyde oxidoreductase (AOR) and alcohol dehydrogenase (ADH). Carbon monoxide dehydrogenase (CODH) activity is also shown. Values represent measured enzyme activity levels in cell-free extracts of *C. ragsdalei* (μmol min^{-1} mg^{-1}). The proposed reaction is mediated by ferredoxin (Fd) and nicotinamide adenine dinucleotide (NADH)

to 207 kPa every 48 h) in an optimized medium [26] at an initial pH of 5.3. Under these conditions, *C. ragsdalei* reduced 97 % of the propionic acid to 1-propanol (Table 3). This represents an improvement in conversion efficiency of 34.7 %. Fed-batch conditions were used to provide a continuous supply of reductant (CO) for the conversion of propionate to 1-propanol. The optimized medium was developed previously to include metals that were required by the metalloenzymes involved in alcohol production [26]. The lower starting pH is reflective of previous work that shows a decrease in initial pH or a lowering of pH during fermentation enhances and can even initiate alcohol production [36]. *C. carboxidivorans* [18] and *A. bacchi* [1], two solventogenic syngas-fermenting acetogens, were also assessed for conversion of propionic acid to 1-propanol. *C. carboxidivorans* and *A. bacchi* reduced propionic acid to 1-propanol at a conversion efficiency of 100 % (Table 3). While conversion efficiency is used frequently in the literature, this measurement does not account for the amount of 1-propanol produced from the total amount of propionic acid added to the bacterial culture. The percent theoretical maximum, however, is the amount of 1-propanol produced from the total added propionic acid. *C. carboxidivorans* produced 77 % of the theoretical maximum 1-propanol from the added propionic acid, whereas *A. bacchi* only converted 10 % of the added propionic acid to 1-propanol. *C. ragsdalei,* on the other hand, converted 86 % of the theoretical maximum propionic acid to 1-propanol. The low theoretical maximum 1-propanol production in *A. bacchi* could be due to the lack of defined fermentation parameters used in this study, which is an assumption further supported by studies in which defined fermentation parameters were used to successfully increase acid conversion efficiency in *A. bacchi* strains [19]. In these previous studies, *A. bacchi* strain CP15 produced approximately, two-fold more 1-propanol than strain $CP11^T$ with a conversion efficiency of 37 % (Table 3) [19]. When a mixture of *A. bacchi* strains and *Clostridium propionicum* was used the conversion efficiency, again, more than doubled (Table 3) [19]. *C. ragsdalei* and *C. carboxidivorans* had similar 1-propanol production rates (3 mM d^{-1}), second only to the well-studied industrial biocatalyst *C. acetobutylicum* (Table 3) [13]. *C. ljungdahlii* produced 10.44 ± 1.69 mM 1-propanol from added propionic acid after 400 h incubation at conversion efficiency of 92 % (Table 3) [22]. *C. ljungdahlii* has also been reported to convert butyric, pentanoic, and hexanoic acids to the corresponding alcohols at conversion efficiencies of 68, 52, and 46 %, respectively [22]. Gas stripping was purportedly responsible for conversion inefficiency, since incubations were conducted under a constant flow of syngas [22]. It remains unclear in the previous studies and this study, why conversion efficiencies decrease as *n*-fatty acid carbon chain length increases or where the carbons from the consumed *n*-fatty acids, which are not converted to alcohols, end up. In this research, *C. ragsdalei* grown on a syngas analog converted propionic, butyric, pentanoic and hexanoic acid, and acetone to the corresponding alcohols at conversion efficiencies of 97, 100, 82, 62 and 100 % (Table 2). This is an improvement in the reduction of *n*-fatty acids to alcohols by *C. ragsdalei* from previous reports. The cost of cleaning syngas feedstocks for metal catalysis is typically higher than for biocatalytic processes. Abiotic methods for synthesizing solvents also tend to be performed at much higher temperatures, so energy input requirements are generally larger for these processes versus biological fermentations. However, this higher temperature generally results in faster kinetics [25]. Additionally, recent advances in bioengineering have eliminated some of the pitfalls of using biocatalysts [3]. Developing a better understanding of the biochemical pathways that produce alcohols from *n*-fatty acids, such as the AOR/ADH pathway presented here, is useful for further advancing bioengineering of sturdy biocatalysts for the production of biofuels.

Conclusions

Whole cells of "*Clostridium ragsdalei*" reduced acetic, propionic, butyric, pentanoic and hexanoic acid, and acetone to the corresponding alcohols. Acetone and butyric acid

Table 3 Comparison of biocatalysts for the conversion of propionic acid to 1-propanol by highest final propanol concentration (mM), highest reported conversion efficiency (%), and the mean propanol production rate (mM d^{-1})

Biocatalyst	Final propanol concentration (mM)	Conversion efficiency (%)[a]	Propanol production rate (mM d^{-1})	Source
"*Clostridium ragsdalei*"	29	97	3.6	This study
Clostridium carboxidivorans strain $P7^T$	23	100	3.3	This study
Clostridium acetobutylicum	19	100	17	[13]
Mixed *Alkalibaculum bacchi* strains and *Clostridium propionicum*	17	83	1.85	[19]
Clostridium ljungdahlii	10	92	0.6	[22]
Mixed anaerobic cultures	8.1	50	0.5	[30]
A*lkalibaculum bacchi* strain CP15	6.7	37	0.7	[19]
Alkalibaculum bacchi strain $CP11^T$	2.8	100	0.4	This study

[a] Calculated as the percent of consumed propionic acid converted to 1-propanol

were reduced to 2-propanol and 1-butanol, respectively, at a conversion efficiency of 100 %. Resting cells of *C. ragsdalei* converted propionic acid to 1-propanol with CO as the reductant. An AOR-like enzyme was used by *C. ragsdalei* to reduce propionic acid to 1-propanol. AOR activity was also measured in *C. ragsdalei* cell-free extracts for lactic and octanoic acid reduction even though whole cells did not reduce these fatty acids to the corresponding alcohols. Whole cells of *C. ragsdalei* converted propionic acid to 1-propanol at a conversion efficiency of 97 % with the highest measured final 1-propanol concentration reported in this study and in the literature for acetogenic biocatalysts.

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