Engineering electron metabolism to increase ethanol production in *Clostridium thermocellum*

Jonathan Lo^{a,b}, Daniel G. Olson^{a,b}, Sean Jean-Loup Murphy^{a,b}, Liang Tian^{a,b}, Shuen Hon^{a,b}, Anthony Lanahan^{a,b}, Adam M. Guss^{b,c}, Lee R. Lynd^{a,b,*}

^aThayer School of Engineering, Dartmouth College, Hanover, NH 03755, USA

^bBioEnergy Science Center, Oak Ridge National Laboratory, Oak Ridge, TN 37830, USA

^cBiosciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 37830

* Corresponding author: Lee R. Lynd, Lee.R.Lynd@Dartmouth.edu

Notice: This manuscript has been authored by Dartmouth College under Subcontract No. 4000115284 and Contract No. DE-AC05-00OR22725 with U.S. Department of Energy. The United States Government retains and the publisher, by accepting the article for publication, acknowledges that the United States Government retains a non-exclusive, paid-up, irrevocable world-wide license to publish or reproduce the published form of this manuscript or allow others to do so, for United States government purposes. (End of Notice)

Abstract

The NfnAB (NADH-dependent reduced ferredoxin:NADP⁺ oxidoreductase) and Rnf (*Rhodobacter* nitrogen fixation) complexes are thought to catalyze electron transfer between reduced ferredoxin and NAD(P)⁺. Efficient electron flux is critical for engineering fuel production pathways, but little is known about the relative importance of these enzymes *in vivo*. In this study we investigate the importance of the NfnAB and Rnf complexes in *Clostridium thermocellum* for growth on cellobiose and Avicel using gene deletion, enzyme assays, and fermentation product analysis. The NfnAB complex does not seem to play a major role in metabolism, since deletion of *nfnAB* genes had little effect on the distribution of fermentation products. By contrast, the Rnf complex appears to play an important role in ethanol formation. Deletion of *rnf* genes resulted in a decrease in ethanol formation. Overexpression of *rnf* genes resulted in an increase in ethanol production of about 30%, but only in strains where the *hydG* hydrogenase maturation gene was also deleted.

Keywords

Clostridium thermocellum; ethanol; *rnf*; *nfnAB*; ferredoxin; electron metabolism

1. Introduction

Clostridium thermocellum is a thermophilic anaerobe that rapidly consumes cellulose and produces lactate, acetate, and ethanol as its main fermentation products [1]. It is a candidate for consolidated bioprocessing (CBP), which is the single step deconstruction and conversion of lignocellulose into useful products, including ethanol [2,3].

There are several reports in the literature describing attempts to increase ethanol production in *C. thermocellum* by disrupting competing pathways for carbon flux (i.e., acetate or lactate) [4–7]. In all of these cases, ethanol production only increased after the strain had been adapted for improved growth, and the highest yield obtained by this approach thus far is 54% of the theoretical maximum [5]. There are also reports of trying to increase ethanol production by disrupting competing pathways for electron flux (i.e., formate and hydrogen) [8,9]. Of these, the hydrogenase deletions ($\Delta hydG$ and Δech) had the most significant impact on ethanol production, increasing yield to 64% of the theoretical maximum. A recent report showing deletion of genes involved in acetate, lactate, formate, and most of the hydrogen production in a single strain [10] showed similar ethanol yield to the hydrogenase deletion [8], and adaptation of this strain resulted in further improvement of yield to 75% of theoretical [11]. Thus, understanding electron flux is important for controlling product formation in *C. thermocellum*.

C. thermocellum uses mixed-acid fermentation pathways in which glucose and cellodextrins are converted to pyruvate via a modified Embden-Meyerhof-Parnas glycolytic pathway [12,13]. Note that the NADH produced by the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) reaction can be converted to NADPH by the malate shunt [14]. This is indicated by the generalized cofactor specificity (i.e., NAD(P)H instead of NADH) for the glycolysis reaction in the diagram. Pyruvate is converted to acetyl-CoA either by pyruvate-ferredoxin oxidoreductase (PFOR) or pyruvate-formate lyase (PFL). Acetyl-CoA is converted to acetate by the combined actions of phosphotransacetylase (PTA) and acetate kinase (ACK). Acetyl-CoA can also be reduced to ethanol by the combined actions of aldehyde dehydrogenase (ALDH) and alcohol dehydrogenase (ADH). The reduced ferredoxin generated by the PFOR reaction can be reoxidized either by H_2 generation or electron transfer to $NAD(P)^+$, generating NAD(P)H. The generalized model is presented in Figure 1 and Table 1.

Figure 1. Generalized model of mixed acid fermentation in *C. thermocellum*. Reactions involving carbon flux are indicated with black arrows. Reactions involving electron flux are indicated with blue arrows. The Fe-Hyd and Bifur-Hyd hydrogenases are both inactivated by deletion of the *hydG* gene, which encodes a hydrogenase maturation protein. For simplicity, only the reduced form of electron carriers is shown (i.e. NADH, NADPH and Fd_{red} are shown, but NAD^+ , $NADP^+$ and Fd_{ox} are not shown). Note that the NADH produced in upper glycolysis can be converted to NADPH by the malate shunt [14]. Also note that strains with the $\Delta hydG$ mutation also carry the $AdhE_{D494G}$ mutation, which allows the use of NADPH in addition to NADH as a cofactor for the ADH reaction [15].

Name	Reaction
Fe-Hyd	$Fd_{red} + 2H^+ \rightarrow Fd_{ox} + H_2$
Bifur-Hyd	$Fd_{red} + NAD(P)H + 3H^+ \rightarrow Fd_{ox} + NAD(P)^+ + 2H_2$
ECH	$Fd_{red} + 2H^+ \rightarrow Fd_{ox} + H_2 + \Delta\mu H^+$
FNOR	$Fd_{red} + H^+ + NAD(P) \rightarrow Fd_{ox} + NAD(P)H$
RNF	$Fd_{red} + ion + NAD^+ \rightarrow Fd_{ox} + NADH + \Delta\mu_{ion}$
NFN	$Fd_{red} + 2NADP^+ + NADH + H^+ \rightarrow Fd_{ox} + 2NADPH + NAD^+$
PFOR	$Fd_{ox} + pyruvate + HSCoA \rightarrow Fd_{red} + acetyl-SCoA + CO_2 + H^+$

Table 1: Ferredoxin-linked reactions in C. thermocellum. For the RNF reaction, the particular ion species (Na^+ or H^+) is not known.

The FNOR (ferredoxin-nicotinamide oxidoreductase) reaction describes the general transfer of electrons from reduced ferredoxin (Fd) to oxidized NAD(P)^+ . Under standard conditions, this reaction is highly exergonic (-15.5 kJ/mole). To conserve energy, many organisms couple the oxidation of ferredoxin with some other endergonic reaction [16]. Based on sequence homology, there is evidence for two FNOR reactions in *C. thermocellum*: RNF and NFN. In the case of RNF, the exergonic reduction of NAD^+ with Fd_{red} is coupled with the endergonic reaction of pumping ions (Na^+/H^+) across the cell membrane, generating an electrochemical gradient. In the case of NFN, the exergonic reduction of NADP^+ with Fd_{red} is coupled with the transhydrogenation of NADH to NADPH [16], using the very negative redox potential of Fd (-450 mV) [16] to drive electron transfer from NADH to produce NADPH.

The supply and demand of nicotinamide cofactors (NADH , NAD^+ , NADPH and NADP^+) are important for ethanol production. The GAPDH and FNOR-type reactions are responsible for the supply of reduced cofactors. Ethanol and hydrogen production are responsible for much of the demand for these reduced cofactors. Understanding the mechanisms of electron transfer between nicotinamide cofactors and Fd will be critical for future metabolic engineering of *C. thermocellum* for CBP. Thus, the goal of this work is to understand the reactions that transfer electrons from ferredoxin to NAD(P)^+ and use this understanding to improve ethanol production in *C. thermocellum*.

2. Materials and Methods

2.1. Strain construction and molecular techniques

All the chemicals ordered were from Sigma-Aldrich (St. Louis, MO) or Fischer Scientific (Pittsburgh, PA) and were of molecular grade unless otherwise stated. Primers were ordered from Integrated DNA Technologies (Coralville, IA). Primers, plasmids, and strains can be found in Tables 2, 3 and 4. Transformation and deletion of *rnfDG* (Clo1313_0062-63) and *nfnAB* (Clo1313_1848-49) was accomplished using plasmids pJLO13 and pJLO17 respectively, using previously described methods [17,18]. Plasmid sequences are available from Genbank (see accession numbers in Table 3). All strains are derived from M1354, which has a deletion of the hypoxanthine phosphoriboxyltransferase (*hpt*) gene, which is necessary for to allow genetic modification [17]. All strains that include the ΔhydG genotype are derived from strain LL350 ($\Delta\text{hpt } \Delta\text{hydG}$) and contain the $\text{adhE}^{\text{D494G}}$ genotype, which expands cofactor specificity of *adhE* to utilize both NADH and NADPH [8,15]. Genetic modification was confirmed by PCR.

2.2. Strains, plasmids and primers

Name	Parent strain	Description	Reference
DSM1313		Wild type, aka LL1004 in the Lynd Lab strain collection	DSMZ
M1354	DSM1313	Δhpt , aka LL345 in the Lynd Lab strain collection	[5]
LL1087	M1354	$\Delta\text{hpt } \Delta\text{rnfDG}$	this work
LL1152	M1354	$\Delta\text{hpt } \Delta\text{rnf}$	this work
LL1272	M1354	$\Delta\text{hpt } \text{P}_{2638}\text{-rnf}$	this work

LL1273	M1354	Δhpt P ₀₈₁₅ - <i>rnf</i>	this work
LL1084	M1354	$\Delta hpt \Delta nfnAB$	this work
LL1085	LL1087	$\Delta hpt \Delta rnfDG \Delta nfnAB$	this work
LL350	M1354	$\Delta hpt \Delta hydG$	[8]
LL1083	LL350	$\Delta hpt \Delta hydG \Delta rnfDG$	this work
LL1275	LL350	$\Delta hpt \Delta hydG$ P ₂₆₃₈ - <i>rnf</i>	this work
LL1276	LL350	$\Delta hpt \Delta hydG$ P ₀₈₁₅ - <i>rnf</i>	this work
LL1086	LL350	$\Delta hpt \Delta hydG \Delta nfnAB$	this work
LL1114	LL1083	$\Delta hpt \Delta hydG \Delta rnfDG \Delta nfnAB$	this work
LL1147	LL350	$\Delta hpt \Delta hydG \Delta ech$	[8]
LL1277	LL1147	$\Delta hpt \Delta hydG \Delta ech$ P ₀₈₁₅ - <i>rnf</i>	this work
LL1387	LL1084	$\Delta hpt \Delta nfnAB$ pDGO144(empty)	this work
LL1388	LL1084	$\Delta hpt \Delta nfnAB$ pDGO144(P ₂₆₃₈ - <i>nfnAB</i>)	this work

102 Table 2. Strains used in this work.

Name	Description	Genbank accession	Reference
pJLO13	Deletion of <i>nfnAB</i>	(add before publication)	this work
pJLO17	Deletion of <i>rnfDG</i>	(add before publication)	this work
pJLO21	Complete deletion of <i>rnf</i> operon	(add before publication)	this work
pSM03	Insertion of promoter P ₂₆₃₈ upstream of the <i>rnf</i> operon	(add before publication)	this work
pSM04	Insertion of promoter P ₀₈₁₅ upstream of the <i>rnf</i> operon	(add before publication)	this work
pDGO144	<i>C. thermocellum</i> expression plasmid	KX259111	[19]
pLL1179	Plasmid pDGO144 expressing <i>C. thermocellum</i> <i>nfnAB</i> genes under controls of the P ₂₆₃₈ promoter	(add before publication)	this work
pLT-18	Expression of <i>C. thermocellum</i> ferredoxin in <i>E. coli</i>		[20]
pRKS2	Expression of <i>Carboxydotherrmus hydrogenoformans</i> carbon monoxide dehydrogenase (CODH) in <i>E. coli</i>		[21]
pRKISC	Isc operon overexpression, for improved production if iron-sulfur clusters in <i>E. coli</i>		[22]

103 Table 3. Plasmids used in this work.

Name	Sequence (5' to 3' direction)	Description
Nfn.ext.F	ATCGAGTTTATTCCGTCAGGATCA	Amplifies <i>nfnAB</i> genomic region
Nfn.ext.R	TTAAAAGTGACTGGTCGCCAGG	for PCR confirmation of strain
Nfn.int.F	ACCACTTCTTATGAAAAGCC	Amplifies an internal fragment of
Nfn.int.R	AAGTCTTTTTGCGCTTCTTG	<i>nfnAB</i> , for PCR confirmation of
Rnf.ext.F	CACTCTAGTCGAACAGCTTGCTACAG	strain
		Amplifies <i>rnf</i> genomic region, for

Rnf.ext.R	GTGGAAAACAACACTCTTTGAGCAAACCG	PCR confirmation of strain Amplifies an internal fragment of <i>rnfDG</i> , for PCR confirmation of strain
Rnf.int.F	GCTGTTTGGAGGAATGGGA	
Rnf.int.R	GCCTGGACTGCTTTTGTTA	
nfn.up.rec.F.new	AACCTATAAAAATAGGCGTATCACGAGGCGATA	To clone <i>C. thermocellum nfnAB</i> 5' flank for deletion plasmid pJLO13
nfn.up.rec.R	TTTGTAGCCAATTTTATATCATTCAATTCAATAAAA CTAGCTTAGGACCTCCCTTTCTCAATCGTAAGG AATAACCTTACGATTGAGAAAAGGGAGGTCCTA	
nfn.dn.rec.F	AGCTAGTTTTATTGAATTGAATGATATAAAATTG GC	
nfn.dn.rec.R.new	GCAAAAAAATCTTTTCCATGCCTATTCCCACGATT GTCCCTTTTATACCTGGGAACAATTTTCGGC	To clone <i>C. thermocellum nfnAB</i> 3' flank for deletion plasmid pJLO13
PgapD.68.begin.F	GTGGGAATAGGCATGGAAAAGATTTTTTGC	
XD431	CTGAACTACTGGGCCAGGTATGTATTAAGCATG GGGAGGGCGTGAATGTAA	
nfn.int.rec.F.new	CCATGCTTTAATACATACCTGGCCCAGTAGTTCA GACCACTTCTTATGAAAAGCC	To clone <i>C. thermocellum nfnAB</i> 3' flank for deletion plasmid pJLO13
nfn.int.rec.R.new	CGGCATTATCCCTGATTTTTTCACTACTATTAGCA GAAGTCTTTTGCCTTCTTG	
nfnA-fwd	GCGAGCTCACAAGACCCTAC	
nfnA-rev	AACTTGATTTACCGCCAAC	RT-qPCR primer to amplify nfnA
recA-fwd	TTTACGGCCAGGGTATTTC	
recA-rev	GCCAATCTTCTGACCGTTGT	
rnfB-fwd	GAGGGAAAATGCAAACCAAA	RT-qPCR primer to amplify rnfB
rnfB-rev	CACATAACCCTGGCAACCTT	
rnfC-fwd	GCCGTGTGAACCCTGTGT	
rnfC-rev	AGCCACATCGTTTACCGTTC	RT-qPCR primer to amplify rnfC
rnfD-fwd	GGACCTTTTCTTTGGCAACA	
rnfD-rev	TCCGGGCCTATAACCTTTCT	
rnfG-fwd	TCCCAGTTTGAGGGTATTGC	RT-qPCR primer to amplify rnfG
rnfG-rev	TTACCGCCCTGGAAGTTATG	

Table 4. PCR primers used in this work.

2.3. Media and growth

Strains were grown anaerobically at 55°C for all experiments. For biochemical characterization and transformation, strains were grown in a modified rich DSMZ M122 medium containing 5 g/L cellobiose (also known as CTFUD)[17] in a COY (Ann Arbor, MI) anaerobic chamber with an 85% N₂, 10% CO₂, and 5% H₂ atmosphere. Oxygen was maintained at < 5 ppm using a palladium catalyst. For quantification of fermentation products, strains were grown in sealed serum bottles on 5 g/L cellobiose in 50 mL of defined MTC (medium for thermophilic clostridia) medium for 72 hours with a N₂ headspace as previously described [12]. g/L

The MTC medium contained 5 g/L cellobiose, 9.25 g/L MOPS (morpholinepropanesulfonic acid) sodium salt, 2 g/L urea, 2g/L potassium citrate monohydrate, 1.25 g/L citric acid monohydrate, 1 g/L Na₂SO₄, 1

g/L KH_2PO_4 , 2.5 g/L NaHCO_3 , 1 g/L $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.2 g/L $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, 0.1 g/L $\text{FeCl}_2 \cdot 6\text{H}_2\text{O}$, 1 g/L L-cysteine HCl monohydrate, 0.02 g/L pyridoxamine HCl, 0.004 g/L p-aminobenzoic acid (PABA), 0.002 g/L D-biotin, 0.002 g/L Vitamin B12, 0.0005 g/L $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.0005 g/L $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.0002 g/L ZnCl_2 , 0.0001 g/L $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 0.0001 g/L H_3BO_3 , 0.0001 g/L $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.0001g/L $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$. The medium components were prepared as separate solutions, A-F. Solution A, concentrated 2.5-fold, contained cellobiose, MOPS sodium salt and distilled water. Solution B, concentrated 25-fold, contained potassium citrate monohydrate, citric acid monohydrate, Na_2SO_4 , KH_2PO_4 , NaHCO_3 and distilled water. Solution C, concentrated 50-fold, contained urea and distilled water. Solution D, concentrated 50-fold, contained $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, $\text{FeCl}_2 \cdot 6\text{H}_2\text{O}$, L-cysteine HCl monohydrate. Solution E, concentrated 50-fold, contained thiamine, pyridoxamine HCl, p-aminobenzoic acid (PABA), D-biotin, Vitamin B12. Solution F, concentrated 1000-fold, contained $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, ZnCl_2 , $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, H_3BO_3 , $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$.

All of six solutions were sterilized through a 0.22 μm filter (Corning, #430517) and kept under nitrogen atmosphere and combined prior to use. The final pH was adjusted to 7.2.

For experiments with 50 g/L Avicel, 200 ml serum bottles were used, with a working volume of 28 ml and ~10% inoculum. The vitamin solution E was doubled.

2.4. Quantification of gene expression

Cultures were grown to mid-exponential phase, RNeasy Protect (Qiagen) was added to prevent RNA degradation, then cells were pelleted and lysed by digestion with lysozyme (15 mg/ml) and proteinase K (20 mg/ml). RNA was isolated with an RNeasy minikit (Qiagen) and digested with DNase (Qiagen) to remove contaminating DNA. cDNA was synthesized from 500 ng of RNA using the iScript cDNA synthesis kit (BioRad). qPCR was performed using cDNA with SsoFast EvaGreen Supermix (BioRad) at an annealing temperature of 55°C to determine *rnfD* and *rnfG* RNA levels and normalized to *recA* RNA levels. All reactions were performed in triplicate. In order to confirm removal of contaminating DNA from RNA samples, cDNA was synthesized in the presence and absence of reverse transcriptase followed by qPCR using *recA* primers to ensure only background levels were detected in the samples lacking reverse transcriptase. Standard curves were generated using a synthetic gBlock (IDT) containing a single copy of each of the amplicons.

2.5. Preparation of cell-free extracts

During the preparation of cell-free extracts for enzyme assays, all steps were performed in a COY anaerobic chamber, except spinning down of cells which was done outside the chamber in closed centrifuge tubes. Cells were harvested in mid-exponential phase, and separated from medium by centrifugation. Cells were lysed by resuspending the pellet in an anaerobic lysis buffer consisting of 50 mM morpholinepropanesulfonic (MOPS) sodium salt (pH 7.5), 5 mM dithiothreitol, Ready-Lyse lysozyme (Epicentre Biotechnologies, Madison, WI), and DNase I (Thermo Scientific, Waltham, MA) similar to previously described methods [12,23]. Cell debris was separated from cell-free extract by centrifuging for 15 minutes at 13,000 x g.

2.6. NADH:BV assay

Since the Rnf complex is a membrane associated protein, the harvested cells were frozen and thawed to help break the membrane. Then cell-free extracts were prepared as above. The reaction mixture contained 20 mM MOPS (pH 7.5), 0.2 mM NADH and 1 mM benzyl viologen. The reaction was started with NADH and the benzyl viologen reduction was followed by photometrical observations at 578 nm ($\epsilon = 7.8 \text{ mM}^{-1}\text{cm}^{-1}$) [24].

2.7. Reduced ferredoxin:NAD⁺ assay (Fd:NAD⁺)

Since benzyl viologen is a promiscuous electron acceptor, we also performed FNOR assays with NAD⁺ as the electron acceptor and reduced ferredoxin as the electron donor, as described previously [20]. The reaction mixture contained 50 mM MOPS/NaOH (pH 7.5), 2 mM dithiothreitol (DTT), 4 mM NAD⁺, 3 μM purified ferredoxin, 0.5 μM purified carbon monoxide dehydrogenase (CODH) enzyme in a 200 μl final volume. The cuvette headspace was purged with CO for 30-90 seconds to saturate the liquid with CO. Then the reaction was started with NAD⁺ and the formation of NADH was followed by photometrical observation at 340 nm ($\epsilon = 6.2 \text{ mM}^{-1}\text{cm}^{-1}$) [25].

2.8. Purified ferredoxin and CODH proteins

Purified proteins were generated as described previously [20], briefly, ferredoxin from *C. thermocellum* (Clo1313_2761) was expressed in *E. coli* using the pLT-18 plasmid. Carbon monoxide dehydrogenase from *Carboxydotherrmus hydrogenoformans* was expressed in *E. coli* using the pRK52 plasmid. Expression plasmids were transformed into *E. coli* strain BL21 harboring the pRKISC plasmid (for correct assembly of iron-sulfur clusters in ferredoxin). Both proteins were purified using His-tag affinity columns in an anaerobic chamber. The concentration of ferredoxin was 198 ng/ μl . The concentration of CODH was 754 ng/ μl . Molar quantities were calculated assuming a molecular weight of 6 kDa for ferredoxin and 67 kDa for CODH. NAD(P)H:BV native PAGE assay

Separating proteins by gel electrophoresis was based on previously described methods with minor modifications [26]. 20 μl of 2 mM sodium dithionite was added to wells 20 minutes prior to loading cell free extract. Approximately 0.1 mg of cell free extract protein mixed with 5X native loading buffer containing 25 mM Tris-HCl (pH 8.5) and 192 mM glycine was loaded into a 4-20% non-denaturing polyacrylamide gel (Bio-Rad). The gel was run for 80 minutes at 200V. Afterwards, the gel was placed in a buffer containing 50 mM MOPS (pH 7.5) and 8 mM benzyl viologen (BV). The reaction was started with the addition of 1.5 mM NADH (NADH-TTC assay) or 1.5 mM NADPH (NADPH-TTC assay) and incubated at 55°C until bands developed (approximately 2 hours). These bands were then fixed by adding 24 mM triphenyltetrazolium chloride (TTC). A control reaction was performed without NADH or NADPH (TTC assay).

2.9. Analytical Techniques and Calculations

Headspace gas composition was measured with a gas chromatograph (SRI Instruments, Torrence, CA) with a HayeSep D packed column with a thermal conductivity detector. Nitrogen was used as the carrier gas. Gas measurements were corrected for pressure differences using a digital pressure gauge (Ashcroft, Stratford, CT). Fermentation products in the liquid media were measured using a Waters HPLC (Milford, MA) with a HPX-87H column as described previously [27]. To measure cell biomass,

pellet carbon and nitrogen were measured using a Shimadzu TOC-V CPH elemental analyzer with TNM-1 and ASI-V modules (Shimadzu Corp., Columbia, MD) [28].

3. Results

3.1. Genetic manipulations

To understand the ways that *C. thermocellum* transfers electrons from ferredoxin to nicotinamide cofactors (NAD⁺ and NADP⁺), we made deletions of *rnf* and *nfnAB*, the two gene clusters that are thought to mediate this reaction. We also deleted *rnf* and *nfnAB* in a *hydG* deletion strain [8], since hydrogenases are a major sink for electrons from ferredoxin. This first set of 7 strains includes all possible combinations of these gene deletions (LL350, LL1083, LL1084, LL1085, LL1086, LL1087, LL1114).

To inactivate the *rnf* gene cluster, we initially deleted just the D and G subunits, which we suspected would be sufficient. Later, after we had already constructed several strains with the *rnfDG* deletion, we tested whether deletion of the D and G subunits inactivated the enzyme. We constructed strain LL1152 ($\Delta hpt \Delta rnf$), where all of the subunits of *rnf* were deleted (C, D, G, E, A and B). Strain LL1152 ($\Delta hpt \Delta rnf$) behaved similarly to LL1087 ($\Delta hpt \Delta rnfDG$); therefore, we concluded that the *rnfDG* deletion completely inactivates Rnf.

We then constructed another set of strains where we attempted to overexpress *rnf* by inserting a known promoter upstream of the *rnf* operon. For this work, we used the previously-characterized promoters from the Clo1313_2638 (P₂₆₃₈) and Clo1313_0815 (P₀₈₁₅) genes [29]. We attempted to insert both of these promoters into 3 strain backgrounds, M1354(Δhpt), LL350($\Delta hpt \Delta hydG$) and LL1147($\Delta hpt \Delta hydG \Delta ech$). This would have resulted in 6 strains; however, we were not able to insert the P₂₆₃₈ promoter in the LL1147 strain background. The 5 strains that were successfully constructed were LL1272, LL1273, LL1275, LL1276 and LL1277.

3.2. Measurement of gene expression

To test expression of *rnf* with the new promoters, expression of *rnfC*, *D*, *G*, *B* and *nfnA* genes was measured by RT-qPCR. Integration of promoters P₂₆₃₈ or P₀₈₁₅ upstream of *rnf* increased its expression (Figure 2), with promoter P₂₆₃₈ increasing gene expression to a greater extent than P₀₈₁₅. The promoter insertions had a larger effect in strains where *hydG* or *hydG* and *ech* had already been deleted. The expression of *nfnA* was fairly constant regardless of the genetic background.

Figure 2. RT-qPCR data for components of the *rnf* operon. Expression was normalized to *recA*. Error bars represent one standard deviation, *n*=3. The *rnf* genes are listed in the order in which they appear on the genome.

3.3. Measurement of enzyme activity

Electron transfer activity was measured both with benzyl viologen (NADH:BV) and reduced ferredoxin (Fd:NAD⁺) assays (Figure 3). Since the ferredoxin assay was more time-consuming, and showed a similar pattern of activity, compared to the NADH:BV assay, we only tested selected strains with the Fd:NAD⁺ assay.

In strains where *rnf* genes were deleted, there was generally no change in activity. Overexpression of the *rnf* operon with either the P₀₈₁₅ or P₂₆₃₈ promoter resulted in increased activity (both for the NADH:BV and Fd:NAD⁺ assays), and the strains with P₂₆₃₈ promoter had higher activity compared to the same strain with the P₀₈₁₅ promoter, which corresponds to what we observed with the gene expression data. Even in strain LL1114, which has deletions of *hydG*, *rnf* and *nfnAB*, FNOR activity is still present at levels similar to that of the wild type strain (M1354). These results suggest the presence of an as-yet-unidentified FNOR-type enzyme which can functionally complement the *rnf* deletion.

Figure 3. FNOR activity as measured by NADH:BV assay and reduced ferredoxin:NAD⁺ assay.. ND means not determined. Error bars represent one standard deviation, n=3. Raw data presented in Supplementary information Table S1. For the Fd:NAD⁺ assay, a representative experiment is shown (Supplementary Table S1, replicate 2), however data for both replicates is given in the supplement.

We were unable to detect significant NADPH:BV activity for any of our strains with our standard cuvette-based assay, possibly due to competing reactions that could use BV as an electron donor. Instead, we tested activity using a native-PAGE NADPH:BV assay based on previous techniques [30] (Figure 4). The *nfnAB* genes were expressed on a plasmid in strain LL1084 ($\Delta hpt \Delta nfnAB$). By comparing lanes 4 and 5 (empty plasmid control vs. *nfnAB* expression plasmid, Figure 4, panel D), NADPH:BV activity can be observed from a protein running just below the 480 kDa marker protein. This protein is absent from all of the *nfnAB* deletion strains (LL1084, LL1387, LL1085 and LL1114). It is present at low concentrations in strains LL1083 and LL1087. The protein is not observed for the wild type strain (M1354), but this may be due to extremely low expression. Another set of proteins is observed around 66 kDa, but the activity associated with these proteins is not specific for NADPH (compare panel C vs. D). Another set of proteins is observed around 242 kDa, but the activity associated with these proteins appears even in the absence of any nicotinamide cofactor (compare panel B vs. D). These proteins may exhibit hydrogenase activity, due to the presence of 5% H₂ in the anaerobic chamber in which the experiment was performed.

Figure 4. Detection of NfnAB from cell free extracts on Native PAGE gel. Cell free extracts were run on a 4-20% gel at 200V for 80 minutes in an anaerobic chamber. Gels were run simultaneously and then treated in one of 4 conditions: Coomassie stain (to show protein levels and molecular weight ladder), panel A; TTC assay, to show hydrogenase activity, panel B; NADH-TTC assay, to show NADH-linked FNOR activity, panel C; NADPH-TTC assay, to show NFN activity, panel D. Two molecular weight ladders were run: the Bio-Rad Precision Plus Dual Color ladder (lane B) and the Novex NativeMark Unstained ladder (lane N). The NativeMark bands only appear in the Coomassie-stained gel. Lane 1, M1354 (Δhpt); lane 2, LL1083 ($\Delta hpt \Delta hydG \Delta rnfDG$); lane 3 LL1084 ($\Delta hpt \Delta nfnAB$); lane 4, LL1387 ($\Delta hpt \Delta nfnAB$ *pDGO144(empty)*); lane 5, LL1388 ($\Delta hpt \Delta nfnAB$ *pDGO144(P₂₆₃₈-nfnAB)*); lane 6, LL1085 ($\Delta hpt \Delta rnfDG \Delta nfnAB$); lane 7, LL1087 ($\Delta hpt \Delta rnfDG$); lane 8, LL1114 ($\Delta hpt \Delta hydG \Delta rnfDG \Delta nfnAB$). A band corresponding to the NfnAB protein complex can be seen just below the 480 kDa marker.

3.4. Effect of genetic modifications on fermentation products

To determine the effect of the deletions on metabolism, we grew the strains in sealed bottles with 5 g/L cellobiose and measured the resulting fermentation products. In the M1354 (Δhpt) strain background, deletion of *rnfDG* resulted in a decrease in ethanol production of about 45% (Figure 5, compare strain LL1087 with M1354). Overexpression of *rnf* in the M1354 strain background also resulted in reduced ethanol production (compare strains LL1275 and LL1276 with M1354).

In the LL350 ($\Delta hpt \Delta hydG$) strain background, deletion of *rnfDG* reduced ethanol production by about 10% and overexpression of *rnf* increased ethanol production by about 30% (Figure 5, compare strains LL1083, LL1275 and LL1276 with LL350). Deletion of *rnfAB* had no effect on ethanol production.

In the LL1147 ($\Delta hpt \Delta hydG \Delta ech$) strain background, overexpression of *rnf* did not significantly increase ethanol production. Although this strain (LL1277) shows the highest ethanol yield of all of the strains in this study (72% of theoretical maximum), the effect seems more likely due to the *ech* deletion (compare LL1277 vs LL1276) than the *rnf* overexpression (compare LL1277 vs LL1147), and thus it appears that the *ech* deletion masks the effect of the *rnf* overexpression.

Deletion of *rnfAB* on its own appeared to have no effect on ethanol production under normal fermentation conditions, with M1354(Δhpt) and LL1084($\Delta rnfAB$) producing similar fermentation product amounts. Deletion of *rnfAB* only impacted ethanol yield when combined with $\Delta hydG$ and Δrnf , where the ethanol yield decreased by 30%. The complete set of fermentation data is presented in the supporting information (Table S2)

Figure 5. Fermentation products of engineered strains. The theoretical maximum yield for ethanol, acetate and formate is 4 moles per mole cellobiose. For hydrogen, the theoretical maximum yield is 8 if the hydrogenase is bifurcating, and 4 if it is not. Error bars represent one standard deviation, $n \geq 3$. Raw data is presented in Supplementary Information Table S2.

3.5. Ethanol production of high-yielding strains at high substrate loadings

For initial characterization of metabolism, strains were grown on 5 g/L cellobiose. For commercial applications, strains will need to consume cellulose, and do so at high substrate loadings. To get an indication of the performance under more commercially-relevant conditions, our highest yielding strains (LL1275, LL1276 and LL1277), along with relevant control strains (LL350 and LL1147) were grown on 50 g/L Avicel microcrystalline cellulose (Figure 6). In the LL350 strain background, overexpressing *rnf* led to more than a 2-fold increase in final ethanol titer. In the LL1147 background, overexpressing *rnf* did not

affect final titer, however the yield increased slightly (note the lower Avicel consumption). The ethanol yield for most strains was lower due to substantial amounts of glucose production.

Figure 6. Ethanol production from 50 g/L of Avicel microcrystalline cellulose. Error bars represent one standard deviation, n=2. Raw data is presented in Supplementary information Table S3.

4. Discussion

4.1. Role of RNF and NFN reactions in *C. thermocellum* metabolism

Electron flux is as critical as carbon flux during fermentation and biofuel production. Here, we begin to characterize how electrons are transferred between NADH, NADPH, and Fd in *C. thermocellum*. Ethanol production in wild type *C. thermocellum* requires 2 moles of NADH per mole of acetyl-CoA. In glycolysis, the GAPDH reaction generates 1 mole of NADH per mole of acetyl-CoA (1/4 cellobiose → 1 acetyl-CoA). Thus without the RNF reaction, ethanol yield is limited to 2 moles/mole cellobiose (i.e. 50% of the maximum theoretical yield), however with the RNF reaction, the ethanol yield could reach 4 moles/mole cellobiose (i.e. 100% of the theoretical yield). Thus, we would expect increased RNF activity to result in increased ethanol production. Indeed, we see exactly this, with deletion of *rnf* generally decreasing and overexpression generally increasing ethanol yield.

The *rnf* operon is well-expressed (approximately at the same level as *recA*, see Figure 2) in wild-type *C. thermocellum*. In strains that are wild type at hydrogenase loci (i.e., wild type for *hydG* and *ech*), the metabolic role of the RNF reaction is indeterminate. However in most instances the loss of *rnf* reduced ethanol and increased acetate and H₂ formation, in contrast to some *in silico* predictions [31] and in support of others [32]. Although we were able to successfully increase the level of activity (Figure 3), this

did not result in improved ethanol production in the wild type background. One explanation for these results is that in the wild type strain, the RNF reaction may operate close to equilibrium [33] and may allow electron transfer from NADH to Fd. In strains with hydrogenase deletions, on the other hand, the Fd pool may be in a more reduced state, driving the RNF reaction towards NADH production, which is subsequently used for ethanol production. Interestingly, in the strain lacking all functional hydrogenases, the *rnf* operon was poorly expressed compared to other strains (Figure 2). While *rnf* appears to be highly expressed in most instances under its native promoter, little is known about the regulation of this operon. For metabolic engineering it may be better to place this critical gene under control of a constitutive promoter. Indeed, a previous study has shown that the *rnf* operon in *C. thermocellum* is downregulated when subjected to ethanol stress [34], which may explain the better ethanol production of *C. thermocellum* strains with altered promoters in Figure 6.

The NFN reaction does not seem to play a major role in fermentation by *C. thermocellum*, at least under the conditions tested. Although we observed gene expression levels similar to that of the *rnf* operon (Figure 2), we were not able to detect NADPH:BV activity using our standard assay, and it was only by using a native-PAGE technique that activity was seen. Even then, the activity appeared to be relatively low (although difficult to quantify precisely, Figure 4). Furthermore, deletion of *nfnAB* did not affect the distribution of fermentation products in most strains (Figure 5). Deletion of *nfnAB* did have a negative impact in the $\Delta hydG \Delta rnf$ mutant, where additional deletion of *nfnAB* decreased ethanol yield. Interestingly, this decrease was not seen in $\Delta rnf \Delta nfnAB$ relative to Δrnf alone (wild type at *hydG* locus). In the $\Delta hydG$ background, NfnAB may play more of a role in ethanol production because this strain happens to contain a point mutation in *adhE* that allows it to utilize both NADH and NADPH as an electron source for the reduction of acetaldehyde to ethanol [15], and NfnAB can facilitate the transfer of a portion of the electrons on Fd to NADPH.

4.2. Theoretical calculations based on formate, acetate, ethanol and hydrogen measurements

In *C. thermocellum*, pyruvate ferredoxin oxidoreductase (PFOR) is thought to be a key route for pyruvate to acetyl-CoA conversion [35]. Although flux through this pathway cannot be measured directly, it can be inferred by the following equation: ethanol + acetate – formate, because the acetyl-CoA needed for ethanol and acetate production is derived from pyruvate either via PFOR or PFL. We would expect that eliminating pathways for re-oxidation of ferredoxin would eventually inhibit PFOR flux, due to the accumulation of reduced ferredoxin, and that formate production would increase. We see this to a certain extent in strain LL1114, where formate production has increased and PFOR flux has decreased, although the PFOR flux (1.42 mol/mol cellobiose) is still more than twice the PFL flux (0.87 mol/mol cellobiose) (Table 5).

Calculated metabolic fluxes (mol/mol cellobiose)

Strain	Genotype	PFOR	PFL	Hyd	FNOR
LL1004	wt	2.41	0.24	2.30	*
M1354	Δhpt	2.43	0.22	2.43	*
LL1087	$\Delta hpt \Delta rnfDG$	2.17	0.22	3.12	*
LL1152	$\Delta hpt \Delta rnf$	2.10	0.17	3.27	*
LL1084	$\Delta hpt \Delta nfnAB$	2.44	0.13	2.44	*

LL1085	$\Delta hpt \Delta rnfDG \Delta nfnAB$	2.08	0.34	3.23	*
LL350	$\Delta hpt \Delta hydG$	2.02	0.39	0.35	1.67
LL1083	$\Delta hpt \Delta hydG \Delta rnfDG$	2.03	0.55	0.49	1.54
LL1275	$\Delta hpt \Delta hydG P2638-rnf$	2.24	0.72	0.21	2.03
LL1276	$\Delta hpt \Delta hydG P0815-rnf$	2.26	0.69	0.17	2.09
LL1086	$\Delta hpt \Delta hydG \Delta nfnAB$	2.27	0.40	0.19	2.08
LL1114	$\Delta hpt \Delta hydG \Delta rnfDG \Delta nfnAB$	1.42	0.87	0.72	0.70
LL1147	$\Delta hpt \Delta hydG \Delta ech$	2.49	0.54	0.00	2.49
LL1277	$\Delta hpt \Delta hydG \Delta ech P0815-rnf$	2.51	0.57	0.00	2.51

Table 5. Calculated flux values for electron transfer reactions.

* values cannot be uniquely determined when both Fe-Hyd and Bifur-Hyd reactions are present

4.3. The role of Rnf in supplying NADH for ethanol production

In *C. thermocellum*, there are two enzymes thought to be responsible for the majority of the NADH that is needed for ethanol production: GAPDH and RNF. The NADH produced by the GAPDH reaction can be converted to NADPH by the transhydrogenation activity of the malate shunt [12]. If the malate shunt carries all of the glycolytic flux, all of the NADH is converted to NADPH. If the malate shunt carries no flux (i.e. the pyruvate phosphate dikinase reaction (PPDK) is used instead), then there is no transhydrogenation. Since the PPDK pathway can be disrupted, but the malate shunt cannot be (unless an alternative route is provided for phosphoenolpyruvate to pyruvate conversion) [14], we suspect that the malate shunt carries more flux than the PPDK pathway, although this is difficult to quantify.

In addition to the problem of quantifying NADH supply, there are also problems quantifying the NADH demand. Wild type *C. thermocellum* uses NADH for both the aldehyde dehydrogenase (ALDH) and alcohol dehydrogenase (ADH) reactions [15]. All of the strains which carry the $\Delta hydG$ deletion, however, are descended from strain LL350 ($\Delta hpt \Delta hydG$) and thus carry the $AdhE_{D494G}$ mutation which expands cofactor specificity of the ADH reaction to allow use of both NADH and NADPH. The current genetic tools for *C. thermocellum* do not allow straightforward elimination of point mutations such as these [19]. Thus, if we want to do a quantitative analysis of NADH supply and demand in *C. thermocellum*, we would need to both delete *ppdk* and eliminate the $AdhE_{D494G}$ mutation.

4.4. Evidence for another enzyme to transfer electrons from ferredoxin to nicotinamide cofactors

In all of the strains where *hydG* has not been deleted, hydrogen production is usually greater than PFOR flux (as calculated in section 4.2). This suggests that hydrogen is being produced by the bifurcating hydrogenase (Table 1, Bifur-Hyd). Eliminating the bifurcating hydrogenase by deleting *hydG* dramatically reduces hydrogen production. The remaining hydrogen has been shown to be produced by ECH [8]. ECH produces 1 mole of H_2 for each mole of reduced ferredoxin; therefore, H_2 flux should always be less than or equal to PFOR flux in strains where Ech is the only functional hydrogenase, which agrees with our experimental data. If FNOR activity (i.e., transfer of electrons from ferredoxin to $NAD(P)^+$) were eliminated, then H_2 production and PFOR flux should be almost identical (neglecting a small amount of ferredoxin that could be oxidized for biomass formation). However, H_2 flux is almost 2-fold less than PFOR flux, even in strain LL1114, suggesting that there may be FNOR activity not catalyzed by either the Rnf or NfnAB protein complexes (i.e., a flux of 0.70 mol/mol cellobiose, Table 5).

Alternatively, there may be a large, unidentified sink for electrons from Fd that would similarly skew the ratios.

In addition to the flux calculations presented above (section 4.1), data from our NADH:BV and ferredoxin:NAD⁺ assays suggests another route for electron transfer from ferredoxin to NAD⁺, since we see a large amount of activity remaining after deletion of *rnfDG*. This FNOR activity could be the result of a currently unknown type of FNOR enzyme, or could be the result of a series of reactions that form a cycle which has the net effect of FNOR activity.

5. Conclusions

For high yields of ethanol in *C. thermocellum* using PFOR, electrons from reduced ferredoxin must be used for making ethanol. Previous studies had identified two possible enzymes for transferring electrons from reduced ferredoxin to nicotinamide cofactors: Rnf and NfnAB. Here we clarify the importance of these two enzymes in *C. thermocellum* metabolism and ethanol formation. In *C. thermocellum*, the RNF reaction plays a key role in ethanol production. Deletion of *rnf* genes reduced ethanol production, and overexpression of the *rnf* operon increased ethanol production in strains with hydrogenase deletions. In some cases, the *ech* hydrogenase deletion masked the effect of *rnf* overexpression. This appears to be due to the presence of cryptic FNOR activity. In addition to improving our understanding of electron flux, we were able to increase ethanol yield by 30% (from 51% to 66% of the theoretical maximum) and achieve an ethanol titer of 5.1 g/L from Avicel.

In contrast to Rnf, NfnAB appears to play at most a small role in end production formation under our standard fermentation conditions, as deletions of *nfnAB* do not appear to significantly change fermentation products in most of the genetic backgrounds tested. The only case where the *nfnAB* deletion played even a small role was in a strain that can use NADPH for the reduction of acetaldehyde to ethanol (i.e LL1114 vs LL1083 showed a decrease in ethanol production).

Genes encoding NfnAB and Rnf are widespread throughout genomes of anaerobic microorganisms, including many being investigated for biofuel production. Understanding and manipulating their function may be important for generating high yields of desirable products, but much is unknown about their function. Here we show that Rnf is important for ethanol production and that overexpression of *rnf* can be used to increase ethanol yield and titer.

6. Acknowledgements

We thank Johannes P. Van Dijken for his useful comments and discussions of metabolism. The BioEnergy Science Center is a U.S. Department of Energy Bioenergy Research Center supported by the Office of Biological and Environmental Research in the DOE Office of Science. The manuscript has been authored by Dartmouth College under sub-contract no. 4000115284 and contract no. DE-AC05-00OR22725 with the U.S. Department of Energy.

7. References

1. Demain AL, Newcomb M, Wu JHD: **Cellulase, clostridia, and ethanol**. *Microbiol. Mol. Biol.*

- 420 *Rev.* 2005, **69**:124–154.
- 421 2. Olson DG, McBride JE, Shaw AJ, Lynd LR: **Recent progress in consolidated bioprocessing.**
422 *Curr. Opin. Biotechnol.* 2012, **23**:396–405.
- 423 3. Akinoshio H, Yee K, Close D, Ragauskas A: **The emergence of *Clostridium thermocellum* as**
424 **a high utility candidate for consolidated bioprocessing applications.** *Front. Chem.* 2014,
425 **2**.
- 426 4. Tripathi SA, Olson DG, Argyros DA, Miller BB, Barrett TF, Murphy DM, McCool JD, Warner AK,
427 Rajgarhia VB, Lynd LR, et al.: **Development of *pyrF*-based genetic system for targeted**
428 **gene deletion in *Clostridium thermocellum* and creation of a *pta* mutant.** *Appl. Environ.*
429 *Microbiol.* 2010, **76**:6591–6599.
- 430 5. Argyros DA, Tripathi SA, Barrett TF, Rogers SR, Feinberg LF, Olson DG, Foden JM, Miller BB,
431 Lynd LR, Hogsett DA, et al.: **High ethanol titers from cellulose by using metabolically**
432 **engineered thermophilic, anaerobic microbes.** *Appl. Environ. Microbiol.* 2011, **77**:8288–
433 94.
- 434 6. van der Veen D, Lo J, Brown SD, Johnson CM, Tschaplinski TJ, Martin M, Engle NL, van den
435 Berg RA, Argyros AD, Caiazza NC, et al.: **Characterization of *Clostridium thermocellum***
436 **strains with disrupted fermentation end-product pathways.** *J. Ind. Microbiol. Biotechnol.*
437 2013, **40**:725–34.
- 438 7. Biswas R, Prabhu S, Lynd LR, Guss AM: **Increase in Ethanol Yield via Elimination of**
439 **Lactate Production in an Ethanol-Tolerant Mutant of *Clostridium thermocellum*.** *PLoS*
440 *One* 2014, **9**:e86389.
- 441 8. Biswas R, Zheng T, Olson DG, Lynd LR, Guss AM: **Elimination of hydrogenase active site**
442 **assembly blocks H₂ production and increases ethanol yield in *Clostridium***
443 ***thermocellum*.** *Biotechnol. Biofuels* 2015, **8**.
- 444 9. Rydzak T, Lynd LR, Guss AM: **Elimination of formate production in *Clostridium***
445 ***thermocellum*.** *J. Ind. Microbiol. Biotechnol.* 2015, doi:10.1007/s10295-015-1644-3.
- 446 10. Papanek B, Biswas R, Rydzak T, Guss AM: **Elimination of metabolic pathways to all**
447 **traditional fermentation products increases ethanol yields in *Clostridium***
448 ***thermocellum*.** *Metab. Eng.* 2015, **32**:49–54.
- 449 11. Tian L, Papanek B, Olson DG, Rydzak T, Holwerda EK, Zheng T, Zhou J, Maloney M, Jiang N,
450 Giannone RJ, et al.: **Simultaneous achievement of high ethanol yield and titer in**
451 ***Clostridium thermocellum*.** *Biotechnol. Biofuels* 2016, **9**:116.
- 452 12. Zhou J, Olson DG, Argyros DA, Deng Y, van Gulik WM, van Dijken JP, Lynd LR: **Atypical**
453 **glycolysis in *Clostridium thermocellum*.** *Appl. Environ. Microbiol.* 2013, **79**:3000–8.
- 454 13. Rydzak T, McQueen PD, Krokhin O V, Spicer V, Ezzati P, Dwivedi RC, Shamshurin D, Levin DB,
455 Wilkins JA, Sparling R: **Proteomic analysis of *Clostridium thermocellum* Core**
456 **Metabolism: Relative protein expression profiles and growth phase-dependent**
457 **changes in protein expression.** *BMC Microbiol.* 2012, **12**:214.
- 458 14. Deng Y, Olson DG, Zhou J, Herring CD, Joe Shaw A, Lynd LR: **Redirecting carbon flux**
459 **through exogenous pyruvate kinase to achieve high ethanol yields in *Clostridium***
460 ***thermocellum*.** *Metab. Eng.* 2013, **15**:151–8.
- 461 15. Zheng T, Olson DG, Tian L, Bomble YJ, Himmel ME, Lo J, Hon S, Shaw AJ, van Dijken JP, Lynd
462 LR: **Cofactor specificity of the bifunctional alcohol and aldehyde dehydrogenase**
463 **(AdhE) in wild-type and mutants of *Clostridium thermocellum* and**

- 464 ***Thermoanaerobacterium saccharolyticum*. *J. Bacteriol.* 2015, **197**:JB.00232-15.**
- 465 16. Buckel W, Thauer RK: **Energy conservation via electron bifurcating ferredoxin**
 466 **reduction and proton/Na(+) translocating ferredoxin oxidation. *Biochim. Biophys. Acta***
 467 **2013, **1827**:94–113.**
- 468 17. Olson DG, Lynd LR: **Chapter seventeen - transformation of *Clostridium thermocellum* by**
 469 **electroporation. In *Methods in Enzymology*. Edited by Gilbert HJ. Academic Press;**
 470 **2012:317–330.**
- 471 18. Guss AM, Olson DG, Caiazza NC, Lynd LR: **Dcm methylation is detrimental to plasmid**
 472 **transformation in *Clostridium thermocellum*. *Biotechnol. Biofuels* 2012, **5**:30.**
- 473 19. Hon S, Lanahan AA, Tian L, Giannone RJ, Hettich RL, Olson DG, Lynd LR: **Development of a**
 474 **Plasmid-Based Expression System in *Clostridium thermocellum* and its use to Screen**
 475 **Heterologous Expression of bifunctional alcohol dehydrogenases (*adhEs*). *Metab. Eng.***
 476 ***Commun.* 2016, [no volume].**
- 477 20. Tian L, Lo J, Shao X, Zheng T, Olson DG, Lynd LR: **Identification of a ferredoxin:NAD⁺**
 478 **oxidoreductase enzyme in *Thermoanaerobacterium saccharolyticum* and its role in**
 479 **ethanol formation. *Appl. Environ. Microbiol.* 2016, (in press).**
- 480 21. Jeoung J-H, Dobbek H: **Carbon Dioxide Activation at the Ni,Fe-Cluster of Anaerobic**
 481 **Carbon Monoxide Dehydrogenase. *Science (80-.).* 2007, **318**:1461–1464.**
- 482 22. Takahashi Y, Nakamura M: **Functional Assignment of the ORF2-iscS-iscU-iscA-hscB-hscA-**
 483 **fdx-ORF3 Gene Cluster Involved in the Assembly of Fe-S Clusters in *Escherichia coli*. *J.***
 484 ***Biochem.* 1999, **126**:917–926.**
- 485 23. Lo J, Zheng T, Hon S, Olson DG, Lynd LR: **The bifunctional alcohol and aldehyde**
 486 **dehydrogenase gene, *adhE*, is necessary for ethanol production in *Clostridium***
 487 ***thermocellum* and *Thermoanaerobacterium saccharolyticum*. *J. Bacteriol.* 2015,**
 488 ****197**:1386–1393.**
- 489 24. Wang S, Huang H, Moll J, Thauer RK: **NADP reduction with reduced ferredoxin and NADP**
 490 **reduction with NADH are coupled via an electron-bifurcating enzyme complex in**
 491 ***Clostridium kluyveri*. *J. Bacteriol.* 2010, **192**:5115–23.**
- 492 25. Ma K, Adams MWW, Kelly RM: **Ferredoxin:NADP oxidoreductase from *Pyrococcus***
 493 ***furiosus*. In *Methods in Enzymology*. . Academic Press; 2001:40–45.**
- 494 26. Fournier M, Dermoun Z, Durand M-C, Dolla A: **A New Function of the *Desulfovibrio***
 495 ***vulgaris* Hildenborough [Fe] Hydrogenase in the Protection against Oxidative Stress. *J.***
 496 ***Biol. Chem.* 2004, **279**:1787–1793.**
- 497 27. Zhang Y-HP, Lynd LR: **Regulation of cellulase synthesis in batch and continuous cultures**
 498 **of *Clostridium thermocellum*. 2005, **187**:99–106.**
- 499 28. Holwerda EK, Ellis LD, Lynd LR: **Development and evaluation of methods to infer**
 500 **biosynthesis and substrate consumption in cultures of cellulolytic microorganisms.**
 501 ***Biotechnol. Bioeng.* 2013, **110**:2380–8.**
- 502 29. Olson DG, Maloney M, Lanahan AA, Hon S, Hauser LJ, Lynd LR: **Identifying promoters for**
 503 **gene expression in *Clostridium thermocellum*. *Metab. Eng. Commun.* 2015, **2**:23–29.**
- 504 30. Lo J, Zheng T, Olson DG, Ruppertsberger N, Tripathi SA, Tian L, Guss AM, Lynd LR: **Deletion**
 505 **of *nfnAB* in *Thermoanaerobacterium saccharolyticum* and its effect on metabolism. *J.***
 506 ***Bacteriol.* 2015, **197**:JB.00347-15.**
- 507 31. Roberts SB, Gowen CM, Brooks JP, Fong SS: **Genome-scale metabolic analysis of**

- 508 ***Clostridium thermocellum* for bioethanol production.** *Bmc Syst. Biol.* 2010, **4**:17.
- 509 32. Thompson RA, Layton DS, Guss AM, Olson DG, Lynd LR, Trinh CT: **Elucidating central**
- 510 **metabolic redox obstacles hindering ethanol production in *Clostridium thermocellum*.**
- 511 *Metab. Eng.* 2015, **32**:207–219.
- 512 33. Biegel E, Schmidt S, González J, Müller V: **Biochemistry, evolution and physiological**
- 513 **function of the Rnf complex, a novel ion-motive electron transport complex in**
- 514 **prokaryotes.** *Cell. Mol. Life Sci.* 2011, **68**:613–634.
- 515 34. Yang S, Giannone RJ, Dice L, Yang ZK, Engle NL, Tschaplinski TJ, Hettich RL, Brown SD:
- 516 ***Clostridium thermocellum* ATCC27405 transcriptomic, metabolomic and proteomic**
- 517 **profiles after ethanol stress.** *BMC Genomics* 2012, **13**:336.
- 518 35. Lamed R, Zeikus JG: **Ethanol production by thermophilic bacteria: relationship between**
- 519 **fermentation product yields of and catabolic enzyme activities in *Clostridium***
- 520 ***thermocellum* and *Thermoanaerobium brockii*.** *J. Bacteriol.* 1980, **144**:569–78.

521