Ferredoxin:NAD⁺ Oxidoreductase of *Thermoanaerobacterium saccharolyticum* and Its Role in Ethanol Formation

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ABSTRACT

Ferredoxin:NAD⁺ oxidoreductase (NADH-FNOR) catalyzes the transfer of electrons from reduced ferredoxin to NAD⁺. This enzyme has been hypothesized to be the main enzyme responsible for ferredoxin oxidization in the NADH-based ethanol pathway in *Thermoanaerobacterium saccharolyticum*; however, the corresponding gene has not yet been identified. Here, we identified the Tsac_1705 protein as a candidate FNOR based on the homology of its functional domains. We then confirmed its activity *in vitro* with a ferredoxin-based FNOR assay. To determine its role in metabolism, the *tsac_1705* gene was deleted in different strains of *T. saccharolyticum*. In wild-type *T. saccharolyticum*, deletion of *tsac_1705* resulted in a 75% loss of NADH-FNOR activity, which indicated that Tsac_1705 is the main NADH-FNOR in *T. saccharolyticum*. When both NADH- and NADPH-linked FNOR genes were deleted, the ethanol titer decreased and the ratio of ethanol to acetate approached unity, indicative of the absence of FNOR activity. Finally, we tested the effect of heterologous expression of Tsac_1705 in *Clostridium thermocellum* and found improvements in both the titer and the yield of ethanol.

IMPORTANCE

Redox balance plays a crucial role in many metabolic engineering strategies. Ferredoxins are widely used as electron carriers for anaerobic microorganism and plants. This study identified the gene responsible for electron transfer from ferredoxin to NAD⁺, a key reaction in the ethanol production pathway of this organism and many other metabolic pathways. Identification of this gene is an important step in transferring the ethanol production ability of this organism to other organisms.

Ferredoxins are iron-sulfur proteins found in many anaerobic bacteria and archaea and mediate electron transfer in various metabolic processes, including photosynthesis (1, 2), alcohol production (3, 4), nitrogen fixation (5, 6), and hydrogen production (7). Lack of knowledge about these ferredoxin-dependent pathways currently limits our ability to incorporate them into metabolic engineering strategies. The ferredoxin:NAD⁺/NADP⁺ oxidoreductase (FNOR) enzyme (EC 1.18.1.2/EC 1.18.1.3) forms a key bridge in metabolism between the nicotinamide cofactor (i.e., NAD⁺, NADH, NADP⁺, and NADPH)-dependent pathways and ferredoxin-dependent pathways (Fig. 1, equation a) (8–11). Recently, the thioredoxin reductase-like (TrxR-type) FNORs were found, and they are widely distributed among the bacteria and archaea (12–14). Even these types of FNORs are more homologous to bacterial NADPH-TrxRs, but they have catalytic properties similar to those of FNOR (14). FNOR enzymes are widely believed to be of central importance for the bioenergetics of anaerobic bacteria due to their ability to couple electron transport with ion or Na⁺ gradient generation (15) or transhydrogenation. Furthermore, they are the key enzymes for many biochemical and biofuel pathways, including isopropanol, ethanol, and *n*-butanol (3, 4, 16). Since ferredoxin has a lower standard reduction potential than nicotinamide cofactors (−420 mV versus −320 mV) (10), this exergonic reaction is frequently coupled to another endergonic reaction for energy conservation. One coupling reaction is the translocation of proton or sodium ions, resulting in the RNF reaction (Fig. 1, equation b) (8, 17, 18). Another coupling reaction is the transhydrogenation reaction, resulting in the NADH-dependent reduced FNOR reaction (Fig. 1, equation c) (10, 19, 20).

Thermoanaerobacterium saccharolyticum is a thermophilic bac-

terium with the ability to ferment many components of the hemicellulose fraction of lignocellulosic biomass, including xylan, and can produce ethanol at high yields and titers. Engineered strains of *T. saccharolyticum* that can produce over 70 g/liter ethanol at near-theoretical yield have been developed (21), and this has inspired us to study the pathway this organism uses to produce ethanol. A key step in the pathway is the transfer of electrons from ferredoxin to NAD⁺ and/or NADP⁺, which *T. saccharolyticum* performs readily (19). One candidate for this activity is the Nfn complex (Tsac_2085 NfnA and Tsac_2086 NfnB). The deletion of these two genes led to the total loss of NADPH-FNOR activity (10, 19); however, NADH-FNOR activity remained and was sufficient for high-yield ethanol production (83% of the theoretical yield) under certain conditions. This suggested the possibility of an ethanol production pathway that used NADH (i.e., not NADPH) for all redox steps; however, the gene responsible for the NADH-FNOR activity was not known (19).

Since *T. saccharolyticum* is not able to use the cellulose fraction of biomass, we have been working to engineer *Clostridium thermocellum*, an anaerobic thermophilic bacterium that can solubi-

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FIG 1 The stoichiometries of three types of FNOR reactions. a, uncoupled FNOR reaction; b, proton- or Na^+ -translocating FNOR reaction (RNF); c, NADH-dependent FNOR reaction (NFN).

lize the cellulosic fraction of biomass, for improved ethanol production. For *C. thermocellum*, the main factor limiting commercialization is the low titer and yield of ethanol. We think this is caused by a limitation in electron transfer from ferredoxin to NAD^+ (i.e., NADH-FNOR activity).

In this work, we have identified the NADH-FNOR in *T. saccharolyticum* and confirmed its function by heterologous expression in *Escherichia coli*. We then determined its role in *T. saccharolyticum* metabolism by gene deletion. Finally, we demonstrated the utility of this enzyme for metabolic engineering by expressing it in *C. thermocellum*, which improved both ethanol yield and titer.

MATERIALS AND METHODS

Media and growth conditions. All chemicals were reagent grade and obtained from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) unless indicated otherwise. CTFUD rich medium at pH 7.0 and pH 6.0 was used for *C. thermocellum* and *T. saccharolyticum*, respectively (22, 23). The growth temperature was 55°C for both strains. For end product analysis, *C. thermocellum* was grown in chemically defined MTC medium (24), and *T. saccharolyticum* was grown in modified MTC medium (25). *Escherichia coli* strains were grown in LB medium Miller (Acros) with the appropriate antibiotic for maintenance (carbenicillin at 100 mg/liter, kanamycin at 50 mg/liter, and tetracycline at 12 mg/liter) and in TB medium for protein expression.

Strains, primers, and plasmids. The strains, primers, and plasmids used in this study are listed in Table 1, Table 2, and Table 3, respectively.

Markerless gene deletion in *T. saccharolyticum*. To delete the target genes in *T. saccharolyticum*, the markerless gene deletion system reported

for *Thermoanaerobacter ethanolicus* was used (26). The thymidine kinase (*tdk*) gene was deleted in *T. saccharolyticum* to create a background strain LL1305. The high-temperature kanamycin (*htk*) marker was used for positive selection. 5-Fluoro-2'-deoxyuridine (FUDR) was used in the subsequent negative selection step to remove the *htk* marker. Transformation of *T. saccharolyticum* was performed as described previously (27). The primers and plasmids used for genes deletions are listed in Tables 2 and 3, respectively.

Preparation of cell extracts. For *C. thermocellum* and *T. saccharolyticum*, CTFUD rich medium was used (23), and cells were harvested by centrifugation when the optical density at 600 nm (OD_{600}) reached a value of 0.6. The cell pellet was resuspended in lysis buffer (1× BugBuster reagent [EMD Millipore, Darmstadt, Germany] with 0.2 mM dithiothreitol [DTT]). The cells were lysed with Ready-Lyse lysozyme (Epicentre, Madison, WI, USA), and DNase I (New England Biolabs, Ipswich, MA, USA) was added to reduce the viscosity. After incubation for 30 min at room temperature, the resulting solution was centrifuged at $10,000 \times g$ for 5 min. The supernatant was used as cell extract for enzyme assays.

Heterologous expression protein in *E. coli*. Target genes were amplified by PCR with Q5 DNA polymerase (New England Biolabs, Ipswich, MA USA). *T. saccharolyticum* or *C. thermocellum* genomic DNA was used as the template. The primers used for each gene are listed in Table 2. The target genes were inserted into plasmid pD861-NH (DNA2.0 Inc., Menlo Park, CA, USA) and tagged with a 5' His₆ cassette. The vector was transformed into *E. coli* BL21(DE3) harboring the pRKISC plasmid (28). This pRKISC plasmid contained the *E. coli* *isc* (iron-sulfur cluster) locus (28), which has previously been shown to improve the expression of iron-sulfur proteins (29, 30).

Cells were aerobically grown in TB medium at 37°C with a stirring speed of 225 rpm. When the OD_{600} reached 0.6, 4 mM rhamnose and 0.2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) were added to induce the expression of the target gene and *isc* operon, respectively. Cysteine (0.12 g/liter), ferrous sulfate (0.1 g/liter), ferric citrate (0.1 g/liter), and ferric ammonium citrate (0.1 g/liter) were supplemented to enhance the iron-sulfur cluster synthesis. The cells were then grown aerobically for 4 h before harvesting by centrifugation. The cell pellets were washed with 50 mM Tris-HCl–0.5 mM DTT (pH 7.5) and stored at –80°C.

The cell extracts were prepared as described above, and *E. coli* proteins were denatured by incubating at 55°C for 30 min. The denatured proteins were removed by centrifugation at $10,000 \times g$ for 5 min. All steps of purification were performed at room temperature in a Coy anaerobic chamber (Coy Labs, Grass Lake, MI) with an atmosphere of 85% N₂, 10% CO₂, and 5% H₂. His tag affinity spin columns (His SpinTrap; GE Health-

TABLE 1 Strains used in this study

Organism	Strain	Description	Source or reference
<i>T. saccharolyticum</i>	LL1025	Wild-type <i>T. saccharolyticum</i> JW/SL-YS485	40
	LL1025 Δ <i>xynA</i> ::0705 Ery ^r		This work
	LL1025-1705	LL1025 Δ <i>xynA</i> ::1705 Ery ^r	This work
	LL1049	LL1025 Δ <i>pta</i> Δ <i>ack</i> Δ <i>ldh</i> <i>adhE</i> ^{G544D}	41
	LL1144	LL1025 Δ <i>nfnAB</i> ::Kan ^r	19
	LL1145	LL1025 Δ <i>pta</i> Δ <i>ack</i> Δ <i>ldh</i> Δ <i>pyrF</i> Δ <i>nfnAB</i> ::Kan ^r	19
	LL1305	LL1025 Δ <i>tdk</i>	This work
	LL1306	LL1025 Δ <i>tdk</i> Δ1705	This work
	LL1316	LL1025 Δ <i>tdk</i> Δ1705 Δ <i>nfnAB</i> ::Kan ^r	This work
	LL1317	LL1025 Δ <i>tdk</i> Δ <i>nfnAB</i> ::Kan ^r	This work
<i>C. thermocellum</i>	LL1004	Wild-type <i>C. thermocellum</i> strain DSM 1313	DSMZ
	LL1087	LL1004 Δ <i>hpt</i> Δ <i>rnf</i>	This work
	LL1087-1705	LL1004 Δ <i>hpt</i> Δ <i>rnf</i> (pDGO126-1705)	This work
<i>E. coli</i>	T7 Express lysY/lq	Used for heterologous protein expression	New England Biolabs
	DH5α	Used for plasmid screening and propagation	New England Biolabs

TABLE 2 Primers used in this study

Primer	Sequence ^a	Comment
LT_01	CTTTTCCTCCCTCGTCTTC	T.sac_tdk deletion
LT_02	ACTTTTGTGGTTTAACTATTTCTAAGAGGTGGATTATGGCGGATTTT <u>TAAGGAGGT</u>	
LT_03	TCTTCTTCATTGCTGCACCTCCTTAAAAATCCGCCATAATCCACCTCTT <u>AGAAAATAGTT</u>	
LT_04	GGAATACGCAAAAAGATTG	
LT_15	TACACGTACTTAGTCGCTGAAGCTCTTCTATGAGATACGTTGTTAGAGAA <u>AATAGAG</u>	Amplification of <i>tsac_1705</i> for plasmid pD861-tsac1705
LT_16	TAGGTACGAACCTGATTGACGGCTCTTCTACCTCAAAATACTACCTCCCTTGACC	
LT_23	CCACCACAATTACAGCAA	pD861-tsac1705 sequencing
LT_24	AAAAAACCCCTCAAGACCC	
LT_28	CGATCTCGAGAGATACGTTGTTAGAGAAAAATAGAG	Amplification of <i>tsac_1705</i> for plasmid pDGO126'ins'_p2638-1705
LT_29	GTCACCTCGAGCCGGGTTGAACCTACTCTTTAATA	
LT_36	GCAGGCATGCAAGCTTTAATAG	Amplification of vector backbone for plasmid pDGO126'ins'_p2638-1705
LT_37	AGGTCGACTCTAGAGGATCC	
LT_44	TGATGACGAAAAAGCCGA	pDGO126'ins'_p2638-1705 sequencing
LT_45	ATCCCAATAACCTAACTCTCC	
LT_153	ACGGGAACAATACAAAAGGA	<i>tsac_1705</i> deletion verification
LT_154	AATTCCTCCCATCCCTATC	
LT_155	TGTGCTGTTGCATGTTGT	<i>tsac_nfnAB</i> deletion
LT_156	GGTGGAGTAATAATTGGTGGT	
0705 F	ATAAATGTGTACATGCCAAAAAAGTAGAAATATTG	Amplification of <i>tsac_0705</i> for plasmid pTOPO-0705
0705 R	CGACCTGCATTAATCGAGAAGTTGCTTTGATTTTGTG	
Histag 0705 F	CTGGTTCTCATCATCATCATCATCATGGTATAAATGTGTACATGC <u>CAAAAAAGTAGAAATATTG</u>	
1705 F	AGATACGTTGTTAGAGAAAAATAGAGAAATTAGCAATGG	Amplification of <i>tsac_1705</i> for plasmid pTOPO-0705
1705 R	CGACCTGCATCAAAATACTACCTCCCTTGACCAAAATACAGG	
Histag 1705 F	CTGGTTCTCATCATCATCATCATGGTAGATACGTTGTTAGAGAAAAATA <u>GAGAAATTAGCAATGG</u>	
pkan 1705 F	GGTCAAGGGAGGTAGTATTTTGTATGCAGGTCGATAAACCCAGCG	
xynA up F	ATCTTTTCTGGCCTTTAATGGCGC	Amplification of <i>xynA</i> operon and Erm resistance gene for plasmid pTOPO-0705
xynA up R	TGATGATGATGATGATGAGAACCAGACATCTTACTTCTCCCTCAGTAA <u>ATTTAATTTATTG</u>	
pkan 0705 F	CAAAGCAACTTCTCGATTAATGCAGGTCGATAAACCCAGCG	
xynA down R	AGTCAAATGCGACAAAAAACGCC	
xynA up R-2	TCTTACTTCTCCCTCAGTAAATTTAATTTATTG	
pkan F-2	TGCAGGTCGATAAACCCAGCG	
xynA SQ F	GAAATAATTCTAATTCAGTTACCCCG	<i>tsac_0705</i> or <i>tsac_1705 xynA</i> replacement verification
xynA SQ R	GGTGAATTCGAATTTACAGGC	

^a Underlined sequences indicate binding regions.

care Bio-Sciences, Pittsburgh, PA, USA) were used to purify the protein. The column was first equilibrated with binding buffer (50 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.5). Cell extracts were applied to the column, and then the column was washed twice with wash buffer (50 mM sodium phosphate, 500 mM NaCl, 50 mM imidazole, 20% ethanol, pH 7.5). The His-tagged protein was eluted with elution buffer (50 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.5).

Enzyme assay. Except where indicated, all the enzyme assays were performed at 55°C in an anaerobic chamber (85% N₂, 10% CO₂, 5% H₂). When carbon monoxide (CO) was used as a substrate, the cuvettes (still

inside the anaerobic chamber) were closed with a rubber stopper and purged with CO gas.

Assay of carbon monoxide dehydrogenase. The plasmid for overexpression of *Carboxydotherrmus hydrogenoformans* carbon monoxide dehydrogenase (CODH) in *E. coli* was a gift from Holger Dobbek. Expression and purification of this enzyme were performed as previously described (31). To measure CODH activity, a cuvette was filled with buffer containing 20 mM morpholinepropanesulfonic acid (MOPS)-NaOH (pH 7.5), 2 mM DTT, and 2 mM benzyl viologen (BV). The reaction was started by purging with 60% (vol/vol) carbon monoxide (CO balanced with 40%

TABLE 3 Plasmids used in this study

Plasmid	Description	Accession no.
pTOPO-0705	Insertion of <i>tsac_0705</i> at the <i>xynA</i> locus of <i>T. saccharolyticum</i>	KX272607
pTOPO-1705	Insertion of <i>tsac_0705</i> at the <i>xynA</i> locus of <i>T. saccharolyticum</i>	KX272606
pD861-tsac1705	Overexpression of <i>tsac_1705</i> gene in <i>E. coli</i>	KX272605
pLT-26	Markerless deletion of <i>tsac_1705</i> gene in <i>T. saccharolyticum</i>	KX272604
pDGO126'ins'_p2638-1705	Overexpression of <i>tsac_1705</i> gene in <i>C. thermocellum</i>	KX272603
pLT-18	Overexpression of Clo1313_2761 gene in <i>E. coli</i>	KX925513

N₂). The activity was measured at 578 nm ($\epsilon = 7.8 \text{ mM}^{-1} \text{ cm}^{-1}$) (32). Reduction of ferredoxin (20 μM) was assayed at 430 nm ($\epsilon = 13.1 \text{ mM}^{-1} \text{ cm}^{-1}$) (10).

Assay of BV-based FNOR (NADH:BV FNOR). The reaction mixture contained 20 mM MOPS-NaOH (pH 7.5), 0.2 mM NADH or NADPH, and 1 mM benzyl viologen (BV). The reaction was started with enzyme, and the benzyl viologen reduction was followed by photometric observations at 578 nm ($\epsilon = 7.8 \text{ mM}^{-1} \text{ cm}^{-1}$) (32).

Assay of ferredoxin-based FNOR (Fd:NAD⁺ FNOR). 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. Ferredoxin from *C. thermocellum* (Clo1313_2761) was expressed in *E. coli* by using the pLT-18 plasmid. The expression plasmid was transformed into *E. coli* strain BL21 harboring the pRKISC plasmid (for correct assembly of iron-sulfur clusters in ferredoxin). The proteins were purified using His tag affinity columns in an anaerobic chamber.

The reaction mixture contained 20 mM MOPS-NaOH (pH 7.5), 4 mM NAD⁺, 3 μM ferredoxin, and 0.5 μM CODH. The cuvette was purged with CO for 30 s to saturate the liquid with CO. The reaction was then started with the FNOR enzyme (purified from *E. coli*), and the formation of NADH was followed by photometric observation at 340 nm ($\epsilon = 6.2 \text{ mM}^{-1} \text{ cm}^{-1}$) (11).

Analytical methods. Acetate, formate, ethanol, glucose, and residual cellobiose were determined by high-pressure liquid chromatography (HPLC) (Waters, Milford, MA) with refractive index detection using an Aminex HPX-87H column (Bio-Rad, Hercules CA) with a 5 mM sulfuric acid solution eluent. The column was incubated at 55°C, and the mobile-phase flow rate was 0.6 ml/min. H₂ was determined by measuring the total pressure and the H₂ percentage in the headspace. For a 100-ml serum bottle, the culture volume is 50 ml, so the headspace is also 50 ml. The headspace gas pressure in bottles was measured using a digital pressure gauge (Ashcroft, Stratford, CT). The headspace H₂ percentage was measured using a gas chromatograph (model 310; SRI Instruments, Torrance, CA) with a HayeSep D packed column using a thermal conductivity detector with nitrogen carrier gas.

Accession number(s). Newly determined sequences were deposited in GenBank and are listed in Table 3.

RESULTS AND DISCUSSION

Identification of ferredoxin:NAD⁺ oxidoreductase candidates from *T. saccharolyticum*. To identify candidate genes with FNOR activity, we searched for the presence of both iron-sulfur (Fe-S) binding domains and NAD cofactor binding domains in the *T. saccharolyticum* genome using the Pfam online database (33). The two best candidates were *tsac_0705* and *tsac_1705*, which are the only candidates (besides *nfnA*) that have both the oxidoreductase NAD binding domain and the iron-sulfur cluster binding domain (Fig. 2).

These two candidates were overexpressed in *T. saccharolyticum* by insertion of an additional copy at the *xynA* locus. This locus had previously been used for xylose-inducible expression of genes (34). Xylose was added at 5 g/liter as a carbon source and to induce expression of target genes, and the benzyl viologen-based NADH-FNOR (NADH:BV FNOR) was used to measure activity of the cell extract. A 5-fold increase in activity was found when the *tsac_1705* gene was overexpressed (Table 4, strains LL1025, LL1352, and LL1353), suggesting that Tsac_1705 is responsible for NADH-FNOR activity.

To further determine whether the *tsac_1705* gene encodes the enzyme catalyzing NAD⁺-linked reduction of ferredoxin, we expressed it in *E. coli*, purified the resulting protein, and measured FNOR activity. We found that the specific enzyme activities were identical for aerobic and anaerobic cultures; Tsac_1705 is strictly NADH linked, and no activity was found with NADPH. Based on the assay of NADH:BV FNOR, the K_m for benzyl viologen was $0.07 \pm 0.01 \text{ mM}$ and the $V_{\max}$ was $23 \pm 0.3 \text{ U/mg}$. Since the benzyl viologen assay is somewhat promiscuous, we also used a ferredoxin-based assay (Fd:NAD⁺ FNOR). For this assay, the apparent K_m for NAD⁺ was found to be approximately 0.5 mM and the apparent $V_{\max}$ was found to be 3.28 U per mg of protein at 55°C and pH 7.5.

Predicted structure of the Tsac_1705 protein. Although the

FIG 2 Protein functional domain alignment. PF00175 is the hidden Markov model (HMM) consensus sequence of the oxidoreductase NAD binding domain. PF10418 is the HMM consensus sequence of the iron-sulfur cluster binding domain. Uppercase letters indicate conserved residues in the HMM consensus sequence.

TABLE 4 Fermentation products and enzyme assay result of *T. saccharolyticum* and *C. thermocellum*

Strain	Description	Amt of fermentation product (mmol) ^a					Ethanol/acetate ratio (mmol/mmol) ^b	Ethanol yield (%) ^c	FNOR sp act (U/mg) ^d	
		Lactate	Formate	Ethanol	Acetate	H ₂			NADH	NADPH
LL1025	Wild-type <i>T. saccharolyticum</i>	0.16 ± 0.02	0.02 ± 0.00	1.26 ± 0.02	0.76 ± 0.01	1.72 ± 0.05	1.66 ± 0.04	23	0.56 ± 0.13	0.29 ± 0.12
LL1049	JW/SL-YS485							42 (19)	0.18 ± 0.03	0.74 ± 0.15
LL1145	LL1025 Δ pta Δ ack Δ ldh adhE ^{S44D}							42 (19)	0.48 ± 0.04	0.04 ± 0.01
	LL1025 Δ pta Δ ack Δ ldh Δ pyrF Δ nfnAB::Kan ^r									
LL1352	LL1025 Δ xynA::0705 Ery ^r								0.45 ± 0.18	0.27 ± 0.10
LL1353	LL1025 Δ xynA::1705 Ery ^r								2.51 ± 0.44	0.15 ± 0.07
LL1305	LL1025 Δ tdk	0.17 ± 0.02	0.02 ± 0.01	1.28 ± 0.01	0.77 ± 0.01	1.7 ± 0.1	1.67 ± 0.02	23	0.57 ± 0.08	0.32 ± 0.10
LL1306	LL1305 Δ 1705	0.23 ± 0.01	0.02 ± 0.02	1.30 ± 0.02	0.88 ± 0.01	1.9 ± 0.2	1.57 ± 0.03	24	0.18 ± 0.07	0.35 ± 0.11
LL1317	LL1305 Δ nfnAB::Kan ^r	0.22 ± 0.03	0.02 ± 0.00	1.31 ± 0.01	0.91 ± 0.01	2.4 ± 0.2	1.44 ± 0.02	24	0.48 ± 0.10	0.03 ± 0.01
LL1316	LL1305 Δ 1705 Δ nfnAB::Kan ^r	0.26 ± 0.02	0.02 ± 0.01	1.06 ± 0.02	0.99 ± 0.2	2.7 ± 0.1	1.07 ± 0.03	19	0.15 ± 0.04	0.04 ± 0.01
LL1004	Wild-type <i>C. thermocellum</i> strain DSM 1313							16 (22)	0.21 ± 0.04	ND ^e
LL1087	LL1004 Δ hpt Δ nrf	0.72 ± 0.04	1.23 ± 0.03	1.42 ± 0.02	2.11 ± 0.03		0.67 ± 0.02	14	0.27 ± 0.04	ND
LL1338	LL1087 (pDGO126-1705)	0.47 ± 0.02	1.14 ± 0.03	1.83 ± 0.03	1.82 ± 0.02		1.00 ± 0.02	18	0.34 ± 0.06	ND

^a For quantification of all the fermentation products, the working volume was 50 ml on 0.72 mmol cellobiose for *T. saccharolyticum* and 14.4 mmol for *C. thermocellum*, and the headspace volume are 50 ml for a 100-ml serum bottle. Values are means ± standard deviations; n = 3.

^b Values are means ± standard deviations; n = 3.

^c Ethanol yield is in grams per gram of glucose produced from cellobiose. Values are means (reference numbers are given in parentheses); n = 3.

^d Specific activity determined from cell extracts. FNOR activity was determined using the NADH:BV assay. Values are means ± standard deviations; n = 3.

^e ND, not detected.

FIG 3 Homology modeling and docking analysis of Tsac_1705. (A) Substrate binding domain of Tsac_1705 (blue). *T. maritima* NfnA (green) was used to superimpose the NADH, FAD, and [2Fe-2S] cluster. (B) The [2Fe-2S] cluster in *T. maritima* NfnA. (C) The predicted [2Fe-2S] cluster in Tsac_1705.

Tsac_1705 protein was annotated as a dihydroorotate dehydrogenase (DodH) electron transfer subunit and is part of a putative operon for dihydroorotate dehydrogenase, it is similar (32% amino acid sequence identity) to the NfnA protein, one of the subunits of the NfnAB complex from *Thermotoga maritima* with known NFN activity (20). The most similar protein with an available crystal structure is DodH from *Lactococcus lactis*, with a sequence identity of 39% (35). We constructed a protein homology model of Tsac_1705 using the crystal structure of *L. lactis* DodH (Protein Data Bank code 1EP2; root mean square deviation [RMSD] = 9.515; 98 to 98 atoms). The model was aligned to the NfnA of *T. maritima* (Protein Data Bank code 4YRY) to superimpose the NADH, FAD, and [2Fe-2S] cluster from 4YRY to our homology model (Fig. 3A). Tsac_1705 has similar NADH, FAD, and [2Fe-2S] binding domains. Specially, the four residues which bind to the [2Fe-2S] cluster are conserved with NfnA from *T. maritima* (Fig. 3B and C) (35). Interestingly, although we did not find activity with this protein, Tsac_0705 is also very similar to NfnA from *T. maritima*, with a sequence identity at the amino acid level of 28%.

Role of Tsac_1705 in the NADH-linked ethanol production pathway. To further confirm the role of *tsac_1705*, it was deleted individually and in combination with *nfnAB* (Table 4, strains LL1306, LL1317, and LL1316). Note that for the *nfnAB* and *tsac_1705* deletion strains, an additional deletion of *tdk* was introduced for purposes of strain construction; however, the *tdk* dele-

FIG 4 Cofactor-based models for stoichiometric ethanol production in *T. saccharolyticum*. NADH-based ethanol production relies on a ferredoxin:NAD oxidoreductase (NADH-FNOR) to transfer electrons from reduced ferredoxin to NAD^+ . NADPH-based ethanol formation relies on the electron transfer from NADH and reduced ferredoxin to 2NADP^+ . NADH- or NADPH-linked aldehyde dehydrogenase (ALDH) and alcohol dehydrogenase (ADH) were used for different pathway. Blue arrows indicate that cofactor is reduced, while red arrows indicate that cofactor is oxidized. PFOR, pyruvate:ferredoxin oxidoreductase; FNOR, ferredoxin:NADH oxidoreductase; Fd_{ox} , oxidized ferredoxin; Fd_{red} , reduced ferredoxin.

tion did not have an effect on fermentation products or FNOR activity (Table 4, strain LL1305). In comparison with that of the parent strain (Table 4, strain LL1305), nearly 70% of NADH-FNOR activity was lost when *tsac_1705* was deleted (Table 4, strain LL1306), which indicated that *Tsac_1705* is the main NADH-FNOR in *T. saccharolyticum*. A small amount of NADH-FNOR activity (~30%) remained even after deletion of *tsac_1705*. Although we have identified the primary gene responsible for NADH-FNOR activity, there may still be other cryptic FNOR enzymes in *T. saccharolyticum*. Another possibility is the presence of a set of enzymes whose net activity is equivalent to FNOR activity.

As described in a previous study (19), there are two ethanol production pathways in *T. saccharolyticum*, one NADH based and one NADPH based (Fig. 4). Strains with the NADH-based (LL1145) or NADPH-based (LL1049) ethanol pathway can produce ethanol at high yield (Table 4). According to the NADH:BV FNOR assay result, these two strains have different cofactor preferences. We tried to delete the *tsac_1705* gene in strain LL1145. However, no colonies were obtained, which is consistent with our understanding of *Tsac_1705* as the primary FNOR in this strain.

Next, we analyzed the effect of the *tsac_1705* gene deletion on the distribution of fermentation products, particularly ethanol

and acetate (Table 4, strains LL1025, LL1305, LL1306, LL1317, and LL1316). Assuming that glucose is converted to pyruvate by glycolysis and pyruvate is converted to acetyl coenzyme A (acetyl-CoA) by pyruvate ferredoxin oxidoreductase, central metabolism can be described by equation 1 below. Note that only trivial amounts of formate (less than 1 mM) were produced for all 5 strains, so flux from pyruvate formate lyase was omitted from the equations. Bifurcating hydrogenase activity was not considered in the equations either, because in *T. saccharolyticum*, the ferredoxin-dependent [FeFe]-hydrogenase is responsible for hydrogen generation and is not thought to be bifurcating (36). The resulting reduced ferredoxin (Fd_{red}) is either used to produce H_2 or NAD(P)H (equations 2 and 3).

When FNOR converts 100% Fd_{red} to NAD(P)H :

When FNOR is eliminated:

In wild-type strain LL1025, the ethanol-to-acetate ratio was about 1.66, which means FNOR converts only about 25% of Fd_{red} to NAD(P)H (Table 4 and equation 2). The single deletion of either *tsac_1705* or *nfnAB* only slightly influenced this ratio, which means that these two FNOR enzymes can complement the deletion of each other (Table 4, strains LL1306 and LL1317). Meanwhile, the H_2 production was increased in both of these strains. In strain LL1316, which had both the *nfnAB* and *tsac_1705* genes deleted, this ratio decreased to 1.07, which is close to 1, the value we would expect for a strain that does not have FNOR activity (i.e., its metabolism is described by equation 3). The H_2 production in this strain was further increased in comparison with that caused by the single deletion of either *tsac_1705* or *nfnAB*. This provides further confirmation that *tsac_1705* is the gene responsible for NADH-FNOR activity in *T. saccharolyticum*.

Heterologous overexpression of *tsac_1705* in *C. thermocellum*. The best engineered strains of *T. saccharolyticum* can produce up to 70 g/liter ethanol (21); however, the highest ethanol titer of *C. thermocellum* is still less than 30 g/liter (16). FNOR activity is necessary for high-yield production of ethanol (37). The NADH-FNOR activity was found to be 2-fold higher in wild-type

TABLE 5 Transformation results for plasmid pDGO126'ins'_p2638-1705

Strain	Description	Transformants
LL1004	Wild-type <i>C. thermocellum</i> strain DSM 1313	No
LL345	LL1004 Δhpt	No
LL350	LL1004 $\Delta hpt \Delta hydG$	No
LL1147	LL1004 $\Delta hpt \Delta hydG \Delta ech$	No
LL1210	LL1004 $\Delta hpt \Delta hydG \Delta pfl \Delta pta \Delta ldh$	No
LL1087	LL1004 $\Delta hpt \Delta rnfDG$	Yes
LL1083	LL1004 $\Delta hpt \Delta hydG \Delta rnfDG$	Yes
LL1152	LL1004 $\Delta hpt \Delta rnfABCDEG$	Yes

FIG 5 The cycling reaction between RNF and FNOR.

T. saccharolyticum than in wild-type *C. thermocellum* (Table 4, strains LL1025 and LL1004). Furthermore, wild-type *T. saccharolyticum* has NADPH-FNOR activity, which was not found in wild-type *C. thermocellum* (which has only NADH-FNOR activity). Therefore, it is possible that NADH-FNOR activity is currently the limiting step for ethanol production. Since the alcohol dehydrogenase and acetaldehyde dehydrogenase reactions in *C. thermocellum* are NADH linked (Fig. 4, NADH based) (22), the NADH-FNOR is more suitable for cofactor balance in *C. thermocellum* (as opposed to the NADPH-linked NfnAB complex). To test this hypothesis, the *tsac_1705* gene was inserted into plasmid pDGO126 (38), and the resulting plasmid was transformed into *C. thermocellum*. Although transformation was attempted in several strains (Table 5), we obtained colonies only in strains where the native proton-translocating FNOR operon (i.e., *rnf*) was deleted. One possible explanation is that Rnf and Tsac₁₇₀₅ create a futile cycle. The equation shown in Fig. 5 describes this reaction, which could occur when the Rnf complex and Tsac₁₇₀₅ are both present. This cycle would result in a net transfer of protons across the cell membrane, eliminating the proton gradient, which would likely be lethal.

In the *rnf* deletion strain, overexpression of *tsac_1705* gene increased the titer of ethanol by 28% (Table 4, strains LL1087 and LL1338). The production of lactate and acetate both decreased, although formate production was unchanged. If we ignore lactate and formate production, *C. thermocellum* metabolism can be described as follows: glucose $\rightarrow$ 2 acetyl-CoA + 2 CO₂ + 2 NADH + 2 Fd_{red} (identical to the case for *T. saccharolyticum* [see equation 1]). When the bifurcating hydrogenase converts 100% Fd_{red} to H₂, the process is as follows: glucose $\rightarrow$ 2 acetyl-CoA + 2 CO₂ + 2 NADH + 2 Fd_{red} $\rightarrow$ 2 acetate + 2 CO₂ + 4 H₂. When the bifurcating hydrogenase and FNOR contribute equally to ferredoxin oxidation, the process is as follows: glucose $\rightarrow$ 2 acetyl-CoA + 2 CO₂ + 2 NADH + 2 Fd_{red} $\rightarrow$ ethanol + acetate + 2 CO₂ + 2 H₂.

Thus, in *T. saccharolyticum*, a 1:1 ethanol-to-acetate ratio was indicative of a lack of FNOR activity, and in *C. thermocellum*, a 1:1 ethanol-to-acetate ratio can exist even in the presence of substantial flux through FNOR. This analysis is further complicated by the fact that *C. thermocellum* has both bifurcating and nonbifurcating hydrogenases (39). Regardless of the type of hydrogenase, an increase in ethanol production at the expense of acetate production generally indicates an increase in FNOR flux. It is, of course, true that a reduction in bifurcating hydrogenase activity and a corresponding increase in nonbifurcating hydrogenase activity would have the same effect on the ethanol/acetate ratio; however, since we observe increased FNOR activity that corresponded to the introduction of the *tsac_1705* gene, we believe that increased ethanol production caused by FNOR activity from Tsac₁₇₀₅ is the sim-

plest explanation. The ability to use the *tsac_1705* gene to improve ethanol production in *C. thermocellum* demonstrates its utility for metabolic engineering.

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