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The Bifunctional Alcohol and Aldehyde Dehydrogenase Gene, *adhE*, Is Necessary for Ethanol Production in *Clostridium thermocellum* and *Thermoanaerobacterium saccharolyticum*

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ABSTRACT

***Thermoanaerobacterium saccharolyticum* and *Clostridium thermocellum* are anaerobic thermophilic bacteria being investigated for their ability to produce biofuels from plant biomass. The bifunctional alcohol and aldehyde dehydrogenase gene, *adhE*, is present in these bacteria and has been known to be important for ethanol formation in other anaerobic alcohol producers. This study explores the inactivation of the *adhE* gene in *C. thermocellum* and *T. saccharolyticum*. Deletion of *adhE* reduced ethanol production by >95% in both *T. saccharolyticum* and *C. thermocellum*, confirming that *adhE* is necessary for ethanol formation in both organisms. In both *adhE* deletion strains, fermentation products shifted from ethanol to lactate production and resulted in lower cell density and longer time to reach maximal cell density. In *T. saccharolyticum*, the *adhE* deletion strain lost >85% of alcohol dehydrogenase (ADH) activity. Aldehyde dehydrogenase (ALDH) activity did not appear to be affected, although ALDH activity was low in cell extracts. Adding ubiquinone-0 to the ALDH assay increased activity in the *T. saccharolyticum* parent strain but did not increase activity in the *adhE* deletion strain, suggesting that ALDH activity was inhibited. In *C. thermocellum*, the *adhE* deletion strain lost >90% of ALDH and ADH activity in cell extracts. The *C. thermocellum adhE* deletion strain contained a point mutation in the lactate dehydrogenase gene, which appears to deregulate its activation by fructose 1,6-bisphosphate, leading to constitutive activation of lactate dehydrogenase.**

IMPORTANCE

***Thermoanaerobacterium saccharolyticum* and *Clostridium thermocellum* are bacteria that have been investigated for their ability to produce biofuels from plant biomass. They have been engineered to produce higher yields of ethanol, yet questions remain about the enzymes responsible for ethanol formation in these bacteria. The genomes of these bacteria encode multiple predicted aldehyde and alcohol dehydrogenases which could be responsible for alcohol formation. This study explores the inactivation of *adhE*, a gene encoding a bifunctional alcohol and aldehyde dehydrogenase. Deletion of *adhE* reduced ethanol production by >95% in both *T. saccharolyticum* and *C. thermocellum*, confirming that *adhE* is necessary for ethanol formation in both organisms. In strains without *adhE*, we note changes in biochemical activity, product formation, and growth.**

Anaerobic bacteria are being investigated for their ability to produce biofuels from biomass. In particular, *Thermoanaerobacterium saccharolyticum* and *Clostridium thermocellum* are of interest because of their ability to break down components of lignocellulosic biomass and produce alcohols (1). These thermophilic anaerobes normally produce a mixture of organic acids and ethanol. They have been engineered for increased ethanol yield (2–4) and can produce higher alcohols, such as *n*-butanol (5), butanediol, and butanol (6), but gaps remain in understanding their metabolism. In particular, there is uncertainty in the genes responsible for ethanol production. The *T. saccharolyticum* and *C. thermocellum* genomes contain multiple annotated genes for aldehyde dehydrogenase (ALDH) and alcohol dehydrogenase (ADH) activity which could be responsible for alcohol formation, including *adhE*. AdhE is a bifunctional enzyme composed of an aldehyde dehydrogenase at the N-terminal domain and an iron-dependent alcohol dehydrogenase at the C-terminal domain, connected by a small linker sequence between the domains. Thus, AdhE can catalyze the two terminal steps in ethanol formation: the reduction of acetyl coenzyme A (acetyl-CoA) to acetaldehyde (i.e., ALDH) and reduction of acetaldehyde to ethanol (i.e., ADH), with two reduced nicotinamide cofactors (i.e., NADH and NADPH) as electron donors.

The *adhE* gene is thought to be important for alcohol formation, and its function has been studied in a number of other organisms. The *adhE* gene *Escherichia coli* (*Ec_adhE*) is essential for anaerobic growth (7). In *Clostridium acetobutylicum*, two different *adhE* genes are expressed during ethanologenesis (*Ca_adhE*) and solventogenesis (*Ca_adhE2*) (8). Deletion of *adhE* in *Thermoanaerobacter mathranii* (*Tm_adhE*) resulted in the loss of ethanol formation and an increase of lactate and acetate formation. Bio-

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TABLE 1 Strains and primers used in this study

Strain or primer	Description or sequence	Reference, source, or purpose
Strains		
T. saccharolyticum		
JW/SL-YS485	Wild type	Gift from J. Wiegel
LL1076	JW/SL-YS485 Δ*adhE*::*pta ack kanR*	This work
C. thermocellum		
M1354	DSM 1313 Δ*hpt*	2
LL1111	M1354 Δ*adhE ldh*(R157L)	This work
LL1160	LL1111 *adhE*$^{+}$ *ldh*(R157L)	This work
Primers		
T.sac.adhE.ext.F	CCCTCCCCTTGTACCTTTGTGTCC	P1 primer for amplifying external region of *T. saccharolyticum adhE*
T.sac.adhE.ext.R	GAAAACTTTGGCATCGGCGGC	P1 primer for amplifying external region of *T. saccharolyticum adhE*
T.sac.seq.int.adhE.F	GAGCAAAGCTGCGCCATGAG	P2 primer for amplifying internal region of *T. saccharolyticum adhE*
T.sac.seq.int.adhE.R	CGATGAATAGCGCTTTTTTGC	P2 primer for amplifying internal region of *T. saccharolyticum adhE*
adhE.ext.F	GTGTATTGACTTTGATTGTATTAAACGG	P3 primer for amplifying external region of *C. thermocellum adhE*
adhE.ext.R	GGTGTTTACCGTATGGCAGCACGAAG	P3 primer for amplifying external region of *C. thermocellum adhE*
adhE.int.F	TTGACGATGCCCTTGATAAAGC	P4 primer for amplifying internal region of *C. thermocellum adhE*
adhE.int.R1	CCTGTTTTTTCATCAGTAATAACCGC	P4 primer for amplifying internal region of *C. thermocellum adhE*

chemical assays of the *T. mathranii Tm_adhE* deletion strain demonstrated that it was responsible primarily for ALDH activity, as ADH activity was largely unchanged in cell extracts (9). Similarly, deletion of *adhE* in *Thermoanaerobacterium thermosaccharolyticum* (*Tt_adhE*) resulted in the loss of ethanol, butanol, and acetate formation and increased lactate formation (10). Studies in *Thermoanaerobacter pseudethanolicus* 39E have suggested that AdhB is the critical enzyme responsible for ethanol formation rather than AdhE (11, 12). Another study suggests a more complicated relationship between AdhB and AdhE in this organism, where AdhB is responsible for ethanol formation in early growth and AdhE is responsible for ethanol formation later in growth as ethanol concentrations increase (13). The true role of *T. pseudethanolicus adhE* (*Tp_adhE*) in this organism, however, has not yet been confirmed by deletion. A metagenomic study of fermentative bacteria suggests that while *adhE* in the genome is indicative of the ability to form ethanol (14), it is not required, as *Caldanaerobacter subterraneus* subsp. *tengcongensis* makes ethanol without an annotated *adhE* (14, 15). Transcriptomic and proteomic studies in *T. saccharolyticum* and *C. thermocellum* have shown *adhE* to be highly expressed, although other putative *adh* genes were detected as well (16–19). AdhE also has been shown to play a role in ethanol tolerance (20).

To clarify the role of *adhE* in ethanol formation in *T. saccharolyticum* and *C. thermocellum*, we deleted *adhE* in these bacteria and characterized changes in biochemical activity, growth, and fermentation product distribution.

MATERIALS AND METHODS

Biochemical and molecular techniques. All chemicals were obtained from Fisher Scientific (Pittsburgh, PA) and Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Primers and strains can be found in Table 1. The *adhE* gene in *T. saccharolyticum* (*Ts_adhE*; Tsac_0416) was deleted by allelic replacement using standard techniques in parent strain JW/SL-YS485 (21, 22), and the resulting strain was named LL1076 (also known as strain M3223). For this deletion, the genome region from coordinates 447543 to 449414 was replaced with the resistance cassette from plasmid pMU424 (21). Genome coordinates are based on numbering from RefSeq NC_017992.1 (http://www.ncbi.nlm.nih.gov/refseq/). Transformation and deletion of *adhE* in *C. thermocellum* (*Ct_adhE*; Clo1313_1798) was accomplished with plasmid pJLO19 using previously described methods in strain M1354, a Δ*hpt* strain allowing genetic manipulations (2, 23–25). The resulting strain was named LL1111. *Ct_adhE* was reinserted in strain LL1111 using plasmid pSH016, restoring *Ct_adhE* to the wild-type locus and resulting in strain LL1160. Genetic modification was confirmed by PCR, Sanger sequencing, and whole-genome resequencing by the Department of Energy Joint Genome Institute. Genome resequencing data are available from the NCBI Sequence Read Archive (SRA; http://www.ncbi.nlm.nih.gov/Traces/sra).

Media and growth conditions. Strains were grown anaerobically at 55°C for all experiments at an initial pH of 6.3 for *T. saccharolyticum* and pH 7.4 for *C. thermocellum*. Strains grown for transformation, biochemical characterization, and growth curves were cultured in a modified DSMZ M122 medium containing 5 g/liter cellobiose (2). For analysis of fermentation products, strains were grown in serum bottles (Fisher Scientific) on 50 ml of defined MTC medium for 72 h, as previously described (26), with the following modifications for *T. saccharolyticum* strains: urea was replaced with ammonium chloride, and thiamine hydrochloride was supplemented at a final concentration of 4 mg/liter. For growth measurements, strains were grown in 200 μl of medium in a 96-well plate. Growth was measured by monitoring absorbance at 600 nm every 5 min for 72 h in a Powerwave XS plate reader as previously described (24). Data for fermentation products and growth rate are averages from biological triplicate experiments.

Analytical techniques. Fermentation products in the liquid fraction were measured using a Waters (Milford, MA) high-pressure liquid chromatograph (HPLC) with an HPX-87H column with a UV and refractive index detector as previously described (26). H_2 was measured using a model 310 SRI Instruments gas chromatograph (Torrence, CA) with a HayeSep D packed column using a thermal conductivity detector and nitrogen carrier gas. Pellet carbon and nitrogen were measured with a Shimadzu TOC-V CPH elemental analyzer with TNM-1 and ASI-V modules (Shimadzu Corp., Columbia, MD) as previously described (26). Mass spectrometry analysis was performed with an HPLC pump (u3000; Dionex, Sunnvale, CA) coupled to an LTQ XL Orbitrap (Thermo Scientific, Waltham, MA) as previously described (28).

Biochemical assays. Cells for biochemical assays were grown to an optical density at 600 nm (OD_{600}) of 0.3 and harvested by centrifugation at 3,000 × *g* for 30 min at 4°C. Harvested cells were manipulated in a Coy (Ann Arbor, MI) anaerobic chamber, placed in serum bottles, and stored anaerobically at −80°C. Cell extract was made by incubating cells in a lysis buffer with 1× BugBuster reagent (EMD Millipore, Billerica, MA), 100

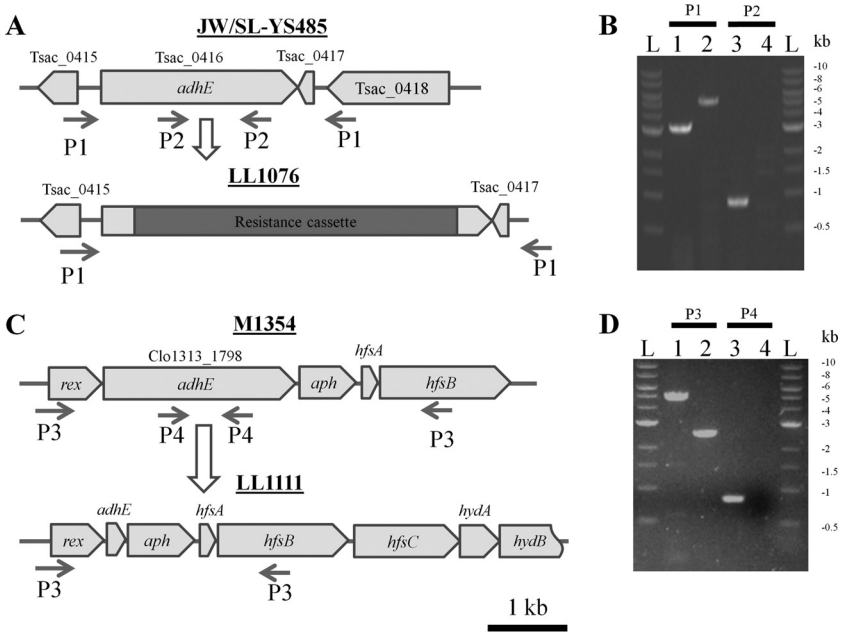

FIG 1 Deletion of *adhE* and PCR confirmation in *T. saccharolyticum* and *C. thermocellum*. Deletion of *T. saccharolyticum adhE* with P1 (external) and P2 (internal) confirmation primer pairs. (A) A resistance cassette is transformed into parent strain JW/SL-YS485, deleting *adhE* and creating strain LL1076. (B) *T. saccharolyticum* strain JW/SL-YS485 in lane 1 shows the expected 3.2-kb fragment with P1 external primers, and lane 3 shows the 900-bp internal P2 fragment of *adhE*. LL1076 in lane 2 shows a larger 5.3-kb band due to the replacement of 1.9 kb with the 4-kb disruption construct, and lane 4 shows the loss of the internal P2 band of *adhE*. (C) Deletion of *C. thermocellum adhE* with P3 external and P4 internal confirmation primer pairs. M1354 was transformed with plasmid pJLO19 and used to delete *adhE* to make strain LL1111. *C. thermocellum* strain M1354 in lane 1 shows the expected P3 external product size of 4.9 kb, and lane 3 shows the 800-bp P4 internal fragment. (D) LL1111 in lane 2 shows a 2.3-kb loss in *adhE* with a P3 product size of 2.6 kb, and lane 4 shows no P4 fragment. The DNA marker is the 1-kb ladder from New England BioLabs and is marked with an L in panels B and D. Primer sequences can be found in Table 1.

mM phosphate buffer (pH 7.0), 5 μM $FeSO_4$, 0.1 mM dithiothreitol (DTT), Ready-Lyse lysozyme (Epicentre Biotechnologies, Madison, WI), and DNase I (Thermo Scientific). Unlysed cells and debris were separated from cell extract by centrifugation for 5 min at 12,000 × *g*. Protein content was measured using Bio-Rad protein dye reagent with bovine serum albumin (Thermo Scientific) as a standard. Typical protein concentrations of the cell extract ranged from 2 to 10 mg/ml.

All biochemical assays were performed at 55°C in a Coy anaerobic chamber with an 85% N_2, 10% CO_2, and 5% H_2 atmosphere maintained under the anoxic conditions using a palladium catalyst. Alcohol, aldehyde, and lactate dehydrogenase activity were measured based on previously described methods after the oxidation of NAD(P)H at 340 nm (ε = 6,220 M^{-1} cm^{-1}) (20, 27). In all cases, the final assay volume was 0.8 ml. For the ADH (acetaldehyde reduction) reactions, the anaerobic reaction mixture contained 100 mM Tris-HCl buffer (pH 7.0), 5 μM $FeSO_4$, 0.25 mM NAD(P)H, 18 mM acetaldehyde, 1 mM DTT, and cell extract as indicated. For the ALDH (acetyl-CoA reduction) reactions, the anaerobic reaction mixture contained 100 mM Tris-HCl buffer (pH 7.0), 5 μM $FeSO_4$, 0.25 mM NAD(P)H, 1.25 mM acetyl-CoA, 1 mM DTT, and cell extract. A decrease in absorbance at 340 nm caused by NAD(P)H oxidation was monitored by an Agilent Technologies (Santa Clara, CA) 8453 UV-visible spectrophotometer with Peltier controlled heating set at 55°C. All of the ALDH activity measurements mentioned in this study refer to the reaction in the acetaldehyde-producing direction. In reactions where 2,3-dimethoxy-5-methyl-p-benzoquinone (ubiquinone-0) or dimethyl sulfoxide (DMSO) was added, ubiquinone-0 was added to a final concentration of 2 mM, and the same volume of 100% DMSO was added in controls. The reaction conditions for lactate dehydrogenase (LDH) activity were 200 mM Tris-HCl (pH 7.3), 0.22 mM NADH, 10 mM sodium pyruvate, and 1 mM fructose 1,6-bisphosphate.

Nucleotide sequence accession numbers. SRA accession numbers for *adhE* deletion strains of *T. saccharolyticum* and *C. thermocellum* are SRX744220 and SRX744221, respectively. GenBank accession numbers for plasmids pJLO19 and pSH016 are KP636798 and KP245915, respectively.

RESULTS

Knockouts of *adhE* in *T. saccharolyticum* and *C. thermocellum*. To delete *Ts_adhE* from *T. saccharolyticum* strain JW/SL-YS485, we used allelic replacement to insert the resistance cassette from plasmid pMU424 (21) into *Ts_adhE* and performed selection with kanamycin to generate strain LL1076. Strain LL1076 has a 1.9-kb deletion in *Ts_adhE* with an insertion of the 4-kb resistance cassette construct in its place, resulting in an increase in size of 2.1 kb at the *Ts_adhE* locus in the mutant strain, which was confirmed by PCR. In *C. thermocellum*, we transformed strain M1354 with plasmid pJLO19 to generate strain LL1111. Strain LL1111 contains a 2.3-kb internal deletion in *Ct_adhE*. Representative deletion strategies and agarose gels of PCRs targeting *Ct_adhE* regions were run, showing successful genetic modifications of *adhE* in strains LL1076 and LL1111 (Fig. 1). To confirm no significant polar effects as a result of the *Ct_adhE* deletion, cell extract samples of LL1111 and M1354 were analyzed by mass spectrometry. Spectral counts of *Ct_adhE* (Clo1313_1798) showed a >95% reduction, confirming the deletion. The spectral counts of proteins encoded by Clo1313_1799 and Clo1313_1795 to Clo1313_1797 were within 15% between the two strains, suggesting that the in-frame deletion did not have significant polar effects (see Fig. S1 in the supplemental material). Complete genome resequencing of *C. thermocellum* strain LL1111 revealed a G470T point mutation in the *ldh* gene, resulting in an amino acid change at position 157 of

TABLE 2 Specific activities of cell extracts from *T. saccharolyticum* and *C. thermocellum*

Reaction	Sp act[a] (μmol · min^{-1} · mg^{-1} protein) of:			
	T. saccharolyticum		*C. thermocellum*	
	JW/SL-YS485 (parent)	LL1076 (Δ*adhE*)	M1354 (parent)	LL1111 [Δ*adhE ldh*(R157L)]
ALDH-NADH	0.09 (0.01)	0.09 (0.01)	2.18 (0.32)	0.04 (0.03)
ALDH-NADPH	0.05 (0.01)	0.09 (0.01)	0.16 (0.04)	0.10 (0.02)
ADH-NADH	1.52 (0.04)	0.01 (0.01)	7.68 (0.13)	0.03 (0.03)
ADH-NADPH	0.68 (0.03)	0.25 (0.01)	0.05 (0.12)	0.07 (0.03)

[a] Standard deviations are in parentheses; $n = 3$. Limit of detection, <0.01.

arginine to leucine. The R157L mutation of *ldh* was named *ldh*(R157L) for simple notation. To determine whether the *ldh*(R157L) mutation had an effect independent of the *Ct_adhE* deletion, *Ct_adhE* was reintroduced into LL1111 at the *Ct_adhE* locus with plasmid pSH016, generating strain LL1160. Strain LL1160 has the wild-type *Ct_adhE* locus with the *ldh*(R157L) mutation.

Enzyme assay for aldehyde and alcohol dehydrogenase activity. To explore biochemical changes caused by inactivation of the *adhE* gene, we assayed for aldehyde (ALDH) and alcohol dehydrogenase (ADH) activity in cell extracts of *adhE* deletion strains and their parent strains in both *T. saccharolyticum* and *C. thermocellum* (Table 2). We measured low ALDH activity in *T. saccharolyticum* parent strain JW/SL-YS485 cell extract, similar to previous results (3), which was essentially unchanged in the *Ts_adhE* deletion strain LL1076. NADH- and NADPH-linked ADH activity was detected in parent strain JW/SL-YS485 cell extracts. In the *Ts_adhE* deletion strain LL1076, NADH-linked ADH activity decreased to basal levels, while NADPH-linked ADH activity decreased by approximately two-thirds from 0.68 to 0.25 U/mg of cell extract protein. It was surprising to see so little ALDH activity in *T. saccharolyticum* JW/SL-YS485 cell extracts, which was unchanged in the *Ts_adhE* deletion strain LL1076. Ethanol is a major fermentation product of *T. saccharolyticum*, and the AdhE protein should have ALDH activity. We wondered whether there were inhibitors present that prevented us from detecting ALDH activity. Gupta and colleagues reported that detection of high levels of ALDH activity required addition of ubiquinone-0 to their enzyme assay reaction (29). With the addition of ubiquinone-0 to our ALDH enzyme assays, we found that the parent strain JW/SL-YS485 had 5-fold higher ALDH activity (Table 3). The source of inhibition is speculative and will be addressed in Discussion.

For *C. thermocellum*, the parent strain M1354 had high NADH-linked alcohol and aldehyde dehydrogenase activity, similar to those in previous reports on ADH and ALDH activity in *C. thermocellum* (20, 30). Both of these activities were largely lost

TABLE 3 Aldehyde dehydrogenase activity of *T. saccharolyticum* cell extract with ubiquinone-0

Substrate	Sp act[a] (μmol · min^{-1} · mg^{-1} protein) of:	
	JW/SL-YS485 (parent)	LL1076 (Δ*adhE*)
DMSO only	0.09 (0.01)	0.08 (0.03)
DMSO plus ubiquinone-0	0.43 (0.02)	<0.01 (0.02)

[a] Standard deviations are in parentheses; $n = 3$. Limit of detection, <0.01.

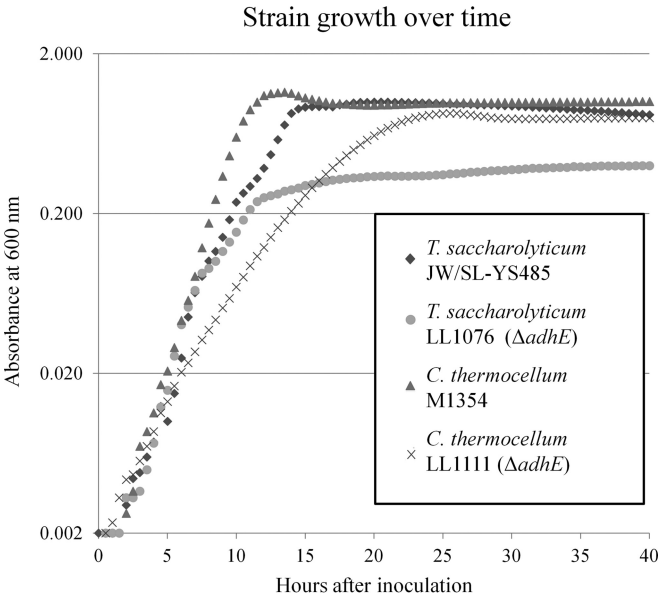

FIG 2 Growth of *C. thermocellum* and *T. saccharolyticum* Δ*adhE* and parent strains over time. Strains were grown in 200 μl of medium in a 96-well plate reader at 55°C for 72 h. Growth was monitored by taking OD_{600} readings every 5 min. Data shown are the averages from three replicates at 30-min intervals for the first 40 h. Significant growth was not seen after 36 h.

(<3% of original NADH-linked activity) in the *Ct_adhE* deletion strain LL1111. Low levels of NADPH-linked alcohol and aldehyde dehydrogenase activities were detected in the parent strain M1354, and these activities were unchanged in the *Ct_adhE* deletion strain LL1111.

Growth and fermentation products. We next compared the growth and product distribution in batch cellobiose fermentations. First, growth was monitored by measuring the change in absorbance every 5 min at 600 nm in a 96-well plate in 200 μl of medium in triplicate for 72 h (Fig. 2). *T. saccharolyticum* parent strain JW/SL-YS485 reached a maximum OD_{600} of 1.0 at 19 h. The *Ts_adhE* deletion strain LL1076 had a similar growth profile for the first 10 h but subsequently exhibited dramatically slower growth, reaching a maximum absorbance of 0.4 after 36 h. The *C. thermocellum* parent strain M1354 grew to a maximum OD_{600} of 1.1 in 12 h, while the *Ct_adhE* deletion strain LL1111 reached a maximum OD_{600} of 0.8 after 25 h. No significant change in OD_{600} in any strain was seen after 36 h.

The slower growth and lower maximum absorbance for the *adhE* deletion strains suggested that the loss of this enzyme has strong effects on metabolism. To further explore metabolic consequences of the loss of *adhE*, we grew the strains for 72 h on 0.72 mmol cellobiose (5 g/liter in a 50-ml working volume) in defined medium in closed bottles (Table 4). In both *T. saccharolyticum* and *C. thermocellum*, loss of *adhE* resulted in a >95% reduction of ethanol formation. *T. saccharolyticum Ts_adhE* deletion strain LL1076 consumed only about 60% of the supplied cellobiose after 72 h. From the 0.44 mmol of cellobiose consumed, the major products were lactate, H_2, and acetate (in descending order). LL1076 pellet C and N, measures of cell mass (31), were approximately a third of the values seen for JW/SL-YS485. The loss of pellet C and N is complementary to the growth data from the 96-well plate assay, which showed a significantly lower maximum

TABLE 4 Fermentation products from *T. saccharolyticum* and *C. thermocellum* from 0.72 mmol cellobiose after 72 h of growth

Product or parameter	Value for[a]:				
	T. saccharolyticum		*C. thermocellum*		
	JW/SL-YS485 (parent)	LL1076 (Δ*adhE*)	M1354 (parent)	LL1111 [Δ*adhE ldh*(R157L)]	LL1160 [*adhE*$^+$ *ldh*(R157L)]
Cellobiose (mmol)	0.01 (0.02)	0.30 (0.08)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
Lactate (mmol)	0.17 (0.07)	0.96 (0.32)	0.02 (0.00)	1.12 (0.02)	0.28 (0.01)
Ethanol (mmol)	1.29 (0.04)	0.05 (0.01)	1.01 (0.07)	0.04 (0.04)	0.77 (0.01)
Acetate (mmol)	0.82 (0.07)	0.36 (0.08)	0.81 (0.04)	0.73 (0.02)	0.73 (0.02)
Formate (mmol)	0.03 (0.02)	0.01 (0.02)	0.23 (0.07)	0.05 (0.00)	0.24 (0.01)
H_2 (mmol)	1.94 (0.14)	0.91 (0.19)	1.67 (0.03)	1.96 (0.19)	1.50 (0.07)
Pellet C (mmol)	0.93 (0.04)	0.33 (0.08)	1.25 (0.04)	1.19 (0.09)	1.20 (0.04)
Pellet N (mmol)	0.24 (0.04)	0.08 (0.03)	0.36 (0.02)	0.31 (0.06)	0.29 (0.02)
Calculated CDW[b] (mg)	25.89	9.19	34.80	33.13	33.41
O/R index[c]	0.9	1	0.8	0.8	0.8
Final pH	4.8	4.7	6.9	6.8	7.1

[a] Standard deviation in are parentheses; $n = 3$.
[b] Cell dry weight, calculated from pellet C and models from Holwerda et al. (31).
[c] O/R, oxidation/reduction.

OD_{600}. The *C. thermocellum adhE* deletion strain LL1111 consumed all of the supplied cellobiose, with the major products being H_2, lactate, and acetate. Strain LL1160, derived from LL1111, with *Ct_adhE* restored to the wild-type genotype and with the *ldh*(R157L) genotype, restores ~75% of the ethanol yield of M1354 (0.77 mmol versus 1.01 mmol). This strain also had increased lactate formation over the parent strain M1354 (0.28 versus 0.02). The increased lactate formation of strain LL1160 over M1354 indicates that the *ldh*(R157L) genotype has an effect on LDH activity.

LDH activity in *C. thermocellum*. To see whether the R157L mutation in *ldh*(R157L) affected LDH activity, we tested for changes in activity in cell extracts (Table 5). Cell extracts of the parent strain M1354 had LDH activity that was activated by fructose 1,6-bisphosphate (F1,6BP), which is consistent with previous reports (27, 32). In contrast, cell extracts of LL1111 had LDH activity that was unaffected by the addition of F1,6BP. The maximum LDH activity in the LL1111 strain was twice the maximum measured in M1354. The LDH activity of cell extract from LL1160, containing only the *ldh*(R157L) mutation, also was unaffected by the addition of F1,6BP and had LDH activity similar to that of M1354 with added F1,6BP. This shows that the R157L mutation in Ldh is responsible for the altered regulation seen in the tested cell extracts. Possibilities for altered Ldh regulation are explored in Discussion.

DISCUSSION

This study shows that the *adhE* gene is necessary for ethanol formation in both *T. saccharolyticum* and *C. thermocellum*. Although other genes are annotated as aldehyde and alcohol dehydrogenases, substantial ethanol production is not observed in the absence of *adhE* in both of these organisms.

TABLE 5 Lactate dehydrogenase activity of *C. thermocellum* cell extract

Condition	Sp act[a] (umol · min^{-1} · mg^{-1} protein) of strain:		
	M1354 (parent)	LL1111 [Δ*adhE ldh*(R157L)]	LL1160 [*adhE*$^+$ *ldh*(R157L)]
With F16BP	0.65 (0.02)	1.44 (0.09)	0.63 (0.12)
Without F16BP	0.11 (0.03)	1.27 (0.01)	0.58 (0.12)

[a] Standard deviations are in parentheses; $n = 3$. Limit of detection, 0.01.

In *T. saccharolyticum*, we found that ubiquinone-0 relieves inhibition of ALDH that is present in the cell extract. Gupta and colleagues showed that in *E. coli*, the inhibition of ALDH activity was due to intermediates of ubiquinone synthesis (29). Ubiquinone synthesis is not believed to occur in anaerobic bacteria (33), although menaquinone synthesis is noted to occur in several related thermophilic anaerobic bacteria (34). Menaquinones are responsible for thiosulfate reduction in *Salmonella* (35), and *T. saccharolyticum* can reduce thiosulfate to elemental sulfur (36), perhaps via a similar mechanism. Menaquinone synthesis has yet to be proven in *T. saccharolyticum*, and whether or not these intermediates interfere with ALDH activity has yet to be proven. The biochemical results nevertheless indicate that the low ALDH value previously observed was due to inhibition, and that AdhE in *T. saccharolyticum* has both ALDH and ADH activity. With the loss of *Ts_adhE*, there was no significant ALDH or NADH-specific ADH activity remaining in cell extracts. However, even with the loss of *Ts_adhE* in strain LL1076, there was significant NADPH-linked ADH activity remaining. A recent proteomic study of *T. saccharolyticum* detected several candidate alcohol dehydrogenase genes for this activity, Tsac_2087, Tsac_1049, and Tsac_0285, with Tsac_2087 being detected among the top 100 peptides in relative abundance (19). Tsac_2087 is predicted to encode a putative AdhA, which shares high identity (88%) to the AdhA from *Thermoanaerobacter ethanolicus* JW200. This AdhA has been shown to be NADPH dependent (37) and could be the source of the remaining NADPH-linked ADH activity. The function of AdhA in *T. saccharolyticum* is unknown.

In *T. saccharolyticum*, deletion of *Ts_adhE* resulted in markedly worse growth in terms of both maximum culture density and time to reach that density. Interestingly, the reduction in growth rate appears more pronounced after an OD_{600} of 0.2. Similarly, the *Tt_adhE* deletion in *T. thermosaccharolyticum* showed a growth phenotype comparable to that of the parent strain at an OD_{600} of <0.3. It also had a slower growth and a final OD_{600} of less than half of that of its parent strain (10). Despite the similarities in the

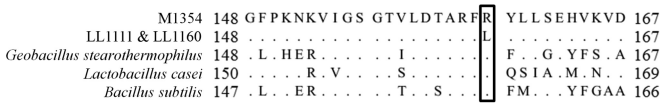

FIG 3 Mutation of Arg157 in lactate dehydrogenase corresponds to the conserved residue shown to interact with F1,6BP. Alignment of amino acid sequences from *C. thermocellum* parent strain M1354, Δ*adhE* strain LL1111, and strain LL1160 and selected lactate dehydrogenases with published structures is shown. Dots represent residues that are identical to those in the top sequence. The boxed residues correspond to a conserved arginine, which is mutated to leucine in strains LL1111 and LL1160.

growth curves of *T. saccharolyticum* strain LL1076 and *T. thermosaccharolyticum* with *Tt_adhE* deleted, there were differences, as the *T. thermosaccharolyticum* strain exhibited complete cellobiose consumption and a complete loss of acetate formation. In the *T. saccharolyticum Ts_adhE* deletion strain LL1076, the distribution of fermentation products changed dramatically, most notably with lactate becoming the major end product. Lactate formation in *T. saccharolyticum* is known to be linked to NADH (40), and perhaps increased lactate formation and incomplete cellobiose consumption reflects an inability of strain LL1076 to oxidize NADH through ethanol formation via AdhE. The inability to consume available cellobiose in *T. saccharolyticum* was previously noted in several hydrogenase mutants (41) and in general may reflect an inability of strains to maintain redox balance.

In *C. thermocellum*, biochemical evidence suggests that *Ct_adhE* is responsible for the majority of both ALDH and ADH activity in cell extracts and that other annotated alcohol/aldehyde dehydrogenases do not play a significant role in ethanol formation. Loss of *Ct_adhE* in *C. thermocellum* strain LL1111 caused a shift from ethanol to lactate production. In *C. thermocellum*, increases in lactate formation have been reported before, particularly in an ethanol-tolerant strain that had a mutated *Ct_adhE* gene (20) and in a phosphotransacetylase (*pta*) deletion strain that could not produce acetate (2). Lactate formation is catalyzed mainly by lactate dehydrogenase in *C. thermocellum* (2), which is strongly activated by F1,6BP (27, 30, 32). The R157L mutation we found in *C. thermocellum* Ldh corresponds to a conserved residue in several well-characterized lactate dehydrogenases (Fig. 3). Structural studies of the *Geobacillus stearothermophilus* Ldh show this arginine residue interacts with the F1,6BP phosphate groups through arginine's positively charged guanidinium group, suggesting that the interaction was disrupted in the mutant Ldh protein (42). Mutation of arginine to glutamine in *G. stearothermophilus* Ldh affected the allosteric regulation of LDH by F1,6BP and stabilized the homotetrameric form, which lowered the K_m for pyruvate (43). Based on the observed accumulation of lactate, loss of F1,6BP activation of Ldh in enzyme assays, mutation of the conserved arginine, and structural data linking the arginine residue to F1,6BP, we believe the observed mutation in the *ldh* of strain LL1111 altered the F1,6BP allosteric regulation in a similar manner. Despite the increased lactate formation in strain LL1160 compared to strain M1354 due to the deregulated Ldh, strain LL1160 had an ethanol yield ~75% of that of strain M1354. This suggests that the primary cause of the increase in lactate production in LL1111 was due to the loss of *Ct_adhE* and not *ldh*(R157L). Although we cannot rule out synergistic effects between *ldh*(R157L) and the loss of *Ct_adhE* for increased lactate formation, it is worth noting that the *T. saccharolyticum Ts_adhE* deletion strain LL1076 produced almost as much lactate as strain LL1111 (0.96 versus 1.12) despite consuming 40% less cellobiose and lacking the *ldh*(R157L) genotype.

The differences in response to the loss of *adhE* in *T. saccharolyticum* and *C. thermocellum* may be indicative of larger differences in metabolism. Previous comparative deletions of *ldh* and *pta* in *T. saccharolyticum* and *C. thermocellum* have resulted in different fermentation phenotypes with regard to ethanol yield. Deletions of *ldh* and *pta* increased ethanol yields in *T. saccharolyticum* (3, 21) but not *C. thermocellum* (2, 27). The genes surrounding *adhE* in *T. saccharolyticum* and *C. thermocellum* suggest major differences between the two strains. *Ct_adhE* in *C. thermocellum* appears to be part of a larger operon, including several genes relevant for electron metabolism. Brown and colleagues identified Cthe_0422-0431 (corresponding to Clo1313_1790-1799 in DSM 1313) as a potential 10-gene operon (20). This set of genes is predicted to encode *rex*, a redox regulator which responds to NADH levels, *hfsB* (also called *hydS*), a putative sensory/regulatory hydrogenase, and *hydABC*, a predicted bifurcating hydrogenase (Fig. 1C). A similar organization recently was identified in a study of *Ruminococcus albus* hydrogenases, where *rex*, *Ra_adhE*, and *hydS* were in a transcriptional unit with a putative ferredoxin-dependent hydrogenase, and this locus is believed to play an important part in the sensory and regulatory mechanism of electron metabolism in *R. albus* (44). In *C. thermocellum*, this gene cluster may play a similar role.

On the other hand, *T. saccharolyticum Ts_adhE* appears to be in its own operon, with the genes surrounding *Ts_adhE* predicted to encode proteins with no predicted role in electron metabolism (Fig. 1A). *T. thermosaccharolyticum* seems to contain a similar genomic locus, with *Ts_adhE*-flanking genes Tsac_0415 and Tsac_0418 sharing 90% and 78% identity, respectively, with the genes surrounding *T. thermosaccharolyticum Tt_adhE*, which may explain the similar growth phenotypes in response to the loss of *adhE*.

Studies have suggested that *C. thermocellum* generates H_2 primarily though bifurcating hydrogenases that use NADH and reduced ferredoxin as electron donors, which generate 2 H_2 molecules per NADH and reduced ferredoxin molecule (45, 46). This would allow *C. thermocellum* to be less reliant on ethanol formation to oxidize NADH generated by glycolysis. Indeed, when *C. thermocellum* was cocultured with H_2-utilizing microbes like methanogens (47) and acetogens (48), ethanol formation dropped by as much as ~90% and ~80%, respectively, indicating that under certain conditions, ethanol formation is unimportant for metabolism. *R. albus* appears to have a similar phenotype: when grown in monoculture, *R. albus* formed ethanol as a major fermentation product, but when cocultured with the H_2-utilizing *Wolinella succinogenes*, ethanol formation was not observed (49). This is believed to be dependent on bifurcating hydrogenase activity in addition to the regulation of genes in the aforementioned transcriptional unit (44). Bifurcating hydrogenase activity has not yet been shown in *C. thermocellum*, and the contribution of bifurcating hydrogenase versus other hydrogenases to overall H_2 formation has not been clearly established. The fermentation data give some evidence in this regard. It was previously predicted that loss of *adhE* in *C. thermocellum* would increase H_2 formation (14), and we did see an increase in H_2 formation in strain LL1111 versus M1354. This is despite a marked increase in lactate formation, which does not generate reduced ferredoxin for H_2 formation.

In contrast, *T. saccharolyticum* H_2 formation is linked with the *hfs* cluster, which encodes a hydrogenase believed to use only reduced ferredoxin as the electron donor (41). Fermentation of sugars results in NADH formation, which is unable to be oxidized by the ferredoxin-dependent Hfs hydrogenase or AdhE bifunctional alcohol/aldehyde dehydrogenase in the *T. saccharolyticum Ts_adhE* deletion strain LL1076. Another option for oxidizing NADH is a putative NfnAB (Tsac_2085-6), which is an electron-bifurcating enzyme that couples the oxidation of NADH and reduced ferredoxin to the reduction of 2 $NADP^+$ (50). We noticed that significant NADPH-linked ADH activity remained despite the loss of *Ts_adhE*, possibly due to AdhA. Interestingly, Tsac_2087, encoding AdhA, is directly upstream of *nfnAB*. This locus may bear particular importance in ethanol formation. In a high-ethanol-yielding *T. saccharolyticum* strain, there was increased NADPH-linked ADH and ferredoxin:NADP oxidoreductase activity (3). These activities could be catalyzed by the enzymes encoded by Tsac_2085-7 and provide an explanation for the seemingly coordinated increase in NADPH-linked activity seen in this high-ethanol-yielding strain. Our biochemical data from cell extracts suggests that ALDH activity still relies on AdhE. Ethanol formation could be more important in *T. saccharolyticum* to maintaining redox balance, as there are fewer metabolic options outside ethanol formation for oxidizing NAD(P)H. The enzymes involved in electron metabolism, especially those involved in ferredoxin reoxidation like NfnAB and hydrogenases, have been poorly studied so far. Understanding their roles in metabolism and product formation will be important for engineering anaerobic microbes for biofuel production.

In conclusion, we have shown that *adhE* is essential for ethanol production in both *C. thermocellum* and *T. saccharolyticum*. In addition, we solved the mystery of low ALDH activity in *T. saccharolyticum* by adding ubiquinone-0 to the enzyme assay mixture. Finally, we found an interesting mutation in *ldh*, which affects its regulation by F1,6BP.

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