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RESEARCH ARTICLE

Both *adhE* and a Separate NADPH-Dependent Alcohol Dehydrogenase Gene, *adhA*, Are Necessary for High Ethanol Production in *Thermoanaerobacterium saccharolyticum*

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ABSTRACT *Thermoanaerobacterium saccharolyticum* has been engineered to produce ethanol at about 90% of the theoretical maximum yield (2 ethanol molecules per glucose equivalent) and a titer of 70 g/liter. Its ethanol-producing ability has drawn attention to its metabolic pathways, which could potentially be transferred to other organisms of interest. Here, we report that the iron-containing AdhA is important for ethanol production in the high-ethanol strain of *T. saccharolyticum* (LL1049). A single-gene deletion of *adhA* in LL1049 reduced ethanol production by ~50%, whereas multiple gene deletions of all annotated alcohol dehydrogenase genes except *adhA* and *adhE* did not affect ethanol production. Deletion of *adhA* in wild-type *T. saccharolyticum* reduced NADPH-linked alcohol dehydrogenase (ADH) activity (acetaldehyde-reducing direction) by 93%.

IMPORTANCE In this study, we set out to identify the alcohol dehydrogenases necessary for high ethanol production in *T. saccharolyticum*. Based on previous work, we had assumed that *adhE* was the primary alcohol dehydrogenase gene. Here, we show that both *adhA* and *adhE* are needed for high ethanol yield in the engineered strain LL1049. This is the first report showing *adhA* is important for ethanol production in a native *adhA* host, which has important implications for achieving higher ethanol yields in other microorganisms.

KEYWORDS AdhA, *Thermoanaerobacterium saccharolyticum*, alcohol dehydrogenase, biofuel, ethanol

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Thermophilic bacteria are excellent candidates for producing ethanol from lignocellulosic biomass. Over the past decade, much work has been dedicated to engineering these organisms for increased ethanol production (1, 2). In particular, *Thermoanaerobacterium saccharolyticum* has been engineered to produce ethanol at ~90% of the theoretical maximum yield and a titer of 70 g/liter (3, 4). However, *T. saccharolyticum* cannot break down cellulose, which accounts for half to a third of plant biomass. Due to the abundance of cellulose in the lignocellulosic biomass, the ideal ethanol producer would be an organism that can rapidly solubilize cellulose. Toward this goal, we have been engineering *Clostridium thermocellum*, one of the most efficient cellulose consumers, for increased ethanol yield. Currently, the highest ethanol yield reported for *C. thermocellum* is 75% of the theoretical maximum (5). One strategy for engineering *C. thermocellum* is to transfer the genes from *T. saccharolyticum* responsible for ethanol production.

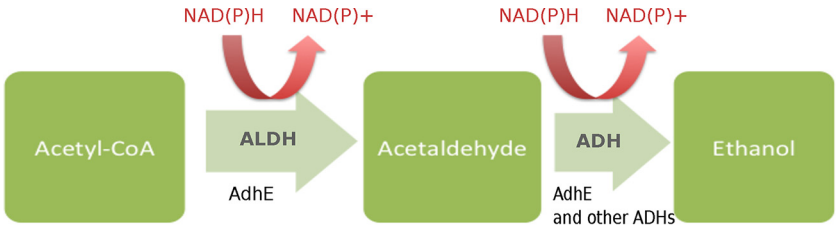

FIG 1 Enzymes and cofactors involved in the *T. saccharolyticum* acetyl-CoA–to–ethanol pathway. Enzymatic reactions and enzymes are shown in black, and cofactors are in red. Note that in the ethanol production pathway, the physiological direction of the ALDH reaction (acetaldehyde dehydrogenase; EC 1.2.1.10) is acetyl-CoA reducing. However, we continue to use the abbreviation ALDH to describe the reaction due to its familiarity. Similarly, the physiological direction of the ADH (alcohol dehydrogenase; EC 1.1.1.1) is acetaldehyde reducing.

In many bacterial organisms, the last two steps of ethanol production are acetyl coenzyme A (acetyl-CoA) to acetaldehyde (the aldehyde dehydrogenase [ALDH] reaction) and acetaldehyde to ethanol (the alcohol dehydrogenase [ADH] reaction) (Fig. 1). While a number of alcohol dehydrogenases can catalyze the ADH reaction, the only known enzyme capable of catalyzing the ALDH reaction is AdhE, a bifunctional enzyme with both ADH and ALDH domains (Fig. 1). Previously, we demonstrated that *adhE* is an important gene for ethanol production in both *C. thermocellum* and *T. saccharolyticum*: deletion of *adhE* reduced the ethanol yield by >95% in both organisms (6). This was not unexpected, since AdhE proteins are essential for anaerobic ethanol production in many organisms (7–11). However, because AdhE is a bifunctional enzyme with two functional domains, the loss of ethanol formation from the *adhE* deletion is not evidence that both domains are essential for ethanol production. As presented below, there is evidence suggesting multiple enzymes could perform the ADH reaction in *T. saccharolyticum*, and AdhE may be necessary only for its ALDH function. We have previously reported the biochemical properties of the *C. thermocellum* and *T. saccharolyticum* bifunctional AdhE: wild-type (wt) AdhE is mostly NADH linked for ADH activity in both organisms (12). Deleting *adhE* in *C. thermocellum* eliminated >90% of the ADH activity in cell extracts, suggesting that AdhE is the primary enzyme contributing to ADH activity. Interestingly, this was not the case for *T. saccharolyticum*. Deleting *adhE* eliminated only NADH-linked ADH activity in *T. saccharolyticum* cell extracts, while significant amounts of NADPH-linked ADH activity remained (6, 12). This suggested that AdhE is not the only enzyme involved in the reduction of acetaldehyde to ethanol and that at least one other NADPH-linked ADH enzyme also plays a role in maintaining cofactor and redox balances in ethanol production.

There are six proteins in *T. saccharolyticum* annotated as "alcohol dehydrogenase" and two as "aldehyde dehydrogenase" in the Pfam protein database (13) (Table 1). Since we had previously characterized the bifunctional alcohol dehydrogenase, AdhE (12), in this work, we focused on understanding the roles of the other putative ALDH and ADH enzymes in an effort to identify the set of genes that are both necessary and sufficient for high ethanol production in *T. saccharolyticum*. In this study, we generated

TABLE 1 Description of deleted genes

Gene locus	Gene name	Seq ID[a]	Length (aa)	Gene product description
Tsac_0218		I3VRV9	404	Alcohol dehydrogenase zinc-binding domain protein
Tsac_2222		I3VXI1	373	Iron-containing alcohol dehydrogenase
Tsac_0285		I3VS20	390	Iron-containing alcohol dehydrogenase
Tsac_1049		I3VU69	394	Iron-containing alcohol dehydrogenase
Tsac_2087	*adhA*	I3VX46	400	Iron-containing alcohol dehydrogenase
Tsac_0416	*adhE*	I3VSF1	860	Aldehyde-alcohol dehydrogenase
Tsac_0219		I3VRW0	468	Aldehyde dehydrogenase

[a]Seq ID, protein sequence ID in Pfam.

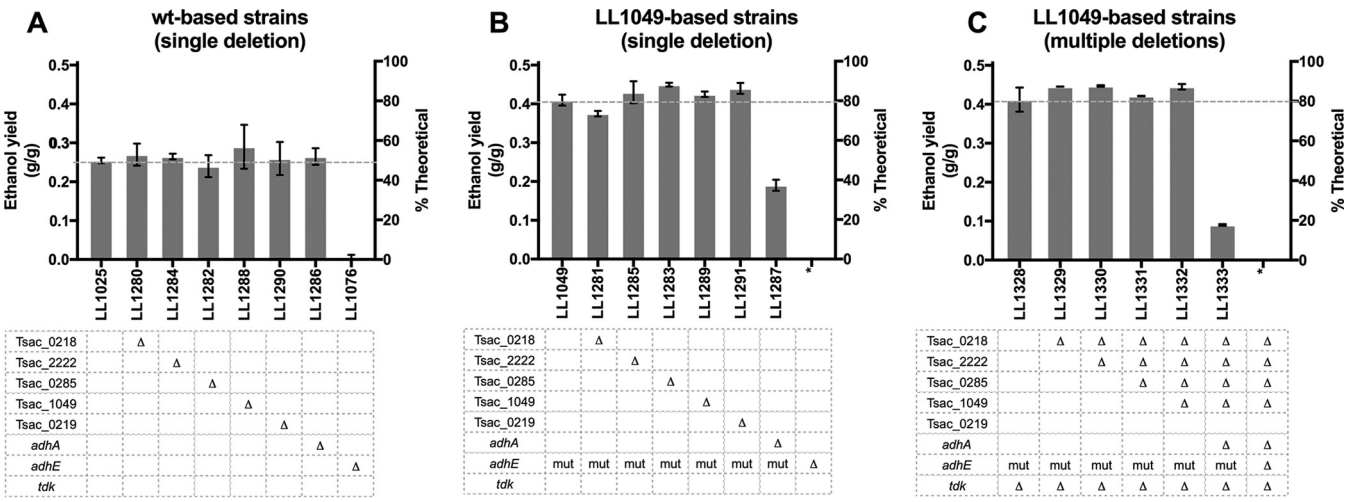

FIG 2 (A and B) Ethanol yields of single-gene deletion strains. (C) Ethanol yields of multiple gene deletions. Ethanol yields are reported in grams of ethanol per gram cellobiose consumed (g/g), and the theoretical maximum yield of ethanol from cellobiose is 0.51 g/g. (B and C) The asterisks indicate *adhE* could not be deleted in strain LL1049, the high-ethanol strain of *T. saccharolyticum*. The dashed lines in the graphs indicate the levels of ethanol yield in parent strains (left bar in each panel). Error bars indicate one standard deviation. The genotype of each strain is shown in the table below each graph, where blanks indicate the wt alleles, Δ indicates disruption of the gene, either by replacement with a *kan* marker or by a markerless deletion, and "mut" refers to the G544D mutation in AdhE that occurred in strain LL1049 (12). Full genotypes of the strains are listed in Table 2.

a series of gene deletions of putative ADH and ALDH genes, the results of which cast light on the importance of one *adh* gene other than *adhE* that contributes to high ethanol production in *T. saccharolyticum*.

RESULTS

Deletion of *adhA* reduced ethanol production in high-ethanol strain LL1049. Individual deletions of putative alcohol and aldehyde dehydrogenases in the wild-type (LL1025) and high-ethanol (LL1049) strains are shown in Fig. 2A and B. In the wild-type strain, *adhE* was the only gene whose deletion affected ethanol production, in agreement with previous reports (6). Deleting the other five putative ADH and ALDH genes had no significant effect on the ethanol yield; the resulting strains grew to high cell densities, similar to their parent strain (wt) (see Table S3 in the supplemental material, pellet C data). However, in the high-ethanol strain (LL1049), deleting *adhA* reduced the ethanol yield by ~50% (Fig. 2B shows a comparison between strains LL1287 and LL1049). Furthermore, the resulting strain LL1287 grew poorly. Deleting the other ADH and ALDH genes in the high-ethanol strain had no significant effect on the ethanol yield or cell growth. We were unable to delete *adhE* in the high-ethanol strain LL1049 (Fig. 2, asterisks) because ethanol is the only significant organic end product of the strain; that is, producing ethanol is the organism's only way to balance redox levels via NAD(P) cofactors and to generate the ATP needed for growth.

The roles of *adhE* and *adhA* for high-yield ethanol production. Having investigated deletions of putative ADH and ALDH genes individually, we now sought to investigate them in combination to control for epistatic effects. The parent strain used for these deletions was LL1328, which is strain LL1049 with the *tdk* gene deleted (necessary for making markerless deletions). The *tdk* deletion did not have any effect on ethanol production, as expected (Fig. 2, LL1328 versus LL1049). Figure 2C shows the results of multiple *adh* deletions. In strain LL1332, Tsac_0218, Tsac_2222, Tsac_0285, and Tsac_1049 have all been deleted, leaving only *adhE* and *adhA* as potential alcohol dehydrogenase genes. The strain had an ethanol yield (0.45 g of ethanol/g of cellobiose consumed [g/g]) similar to that of its parent strain, LL1328 (0.41 g/g), showing no reduction in ethanol and indicating that Tsac_0218, Tsac_2222, Tsac_0285, and Tsac_1049 are not required for high-yield ethanol production and that *adhA* and *adhE* are, in fact, sufficient for high-yield ethanol production.

TABLE 2 Strains used in this study

Strain name	Description	Cloning plasmid(s)[a]	Source and/or reference[b]
LL1025	Wild-type *T. saccharolyticum* strain JW/SL-YS485		Mai et al. (45); GenBank accession no. CP003184
LL1049	Evolved *T. saccharolyticum* Δ(*pta-ack*) Δ*ldh* Δ*or796 ure metE* Δ*eps* strain with mutation G544D in AdhE; high ethanol producer		Also called M1442; Herring et al. (4); SRA accession no. SRA233073
LL1076	Δ*adhE*::(*pta-ack kan*)		Mascoma Corp.; SRA accession no. SRS731880
LL1280	Wild-type *T. saccharolyticum* ΔTsac_0218::*kan* strain	pTZvec001	This study
LL1281	LL1049 *T. saccharolyticum* ΔTsac_0218::*kan* strain	pTZvec001	This study
LL1282	Wild-type *T. saccharolyticum* ΔTsac_0285::*kan* strain	pTZvec002	This study
LL1283	LL1049 *T. saccharolyticum* ΔTsac_0285::*kan* strain	pTZvec002	This study
LL1284	Wild-type *T. saccharolyticum* ΔTsac_2222::*kan* strain	pTZvec003	This study
LL1285	LL1049 *T. saccharolyticum* ΔTsac_2222::*kan* strain	pTZvec003	This study
LL1286	Wild-type *T. saccharolyticum* Δ*adhA*::*kan* strain	pTZvec004	This study
LL1287	LL1049 *T. saccharolyticum* Δ*adhA*::*kan* strain	pTZvec004	This study
LL1288	Wild-type *T. saccharolyticum* ΔTsac_1049::*kan* strain	pTZvec005	This study
LL1289	LL1049 *T. saccharolyticum* ΔTsac_1049::*kan* strain	pTZvec005	This study
LL1290	Wild-type *T. saccharolyticum* ΔTsac_0219::*kan* strain	pTZvec006	This study
LL1291	LL1049 *T. saccharolyticum* ΔTsac_0219::*kan* strain	pTZvec006	This study
LL1328	LL1049 *T. saccharolyticum* Δ*tdk* strain	pTZvec007	This study
LL1329	LL1049 *T. saccharolyticum* Δ*tdk* ΔTsac_0218 strain	pTZvec008, pTZvec009	This study
LL1330	LL1049 *T. saccharolyticum* Δ*tdk* ΔTsac_0218 ΔTsac_2222 strain	pTZvec008, pTZvec009, pTZeco012	This study
LL1331	LL1049 *T. saccharolyticum* Δ*tdk* ΔTsac_0218 ΔTsac_2222 ΔTsac_0285 strain	pTZvec008, pTZvec009, pTZeco012, pTZeco014	This study
LL1332	LL1049 *T. saccharolyticum* Δ*tdk* ΔTsac_0218 ΔTsac_2222 ΔTsac_0285 ΔTsac_1049 strain	pTZvec008, pTZvec009, pTZeco012, pTZeco014, pTZeco013	This study
LL1333	LL1049 *T. saccharolyticum* Δ*tdk* ΔTsac_0218 ΔTsac_2222 ΔTsac_0285 ΔTsac_1049 Δ*adhA*::*kan* strain	pTZvec008, pTZvec009, pTZeco012, pTZeco014, pTZeco013, pTZvec004	This study
LL1334	*T. saccharolyticum* Δ*adhE* strain	pTZvec010	This study
LL1335	*T. saccharolyticum* Δ*adhE* Δ*adhA*::*kan* strain	pTZvec010, pTZvec004	This study
LL1402	LL1049 *T. saccharolyticum* Δ*tdk* ΔTsac_0218 ΔTsac_2222 ΔTsac_0285 ΔTsac_1049 Δ*adhA*::*adhA*$^+$ *erm* strain	pTZvec008, pTZvec009, pTZeco012, pTZeco014, pTZeco013, pTZvec004, pTZvec011	This study
LL1403	*T. saccharolyticum* Δ*adhE*::*adhE*$^+$ *kan* colony 8	pTZvec010, pTZvec012	This study
LL1404	*T. saccharolyticum* Δ*adhE*::*adhE*$^+$ *kan* colony 1	pTZvec010, pTZvec012	This study
LL1405	*T. saccharolyticum* Δ*adhE*::*adhE*$^+$ *kan* colony 3	pTZvec010, pTZvec012	This study
LL1406	*T. saccharolyticum* Δ*adhE*::*adhE*$^+$ *kan* colony 5	pTZvec010, pTZvec012	This study
LL1407	*T. saccharolyticum* Δ*adhE*::*adhE*$^+$ *kan* colony 7	pTZvec010, pTZvec012	This study

[a]Plasmid information is included in Table S1 in the supplemental material; plasmid sequences can be found in GenBank under accession numbers KY110698 to KY110712. The primers used for plasmid construction are listed in Table S2 in the supplemental material.
[b]SRA, Sequence Read Archive.

When we deleted *adhA* in the strain with the other four *adh* deletions (Fig. 2C, LL1333 versus LL1332), we saw a large decrease in ethanol production, confirming the importance of *adhA* in the strain.

Additionally, we considered the possibility that the alcohol dehydrogenase proteins annotated in the Pfam database are not an exhaustive list, in which case another, unidentified *adh* gene could also be necessary for ethanol production. To investigate this possibility, we deleted both *adhE* and *adhA* in the wild-type strain background (Table 2), generating strain LL1335 (LL1025→LL1076→LL1334→LL1335, where arrows indicate the lineage of strain LL1335). If another functional *adh* gene existed, there should be ADH enzymatic activityremaining in strain LL1335. As shown in Table 3, wild-type *T. saccharolyticum* exhibited high ADH activity with both NADH and NADPH cofactors. Deletion of *adhE* (strain LL1334) reduced NADH-linked ADH activity by 98%, but NADPH-linked ADH activity remained high. On the other hand, deletion of *adhA* (strain LL1286) did not significantly affect NADH-linked ADH activity but reduced NADPH-linked activity by 93%. Deletion of both *adhE* and *adhA* eliminated NADH-linked activity and reduced NADPH-linked activity by 93% (LL1335). This strongly suggests that AdhA and AdhE are the two main alcohol dehydrogenases involved in

TABLE 3 ADH activity of Δ*adhE* and Δ*adhA* strains

Strain name	Description	ADH activity (U/mg)[a]	
		NADH	NADPH
LL1025	Wild type	2.64 ± 0.35	0.83 ± 0.08
LL1334	Δ*adhE*	0.06 ± 0.01	0.64 ± 0.21
LL1286	Δ*adhA*	2.19 ± 0.49	0.06 ± 0.01
LL1335	Δ*adhE* Δ*adhA*	0.01 ± 0.01	0.06 ± 0.06

[a]The averages for two biological replicates are shown with their standard deviations.

ethanol production in *T. saccharolyticum*. Furthermore, it suggests that ADH activity from AdhA is NADPH linked.

Complementation of *adhE* and *adhA* deletions. In order to confirm the functions of *adhE* and *adhA* and gauge the effects of secondary mutations, we attempted to complement the *adhA* and *adhE* deletions by introducing the genes back onto the chromosome. In strains LL1287 (single gene deletion) and LL1333 (multiple gene deletions), where *adhA* had been deleted, transformation efficiency was extremely low. After multiple attempts, we were able to insert the *adhA* gene in strain LL1333 but not in strain LL1287—transformations with LL1333 had a single colony, while transformations with LL1287 had none. This colony became the resulting strain LL1402 with *adhA* introduced back into its original locus, producing ethanol at a yield of 0.19 g/g (see Table S4 in the supplemental material). This was a 2-fold increase in ethanol production compared to its parent strain, LL1333, whose ethanol yield was 0.09 g/g. However, reintroducing *adhA* restored ethanol production only to 43% of that of the predeletion strain (LL1332). This suggested the accumulation of secondary mutations as a result of deleting *adhA* and other alcohol dehydrogenase genes.

Similarly, *adhE* was reintroduced into strain LL1334 (Δ*adhE*). The transformation efficiency of strain LL1334 was also low, yielding only five colonies with the desired *adhE* insertion: strains LL1403 to LL1407. All five colonies were able to restore ethanol production to different degrees, with ethanol yields ranging from 0.14 g/g to 0.22 g/g (see Table S4 in the supplemental material). Strain LL1403 had the highest ethanol yield, 0.22 g/g, restoring ethanol production to 88% of that of the wild-type strain.

AdhA is an oxygen-insensitive NADPH-linked alcohol dehydrogenase. To further characterize the biochemical properties of AdhA, we expressed and purified *T. saccharolyticum* AdhA in *Escherichia coli* (see Fig. S2 in the supplemental material). Purified AdhA had high NADPH-linked ADH activity (6.8 ± 1.7 U/mg) and negligible amounts of NADH-linked ADH activity (0.3 ± 0.6 U/mg), which confirmed the results of the gene deletion studies (see "Deletion of *adhA* reduced ethanol production in high-ethanol strain LL1049" above).

In addition, we noted that it was unnecessary to perform anaerobic protein expression and purification for AdhA, as was done previously for AdhE (12). Unlike AdhE, which was extremely sensitive to oxygen, AdhA appeared to be insensitive to the presence of oxygen. It had comparable ADH activities under anaerobic and aerobic expression conditions: 6.9 ± 1.9 U/mg and 6.8 ± 1.7 U/mg, respectively.

DISCUSSION

AdhA proteins in various organisms. Keshav et al. first adopted the name *adhA* in 1990 to describe a gene that encodes a zinc alcohol dehydrogenase in *Zymomonas mobilis* (14). Analysis of the amino acid sequence of *Z. mobilis* AdhA showed that it contained an alcohol dehydrogenase GroES-like domain (ADH_N) and a zinc-binding dehydrogenase domain (ADH_zinc_N) (Fig. 3). Alcohol dehydrogenases in general have been extensively studied in various organisms for many years. However, many of these proteins were given the name AdhA regardless of their homology to the zinc-binding *Z. mobilis* AdhA. In an effort to clarify the differences among the "AdhA" proteins reported in the literature and to avoid confusion, we compiled a nonexhaustive list of AdhA proteins from the literature according to Pfam protein domains and families.

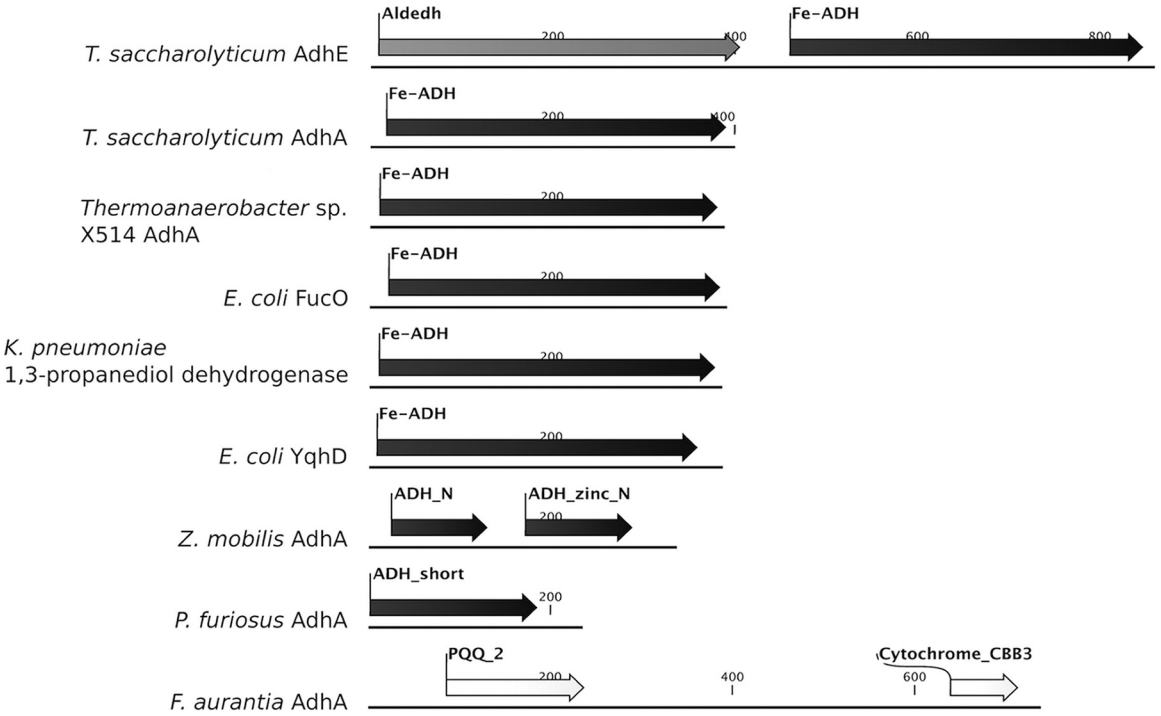

FIG 3 Sequence comparison of proteins that share homology with *T. saccharolyticum* AdhA and other AdhA proteins in the literature. The lines below the arrows indicate the amino acid sequence of each protein, on which the sequence length is marked in 200-amino-acid (aa) units. The length of *T. saccharolyticum* AdhA is 400 aa; *T. saccharolyticum* AdhE is 860 aa, with the Fe-ADH domain from aa 460 to 847. Each sequence is annotated to scale with the appropriate Pfam protein domain/family. ADH-like domains are shown in black, ALDH domains are in gray, and other domains are in white. Aldedh, aldehyde dehydrogenase family; Fe-ADH, iron-containing alcohol dehydrogenase; ADH_N, alcohol dehydrogenase GroES-like domain; ADH_zinc_N, zinc-binding dehydrogenase domain; ADH_short, short-chain dehydrogenase family; PQQ_2, PQQ-like domain; cytochrome_CBB3, cytochrome *c* oxidase, cbb_3 type, subunit III.

AdhA proteins that have sequence similarity to the *Z. mobilis* AdhA include those from *Corynebacterium glutamicum* (15, 16), *Lactococcus lactis* (17), *Picrophilus torridus* (18), and *Rhodococcus erythropolis* (19). "AdhA" was also used to describe the *Pyrococcus furiosus* short-chain dehydrogenase (adh_short) (20, 21) (Fig. 3) and the PQQ-dependent alcohol dehydrogenases from *Frateuria aurantia* (22) (Fig. 3) and *Acetobacter pasteurianus* (23, 24). In many cases, the name "AdhA" simply implies that it was the first alcohol dehydrogenase studied in a given organism.

The above-mentioned AdhA proteins are not homologous to the *T. saccharolyticum* AdhA that we report in this study in terms of protein sequence and domains. *T. saccharolyticum* AdhA consists of an iron-containing alcohol dehydrogenase domain (Fe-ADH); similar AdhAs (>60% protein sequence identity) include those from *Thermoanaerobacter brockii* (25–27), *Moorella* sp. (28), and *Thermoanaerobacter mathranii* (29). We suggest that, for clarification purposes, future studies involving AdhA should specify a distinct protein feature, for example, iron-containing AdhA or PQQ-dependent AdhA.

In addition, it is worth noting that the Fe-ADH family (which includes *T. saccharolyticum* AdhA) encompasses a large group of proteins, some of which are not named alcohol dehydrogenases and have less than 30% protein sequence identity with *T. saccharolyticum* AdhA. The additional Fe-ADH family members include *E. coli* FucO (29% identity) (30, 31), *Klebsiella pneumoniae* 1,3-propanediol dehydrogenase (29% identity) (32), and *E. coli* YqhD (24% identity) (33) (Fig. 3). The ADH domain of *T. saccharolyticum* AdhE is also an iron-containing ADH with 30% protein sequence identity to *T. saccharolyticum* AdhA (Fig. 3).

Oxygen tolerance of AdhA. Protein sequence alignments between *E. coli* AdhE, *T. saccharolyticum* AdhE, and *T. saccharolyticum* AdhA showed that the AdhE proteins had a Glu568 residue, while the *T. saccharolyticum* AdhA had a Lys residue at the corresponding position (see Fig. S1 in the supplemental material). Glu568 has been shown

to be the critical residue responsible for oxygen sensitivity in *E. coli* AdhE: Holland-Staley et al. reported that the Glu568Lys mutation "produced AdhE that was active under both aerobic and anaerobic conditions" (34). This difference in amino acids at position 568 may explain the difference in oxygen sensitivity between AdhE and AdhA.

Identification of cryptic NADPH-ADH activity in *T. saccharolyticum*. Previously it was shown that deletion of *adhE* in *T. saccharolyticum* nearly eliminated NADH-linked ADH activity but only slightly reduced NADPH-linked ADH activity (6). Furthermore, in the high-ethanol-producing strain LL1049, levels of NADPH-ADH in the cell extract were higher than in the wild-type strain (LL1025), whereas levels of NADPH-ADH from purified AdhE were lower than in the wild type (12). The combination of these observations suggested that there was a cryptic *adh* gene responsible for the NADPH-ADH activity that was distinct from *adhE*. Here, we have presented evidence that this cryptic NADPH-ADH gene is, in fact, *adhA*.

Cofactor specificity and ethanol production in *T. saccharolyticum*. Why was *adhA* important for ethanol production only in the high-ethanol-producing strain (LL1049) and relatively unimportant in the wild-type strain (LL1025)? This may be explained by the different NAD(P)H cofactors involved in ethanol production. Previously, we have shown that wild-type *T. saccharolyticum* (strain LL1025) and strain LL1049 appear to utilize different cofactors in the pyruvate-to-ethanol pathway (35). Pyruvate-ferredoxin oxidoreductase (PFOR) has been shown to be the enzyme responsible for the oxidation of pyruvate to acetyl-CoA in *T. saccharolyticum* (36, 37), coupled with the reduction of ferredoxin. In order to maintain electron balance, the cell uses a ferredoxin-NAD(P)$^+$ oxidoreductase (FNOR) to transfer electrons from reduced ferredoxin to NAD(P)$^+$. In the high-ethanol-producing strain, NADPH-FNOR activity is increased relative to the wild type (35), which presumably leads to increased NADPH production. In order to use the electrons from NADPH for ethanol production, high levels of NADPH-ADH activity are needed. Previously, we have shown that the high-ethanol-producing strain (LL1049) has mutations in its *adhE* gene that confer NADPH-ADH activity, but the newly acquired NADPH-ADH activity in the LL1049 AdhE is lower than the ADH activity in wt AdhE (12). In this study, we observed that without AdhA, the NADPH-ADH activity from AdhE is sufficient to produce ethanol only at about 40% of the theoretical maximum yield (strain LL1287). It is only when the gene *adhA* is also present (strain LL1332) that high ethanol yields (~90% of the theoretical maximum) are observed. Thus, NADPH-ADH activity of AdhA is needed to balance the NADPH-FNOR activity (presumably from NfnAB) in strain LL1049. Their proximity on the genome suggests that their cofactor compatibility may not be a coincidence and that they may, in fact, be an operon.

Additionally, acetaldehyde is very reactive and ultimately toxic at thermophilic temperatures (38). Therefore, it is possible that the NADPH-linked ADH activity from the mutated AdhE is not sufficient for effective acetaldehyde conversion and that AdhA is needed to serve as an additional acetaldehyde scavenger.

Importance of the iron-containing AdhA in ethanol production. Alcohol dehydrogenases have long been utilized in engineering strategies for increased biofuel production. Among them, AdhE has been widely studied for ethanol production (7–11), the zinc-binding AdhA was used in isobutanol production (17, 39), and YqhD was used to increase isobutanol (17) and propanediol (40) production. To date, there has only been one study where the iron-containing AdhA was utilized to increase bioethanol production (21). Basen et al. inserted the *adhA* gene from *Thermoanaerobacter* strain X514 (Fig. 3) into the hyperthermophilic archaeon *P. furiosus*, which has a unique aldehyde ferredoxin oxidoreductase (AOR) enzyme that reduces acetate to acetaldehyde but no native alcohol dehydrogenase. Wild-type *P. furiosus* does not produce ethanol; however, as a result of the *adhA* insertion, the engineered strain was able to generate ethanol from acetaldehyde and produced >20 mM ethanol from 5 g/liter cellobiose (21), demonstrating that the iron-containing AdhA can be used for ethanol production. Here, we show that *adhA* is important for ethanol production in a native

adhA host. Although strain LL1049 was engineered for increased ethanol production, none of the genetic modifications targeted *adhA*, and in fact, the role of *adhA* in the strain was largely unknown until this report. The ability of engineered strains of *T. saccharolyticum* to produce ethanol at a high yield (90% of the theoretical maximum) and titer (70 g/liter) has drawn attention to its metabolic pathways. Uncovering the role of *adhA* has shed light on the ethanol production pathway in *T. saccharolyticum* and has provided ideas for improving ethanol production in other organisms, such as *C. thermocellum*.

MATERIALS AND METHODS

Plasmid and strain construction. The strains used in this study are listed in Table 2. Kanamycin marker deletions were made by transforming *T. saccharolyticum* cells with deletion vectors, using a protocol described previously (41). In brief, the deletion vectors consist of a kanamycin marker flanked by upstream and downstream homology regions of the target gene. The vectors were constructed from linear PCR products via Gibson assembly (42), followed by anaerobic incubation with cells at 55°C to allow the naturally competent *T. saccharolyticum* cells to take up the DNA construct. The cells incubated with the deletion vector were harvested at an optical density at 600 nm (OD_{600}) of ~0.6 during the exponential growth phase and plated with 200 μg/ml kanamycin. The Tsac_0218 markerless deletion was made using the *pta-ack* marker removal system, as described previously by Shaw et al. (43); all other markerless deletions were made using the *tdk* (thymidine kinase) marker removal system described by Shao et al. (44). Briefly, the *tdk* gene was deleted in strains LL1049 and LL1076, and the Δ*tdk* strains were used for subsequent transformations. The selection process included a first round of positive selection based on resistance to kanamycin, followed by a second round of negative selection based on resistance to floxuridine (FUDR), as previously described (44). The plasmids and primers used for cloning are listed in Tables S1 and S2 in the supplemental material. *E. coli* competent cells (T7 Express *lysY/I*^{q}; NEB) were transformed as previously described (12), using the pEXP5-NT/TOPO (Invitrogen) plasmid backbone for AdhA protein expression.

Media and growth conditions. For transformations and enzyme assays, *T. saccharolyticum* strains were grown anaerobically to exponential phase (OD_{600}, ~0.6) in CTFUD medium, as previously described (12); for fermentation end product analysis, all the strains were cultivated on MTC-6 defined medium. The MTC-6 medium was prepared as previously described (36), filter sterilized immediately after preparation, transferred to sterilized serum bottles, and purged of oxygen using 20 45-s cycles of ultrahigh-purity N_2 gas and vacuum. Residual pressure in the bottles was released inside an anaerobic chamber using a sterile filter and needle prior to inoculation. All the media used for fermentation purposes were inoculated within 2 h of preparation. All fermentations were incubated for 72 h in an orbital shaking incubator (Innova 4080 incubator shaker; New Brunswick Scientific) held at 55°C and 180 rpm. The fermentations were performed in 125-ml glass serum bottles with a working volume of 50 ml and were inoculated with 2% frozen cell culture.

E. coli cells used for protein expression were grown in lysogeny broth (LB) medium.

Fermentation end product analysis. All fermentation data were calculated from biological replicates and are reported in Table S3 and Table S4 in the supplemental material.

Gas chromatography. The gas composition was analyzed using an SRI 310C gas chromatograph (SRI Inc.), utilizing a nitrogen carrier gas at a flow rate of 8.2 ml/min. The oven temperature was maintained at 151°C and the current at 80 mA. Triplicate injections of 99% hydrogen and 10% carbon dioxide calibration gasses (MESA Specialty Gasses & Equipment) were used for standard curves, using 100-μl and 500-μl injections, respectively. Single 500-μl gas samples from cultures were then analyzed for H_2 and CO_2 concentrations. The absolute H_2 and CO_2 measurements were determined after correction for pressure differences. The cultures were then stored in the dark at 4°C overnight prior to further analysis.

TOCN analysis. Total organic carbon and nitrogen (TOCN) analysis was used to determine pellet carbon (pellet C), which is a proxy for cell biomass production. Ten-milliliter samples were removed from fermentation bottles by syringe, with constant mixing of the contents for homogeneity, and divided into 1-ml aliquots. The aliquots were centrifuged at 15,000 rpm for 5 min, and the pellets were used for TOCN analysis and the supernatant for high-pressure liquid chromatography (HPLC) analysis. The pellets were washed twice with 950 μl of deionized water.

The washed cell pellets were resuspended via vigorous vortexing and were added to the TOCN vials filled with 19.5 ml deionized water. A single standard for the quantification of total organic carbon and nitrogen was prepared by adding 1 ml of a 5-g/liter glycine solution to a vial filled with 19.5 ml deionized water. TOCN analysis was performed using an organic carbon/nitrogen analyzer with a liquid module and automatic sample injection (ASI-V/TOC-V/TNM-1/SSM-5000; Shimadzu Scientific Instruments Inc.). The eluent consisted of 6.67 ml concentrated H_2SO_4 diluted in 10 liters of deionized water. Ultrazero compressed air (AI UZ300; Airgas) was used as the carrier gas at a pressure of 200 kPa and a flow rate of 150 ml/min.

HPLC analysis. Seven hundred microliters of supernatant resulting from the preparation of cell pellets was acidified with 35 μl 10% H_2SO_4 solution. They were mixed gently by inversion and then passed through a Costar SpinX HPLC 0.2-μm nylon microcentrifuge filter (Corning, Inc.) via centrifugation at 15,000 rpm for 5 min.

Fermentation products were quantified using a Waters 2695 separations module fitted with an additional UV detector (for the accurate quantification of pyruvate). The injection volumes were 40 μl,

and the product concentrations were calculated from standard curves. All peaks were reviewed manually for errors.

Enzymatic assays. Cells were harvested and lysed anaerobically as previously described (12). ADH reactions (acetaldehyde reduction direction) were carried out anaerobically at pH 7.0 in a reaction mixture containing 0.2 mM NADH or NADPH, 20 mM acetaldehyde, 100 mM Tris-HCl, 5 μM $FeSO_4$, and cell extract or purified protein solution. The final volume was 1,000 μl, the assay temperature was 55°C, and the assay was started by the addition of acetaldehyde. ADH activity was monitored, and the activity was calculated as previously described (12). One unit of activity (U) is equal to the formation of 1 μmol of product per minute. Specific activities are expressed in units per milligram of protein.

Protein expression and purification. AdhA protein was expressed in *E. coli* with an N-terminal His tag and purified by affinity chromatography. One hundred milliliters of *E. coli* cell culture was grown to an OD_{600} of ~0.6 before induction of protein expression with IPTG (isopropyl-β-D-1-thiogalactopyranoside) at 0.4 mM final concentration. Cells were induced for 2 h before harvesting, after which the pellets were stored at −80°C. Anaerobic protein expression was performed similarly; however, cell cultures were transferred to an anaerobic serum bottle (purged with nitrogen) prior to IPTG induction to maintain anaerobic conditions. After induction, the cells were cultured for 2 h before harvesting, and the pellets were stored anaerobically at −80°C.

Protein purification was carried out using affinity columns (Ni-nitrilotriacetic acid [NTA] spin columns; Qiagen) as previously described (12): aerobic purification was performed under normal atmospheric conditions (i.e., outside the anaerobic chamber), and anaerobic protein purification was performed inside an anaerobic chamber with oxygen maintained at levels below 5 ppm. The purified proteins were visualized, and their sizes were confirmed by SDS-PAGE.

Accession number(s). Plasmid sequences have been deposited in GenBank under accession no. KY110698 to KY110712.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00542-16.

TEXT S1, PDF file, 0.3 MB.

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