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BIOENERGY/BIOFUELS/BIOCHEMICALS - ORIGINAL PAPER

Determining the roles of the three alcohol dehydrogenases (AdhA, AdhB and AdhE) in *Thermoanaerobacter ethanolicus* during ethanol formation

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Abstract *Thermoanaerobacter ethanolicus* is a promising candidate for biofuel production due to the broad range of substrates it can utilize and its high ethanol yield compared to other thermophilic bacteria, such as *Clostridium thermocellum*. Three alcohol dehydrogenases, AdhA, AdhB and AdhE, play key roles in ethanol formation. To study their physiological roles during ethanol formation, we deleted them separately and in combination. Previously, it has been thought that both AdhB and AdhE were bifunctional alcohol dehydrogenases. Here we show that AdhE has primarily acetyl-CoA reduction activity (ALDH) and almost no acetaldehyde reduction (ADH) activity, whereas AdhB has no ALDH activity and but high ADH activity. We found that AdhA and AdhB have similar patterns of activity. Interestingly, although deletion of both *adhA* and *adhB* reduced ethanol production, a single deletion of either one actually increased ethanol yields by 60–70%.

Keywords Bioethanol · Gene deletion · Thermophilic bacteria · Bifunctional alcohol dehydrogenase

Introduction

Biofuel production from lignocellulosic biomass has been proposed as an alternative renewable energy source to reduce fossil fuel dependence. Thermophilic microorganisms in the *Thermoanaerobacter* and *Thermoanaerobacterium* genera are good candidates for bioethanol production due to their ability to ferment a broad range of sugars found in the hemicellulose fraction of lignocellulosic biomass [20, 38, 44]. They are also of interest as companion organisms that could be co-cultured with cellulolytic thermophilic microorganism such as *Clostridium thermocellum* (i.e., an organism that can use the cellulosic fraction of lignocellulosic biomass), in one-step consolidated bioprocessing [1, 28]. Alcohol and aldehyde dehydrogenases play key roles in the metabolic pathways of ethanol production and most thermophiles have multiple alcohol dehydrogenases in their genomes [12, 33]. The final two steps in ethanol production are the reduction of acetyl-CoA to acetaldehyde (i.e., ALDH activity) and the subsequent reduction of acetaldehyde to ethanol (i.e., ADH activity). Both of these reactions can use either NADH or NADPH as an electron donor. The roles of alcohol dehydrogenases during ethanol formation in *Thermoanaerobacter ethanolicus* JW200 have been studied since 1981 [8], but have not been unambiguously identified. Previous efforts mainly focused on three enzymes, which are encoded by the *adhA*, *adhB* and *adhE* genes.

In many thermophilic microorganisms, *adhE* is essential for ethanol production. Organisms that possess *adhE* are usually able to produce ethanol, while organisms without this gene produce ethanol in at most trace amounts [12, 22]. *Thermoanaerobacter tengcongensis* and *Thermoanaerobacter kivui* are two members in *Thermoanaerobacter* spp. that do not have *adhE* in their genome, which correlates with their limited ability to produce ethanol [22, 45]. Deletion of *adhE* frequently results in elimination of ethanol production, and has been confirmed in *Thermoanaerobacter mathranii*, *Thermoanaerobacterium saccharolyticum*

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and *C. thermocellum* [27, 46]. Supporting its role as a key enzyme in ethanol production, several interesting mutations have been found in *adhE* in strains that have been engineered for high ethanol production or tolerance [7, 34, 39, 41, 49].

Based on its amino acid sequence, AdhE is thought to be a bifunctional enzyme, responsible for both the ALDH and ADH reactions [12, 16, 23]. In *T. ethanolicus,* AdhE was initially identified based on its ALDH activity [9], but was later shown to have ADH activity as well [32]. Purified AdhE from *T. saccharolyticum*, *C. thermocellum* and *T. ethanolicus* has been shown to exhibit both ALDH and ADH activities [31, 32, 49]. There is some disagreement in the literature with respect to ALDH activity from the purified AdhE of *T. ethanolicus* JW200. Pei et al. [31] measured 224.8 ± 5.2 U/mg ALDH activity in purified AdhE protein, while Peng et al. [32] measured only 11.0 ± 0.3 U/mg. Both of these studies reported similar ADH activities from purified AdhE. The values are 2.1 ± 0.39 and 2.6 ± 0.2 U/mg, respectively. In the study of Pei et al. [31], ADH activity of AdhE is only 1% of its ALDH activity while in the study of Peng et al. [32], it was almost 20%.

Many of thermophilic microorganisms have other ADH enzymes in addition to AdhE. For example, after deleting *adhE* in *T. saccharolyticum*, Lo et al. [27] found NADPH-dependent ADH activity. This activity was subsequently attributed to the *adhA* gene [48]. There are several reasons for having multiple ADH enzymes. One possibility may be to scavenge aldehyde that would otherwise be toxic to the cell. Another possibility is that different ADHs may have different substrate [10, 11, 46], temperature [9, 31] or pH preferences [31]. A final possibility is that the different ADH isozymes are regulated based on different sets of conditions [9, 31].

In the *Thermoanaerobacter* genus, most species have both AdhA and AdhB enzymes. Previously, these enzymes have been referred to as the primary alcohol dehydrogenase (P-ADH) and secondary alcohol dehydrogenase (S-ADH) (for AdhA and AdhB, respectively). In *T. ethanolicus* JW200, AdhA and AdhB were purified and both were found to be NADPH dependent [9]. AdhA from *T. ethanolicus* JW200 was also purified by Holt et al. who found similar cofactor specificity [23]. AdhA and AdhB were purified from *Thermoanaerobacter pseudoethanolicus* 39E by Burdette et al. [10]. In contrast to *T. ethanolicus*, the AdhA in *T. pseudoethanolicus* 39E has both NADH- and NADPH-dependent activity [10]. AdhB in *T. pseudoethanolicus* 39E was characterized as a bifunctional alcohol and aldehyde dehydrogenase by Burdette et al. [10]. This property of AdhB was subsequently confirmed by Pei et al. [31] in *T. ethanolicus* JW200 as well. However, Pei et al. found that AdhB in *T. ethanolicus* JW200 only had acetaldehyde reduction activity (no acetyl-CoA reduction) under physiological conditions [31].

Despite the characterization of these enzymes, the physiological roles of these enzymes during ethanol formation have not been well studied. Targeted genetic modification is a powerful tool for this kind of study. Yao et al. [46] used targeted gene deletion to study the roles of AdhA and AdhB in ethanol formation in *T. mathranii*. They deleted *adhA* and *adhB* separately and found these strains had a similar ethanol yield compared to the wild type. However, no double or multiple deletions mutants were constructed to further study the roles of each enzyme in ethanol formation. In recent years, significant progress has been made in the development of genetic tools in thermophilic bacteria [29, 35, 37], which allows us to study their physiology [27, 36, 51] as well as to engineer them for applied goals, such as improving ethanol production [14, 38]. With the help of these genetic tools, we aimed to answer the following two questions about alcohol dehydrogenases in *T. ethanolicus* JW200:

1. Among the three alcohol dehydrogenases, which ones are responsible for ALDH activity and which ones are responsible for ADH activity?
2. Is either of the allegedly bifunctional enzymes (AdhB or AdhE) individually able to produce ethanol from acetyl-CoA in vivo?

Materials and methods

Strains, media and growth conditions

Thermoanaerobacter ethanolicus JW200 (ATCC 31550) was obtained from ATCC. All mutants constructed in this study are listed in Table 1.

Genetic modifications of *T. ethanolicus* were performed in CTFUD medium [51] with or without 0.8% (w/v) agar with an initial pH of 7 in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI). For fermentation products analysis, growth of *T. ethanolicus* was performed in MTC-6 medium [51] with addition of 4.5 g/l yeast extract and an initial pH of 7.4. Medium was sterilized through a 0.22 μm filter (Corning, Tewksbury, MA). All fermentation experiments were done in 125 ml serum bottles at 65 °C with a 50 ml working volume, shaking at 250 rpm. To make a nitrogen atmosphere in the serum bottles for fermentation experiments, bottles were vacuumed and then purged by ultra-high purity nitrogen gas for 45 s and this sequence was repeated for 20 cycles. Fermentations were allowed to proceed for 72 h, at which point samples were collected for analysis (see "Analytical techniques" section below).

Specific growth rates and maximal OD of all strains were determined in a 96-well plate incubated at 65 °C in the absence of oxygen as previously described [30]. Each well contained 200 μl MTC-6 medium, which was the

Table 1 Strains and their genotypes

T. ethanolicus Strain	Lynd lab strain ID	Description	Accession number
Wild type	LL1204	Wild type strain, ATCC 31550	SRX2139340
DelA	LL1205	Δ*adhA*::*kan*	SRX2139334
DelB	LL1206	Δ*adhB*::*kan*, *adhE*$^{\#}$	SRX2139335
DelE	LL1207	Δ*adhE*::*kan*	SRX2139341
DelAB	LL1215	Δ*adhA*::*kan*, Δ*adhB*::*tm*	SRX2139086
DelBA	LL1257	Δ*adhB*::*kan,* Δ*adhA*::*tm*, *adhE*$^{\#}$	N/A
DelEA	LL1217	Δ*adhE*::*kan*, Δ*adhA*::*tm*	SRX2139088
DelBE	LL1216	Δ*adhB*::*kan*, Δ*adhE*::*tm*	SRX2139087
DelEAB	LL1379	Δ*adhE*::*kan*, Δ*adhA*::*tm,* Δ*adhB*::*htk*	N/A

Table 2 List of primers used in this study

Name	Sequence	Function
p1	ACATTAACCTATAAAAATAGGCGTATCACGAGATGCATCAGAATATGCGTGAAAGTACCG	*adhA* upstream fwd
p2	CTTACCTATCACCTCAAATGGTTCGCTGGGTTTTACGCAGCCCACACTTTAATTACCTCC	*adhA* upstream rev
p3	TAATCTTTTCTGAAGTACATCCGCAACTGTCCATACTCCAGAACTACGCCTTCTCTGGAC	*adhA* downstream fwd
p4	TCCCCGCGCGTTGGCCGATTCATTAATGATGCATCAGCCATACCTATCTTCACTGCATCT	*adhA* downstream rev
p5	ATTAACCTATAAAAATAGGCGTATCACGAGATGCATCAGACATTCCCCTCTATTATGCCA	*adhB* upstream fwd
p6	TACCTATCACCTCAAATGGTTCGCTGGGTTTTACGCAGTCCAACCGACTTTACCGATACT	*adhB* upstream rev
p7	AATCTTTTCTGAAGTACATCCGCAACTGTCCATACTCCAGATCCTTCCAAACTCGTCACT	*adhB* downstream fwd
p8	GCCTCTCCCCGCGCGTTGGCCGATTCATTAATGATGCATCAGTTTTGAGCCATCGTCGGT	*adhB* downstream rev
p9	ACCTATAAAAATAGGCGTATCACGAGATGCATCAGACTTTTTTATCTCAATCCCCCCTCC	*adhE* upstream fwd
p10	ACCTATCACCTCAAATGGTTCGCTGGGTTTTACGCAGACATCCAGCGTTTCTTTCACTTC	*adhE* upstream rev
p11	CTAATCTTTTCTGAAGTACATCCGCAACTGTCCATACTCCAGCCCAAAGCAGCCGAGAAA	*adhE* downstream fwd
p12	CCCGCGCGTTGGCCGATTCATTAATGATGCATCAGACAGGAAACTACACAAAAACTCGGA	*adhE* downstream rev
p13	AATTCTCTTACTGTCATGCC	Deletion PCR product fwd
p14	GAGAAAGGCGGACAGGTA	Deletion PCR product rev

same recipe as used in the fermentation product analysis. The plate was shaken for 30 s every 3 min, following by measuring the optical density at 600 nm. Specific growth rate was calculated by exponential curve fitting of optical density as a function of time.

Construction of vectors and PCR products for target gene deletion

All deletion vectors used in this study were derived from plasmid pZJ23 [51], in which the erythromycin gene was replaced with a kanamycin resistance gene (*kan*) to create pZJ24. The kanamycin resistance gene in pZJ24 was replaced with a thiamphenicol resistance gene (*tm*) or a high temperature kanamycin resistance gene (*htk*) to create pZJ25 or pZJ26, respectively. The *htk* marker was developed by Hoseki et al. for kanamycin selection at temperatures up to 72 °C [24]. The backbone plasmids were digested by *Pvu*II and column purified. The upstream and downstream regions of the target gene were amplified from *T. ethanolicus* by PCR. The upstream and downstream PCR products were gel-purified. The purified PCR products and the digested backbone plasmid were assembled by Gibson Assembly [18]. Gene deletion PCR products were amplified directly from the Gibson Assembly mixture, column purified, and transformed into target strains. Table 2 shows a list of primers used in this study.

Transformation and mutant selection in *T. ethanolicus* JW200

Transformation was performed via a natural competence protocol as described previously [37]. For selection of mutants with DNA integrated onto the genome by homologous recombination, up to 250 µl transformed culture was mixed with 20 ml CTFUD medium with 0.8% (w/v) agar supplemented with 400 mg/l kanamycin sulfate or 10 mg/l thiamphenicol, poured into a Falcon 100 mm petri dish (Corning, Tewksbury, MA), and then incubated at 55 °C (*kan* and *tm* gene selection) or 65 °C (*htk gene* selection) after solidification. Colonies usually appeared within 2–3 days. Several colonies were analyzed by PCR

to confirm that the desired mutation was present. The general strategy for genetic manipulation of *T. ethanolicus* is described previously [35].

Preparation of cell extract and enzyme assays

Thermoanaerobacter ethanolicus cells were grown in CTFUD medium with an initial pH at 7.4 in an anaerobic chamber (COY labs, Grass Lake, MI) at 65 °C, and harvested in the exponential phase of growth. The procedure of preparing cell-free extract was performed as previously reported [51].

The acetyl-CoA reduction activity was assayed at 340 nm at 55 °C with minor modifications as described before [49, 51]. The assay mixture contained 100 mM Tris-HCl (pH 8.0), 0.3 mM NADH or NADPH and cell extract. This reaction was started by adding 0.25 mM acetyl-CoA. The acetaldehyde reduction activity was assayed under the same conditions but adding 10 mM acetaldehyde instead of acetyl-CoA. The enzyme activity was calculated as described previously [50]. One unit of enzymatic activity is equal to one μ mol of product formed per minute per mg of cell extract protein.

Genome resequencing

Unamplified libraries were generated using a modified version of Illumina's standard protocol. 100 ng of DNA was sheared to 500 bp using a focused-ultrasonicator (Covaris, Woburn, MA). The sheared DNA fragments were size selected using SPRI beads (Beckman Coulter). The selected fragments were then end-repaired, A-tailed, and ligated to Illumina compatible adapters (IDT,Coralville, IA) using KAPA-Illumina library creation kit (KAPA Biosystems, Wilmington, MA). Libraries were quantified using KAPA Biosystem's next-generation sequencing library qPCR kit and run on a Roche LightCycler 480 real-time PCR instrument. The quantified libraries were then multiplexed into pools for sequencing. The pools were loaded and sequenced on the Illumina MiSeq sequencing platform utilizing a MiSeq Reagent Kit v2 (300 cycles) following a 2 × 150 indexed run recipe.

Genomic DNA was submitted to the Joint Genome Institute (JGI) for sequencing with an Illumina MiSeq instrument. Paired-end reads were generated, with an average read length of 150 bp and paired distance of 500 bp. Raw data was analyzed using CLC Genomics Workbench, version 7.5 (Qiagen, USA). First reads were mapped to the reference genome (NC_017992). Mapping was improved by two rounds of local realignment. The CLC Probabilistic Variant Detection algorithm was used to determine small mutations (single and multiple nucleotide polymorphisms, short insertions and short deletions). Variants occurring in less than 90% of the reads and variants that were identical to those of the wild-type strain (i.e., due to errors in the reference sequence) were filtered out.

Analytical techniques

Fermentation products: cellobiose, glucose, acetate, lactate, formate, pyruvate, succinate, malate and ethanol were analyzed by a Waters (Milford, MA) high pressure liquid chromatography (HPLC) system with an Aminex HPX-87H column (Bio-Rad, Hercules, CA). The column was eluted at 60 °C with 0.25 g/l H_2SO_4 at a flow rate of 0.6 ml/min. Cellobiose, glucose, acetate, lactate, formate, succinate, malate and ethanol were detected by a Waters 410 refractive-index detector and pyruvate was detected by a Waters 2487 UV detector. Sample collection and processing were as reported previously [47].

Carbon from cell pellets were determined by elemental analysis with a TOC-V CPH and TNM-I analyzer (Shimadzu, Kyoto, Japan) operated by TOC-Control V software. Fermentation samples were prepared as described with small modifications [42]. A 1 ml sample was centrifuged to remove supernatant at 21,130*g* for 5 min at room temperature. The cell pellet was washed twice with MilliQ water. After washing, the pellet was resuspended in a TOCN 25 ml glass vial containing 19.5 ml MilliQ water. The vials were then analyzed by the TOC-V CPH and TNM-I analyzer.

Hydrogen was determined by gas chromatography using a Model 310 SRI Instruments (Torrence, CA) gas chromatograph with a HayeSep D packed column using a thermal conductivity detector and nitrogen carrier gas. The nitrogen flow rate was 8.2 ml/min.

The carbon and electron balances were determined as previously described [51].

Phylogenetic analysis and protein domain analysis

Evolutionary analysis was conducted in MEGA6 [40]. The protein sequence alignment was conducted using the MUSCLE algorithm [15]. The evolutionary history of alcohol dehydrogenase was inferred using the maximum likelihood method based on a Poisson model with Gamma-distributed rates. The tree with the highest log likelihood (−15078) is shown. Initial trees for the heuristic search were obtained by applying default Neighbor-Join and BioNJ algorithms. Trees were drawn to scale, with branch lengths measured as the number of substitutions per site. Branch length is displayed above the branch and those lengths shorter than 0.01 were hidden.

Protein domain analysis was performed using the Pfam database [17]. Protein sequences of alcohol dehydrogenases were submitted and searched directly using the default search methodology.

Results and discussion

Deletion of *adhA*, *adhB* separately and in combination

To illustrate the roles of *adhA* and *adhB* in ethanol formation, we first deleted *adhA* and *adhB* separately. These two strains were named DelA and DelB, respectively. Surprisingly, both DelA and DelB strains showed a significant increase in ethanol production (Table 2). Ethanol yields increased from 43% of the theoretical maximum (wild type) to 74% (DelA) and 78% (DelB) in the deletion strains. Enzyme assay data showed that both strains still had high NADH-ALDH activity and NADPH-ADH activity (Fig. 2). But the level of ADH activity in the two mutants was different. In the DelA strain, ADH activity increased and was almost five times that of the wild-type strain, whereas in DelB strain, it was 20% lower than that of the wild type. Based on these results, we do not have a good explanation for the phenomenon that a single deletion (either *adhA* or *adhB*) increased ethanol yield. The low biomass (as measured by pellet carbon) is due to the cell lysis in the stationary phase of DelB strain (Table 3). The rapid cell lysis in *Thermoanaerobacter* and *Thermoanaerobacterium* genera was also reported previously when substrate concentration was high or after genetic modifications [4, 20, 21]. But so far, the mechanism has not been determined. Likely, it is a similar mechanism to that of autolysis that has been observed in *Clostridium acetobutylicum* [13, 26].

Next, we decided to investigate the effect of a double deletion of *adhA* and *adhB*. After resequencing the DelB strain, we found a SNP (resulting in the G558D mutation in the amino acid sequence) and a six base pair deletion (starting from position 1838 of the wild type *adhE* gene, GCGGTA was deleted) in the *adhE* gene of the DelB strain. We subsequently refer to an *adhE* gene carrying both of these mutations as *adhE*$^{\#}$. Thus, we made two versions of the double deletion strain: deleting *adhB* in the *adhA* deletion strain and deleting *adhA* in the *adhB* deletion strain, resulting in strains DelAB (no mutations in *adhE*) and DelBA (*adhE*$^{\#}$ mutations are present) (Fig. 1). Both strains showed similar enzyme activity with very little change in ALDH activity and a substantial decline in ADH activity (Fig. 2). Two minor differences were observed between DelAB and DelBA: the presence of low, but detectable, NADPH-linked ALDH activity and a slightly faster growth rate was observed in DelBA. In fact, the detectable NADPH-linked ALDH activity is also present in the DelB strain and is correlated with the *adhE*$^{\#}$ mutation.

One of the reasons for deleting *adhA* and *adhB* simultaneously was to allow us to observe the physiological role of *adhE* in relative isolation. In the DelAB deletion strain, ALDH activity was unchanged, but ADH activity was reduced by 90%, which was almost the same level of ADH activity of the DelEAB strain (Fig. 2). This suggests that AdhE is responsible for almost none of the observed NADPH-linked ADH activity. Although AdhE proteins from other thermophilic bacteria usually have ADH activity [27, 46, 49], our result is in agreement with that of Pei et al. who also found ADH activity only at very low levels in AdhE from *T. ethanolicus* [31]. According to their study, NADH-dependent ADH activity from AdhE is only 1% of its ALDH activity, which would have been below the limit of detection in our study. Therefore, the primary role of AdhE in *T. ethanolicus* seems to be catalyzing acetyl-CoA reduction. Previously, Peng et al. [32] reported the NADH-ALDH activity in cell extracts of 0.17 ± 0.02, which is in the same order of the value 0.41 ± 0.05 reported in this study. They also did not report NADH-ADH activity in the cell extract. The lack of ADH activity from AdhE protein in cell extract is interesting. AdhE from *T. ethanolicus* is closely related to other AdhEs from *T. saccharolyticum*, *T. mathranii* or *C. thermocellum* (Fig. 3), but other AdhEs all have detectable ADH activity in cell extract [46, 49]. Based on a comparison of the AdhE protein sequence from various organisms, we did not see any obvious evidence for the lack of ADH activity.

Another interesting result is that both DelAB and DelBA had very limited ADH activity under the enzyme assay conditions tested, but they can still produce a considerable amount of ethanol, though lower than the wild-type level. One possible explanation is that both strains produce ethanol very slowly, but reasonable titers accumulate over the course of their slow growth. Another possibility is that the ADH activity of AdhE was activated in vivo, but is not active under in vitro condition (possibly due to the absence of a necessary activator or suboptimal assay conditions).

Deletion of *adhE* and combining it with deletion of *adhA* and *adhB*

We deleted the *adhE* gene to determine its role in ethanol production. This resulted in a loss of ALDH activity (Fig. 2) and a ~90% decrease in ethanol yield, which is similar to what is observed when this gene has been deleted in other organisms such as *C. thermocellum* and *T. saccharolyticum* [27], however, neither of these organisms had the *adhB* gene. Previously, it has been claimed that AdhB is a bifunctional enzyme with ALDH and ADH activities, and therefore could theoretically substitute for AdhE [10, 31] Although the results of the *adhE* deletion indicated that this was not likely the case, we constructed a few additional strains for confirmation. First, we constructed the DelEA strain, by deleting *adhA* in the DelE strain (Fig. 1). In this strain, AdhB should be the only enzyme available to mediate the conversion of acetyl-CoA to ethanol, however, ethanol production was only observed at very low

Table 3 Fermentation profile of wild-type and alcohol dehydrogenase mutants

Strain	Fermentation profile[a] Unit: mmol in 50 ml culture									Growth curve measurement in plate reader (in 200 µl culture)	
	Cellobiose consumed	Lactate	Acetate	Ethanol	Pellet Carbon	Hydrogen	Carbon recovery (%)	Electron recovery (%)	% of theoretical ethanol yield (%)	Growth rate (/h)	Maximal OD_{600}
Wild type	0.66	1.24	0.36	1.18	0.60	0.68	115	113	45	0.34	1.13
DelA (Δ*adhA*)	0.66	0.28	0.31	1.95	0.48	0.64	106	104	74	0.23	0.98
DelB (Δ*adhB*)	0.66	0.19	0.28	2.04	0.22	0.58	101	100	78	0.32	1.05
DelE (Δ*adhE*)	0.66	0.93	1.28	0.15	0.91	2.44	103	99	6	0.25	0.94
DelBE (Δ*adhB* Δ*adhE*)	0.66	0.89	1.20	0.12	0.68	2.21	95	91	5	0.12	0.79
DelEA (Δ*adhE* Δ*adhA*)	0.66	1.23	0.96	0.11	0.94	1.93	101	99	4	0.14	0.65
DelAB (Δ*adhA* Δ*adhB*)	0.66	0.77	0.80	0.79	0.42	1.71	98	97	30	0.18	1.01
DelBA (Δ*adhB* Δ*adhA*)	0.66	0.68	0.82	0.92	0.67	1.59	105	102	35	0.23	1.12
DelEAB (Δ*adhB* Δ*adhA* Δ*adhE*)	0.66	1.38	0.91	0.10	0.89	1.72	105	102	4	0.13	0.78

[a] Amount of fermentation end-products are reported in millimoles from 50 ml fermentation. The standard deviations were less than 10% for cellobiose, lactate, acetate, ethanol, which were measured by HPLC. Malate, formate, pyruvate and succinate were also analyzed by HPLC, but the amounts were below the limit of detection. For pellet carbon and hydrogen measurement, the standard deviation was less than 2%. The calculated carbon recovery and electron recovery has a combined standard deviation less than 5%. The growth rate has a standard deviation less than 10%

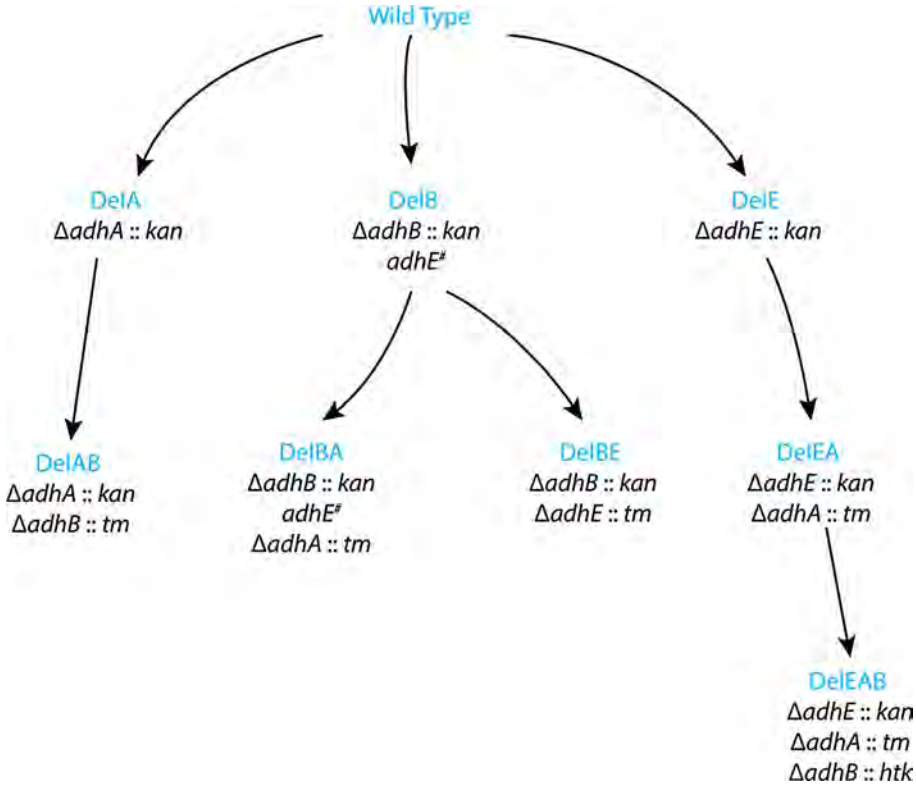


Fig. 1 Lineage of strains of *T. ethanolicus* generated in this study. *adhE*$^{\#}$ is the *adhE* mutant originally found in the DelB strain. It contains a SNP (resulting in the G558D mutation in the amino acid sequence) and six base pair deletion in the *adhE* gene of the DelB strain. Strain names are indicated in *blue*, genotypes in *black*. Mutations are listed in the order of genetic introduction

levels (Table 3), suggesting that AdhB was not able to substitute for AdhE. This was further confirmed by the lack of ALDH activity as measured by enzyme assay (Fig. 2). In the DelEA strain, we subsequently deleted *adhB*. This additional deletion had almost no effect on fermentation products. However, there was a tenfold decrease in NADPH-linked ADH activity (Fig. 2). Since there is no change on the remaining NADPH-dependent ADH activity after comparing the enzyme assay data of the DelEAB strain with that of DelAB and DelBA strains, this activity in these three strains may come from potential ADHs other than AdhA, AdhB and AdhE (Fig. 4).

The remaining ethanol produced by Δ*adhE* mutants may come from the conversion of chemical compounds in yeast extract to acetaldehyde. To avoid this problem, we tried to eliminate yeast extract from the medium recipe, but were unsuccessful in finding a recipe that did not have yeast extract but could still support growth. There are several compounds in yeast extract, however, that could be converted to acetaldehyde and subsequently to ethanol. To check these possibilities, we searched the KEGG reaction database for potential acetaldehyde-producing reactions. *T. ethanolicus* JW200 is not in the KEGG database, so homology searches for JW200 were based on sequences from the closely related *T. pseudoethanolicus* (which *is* listed in KEGG). We think there are three potential pathways for producing acetaldehyde from yeast extract. The first pathway is catalyzed threonine aldolase (EC 4.1.2.5).

Threonine → glycine + acetaldehyde

The enzyme is putatively encoded by Teth_0186 in *T. ethanolicus* JW200. Furthermore, aspartate and lysine can be converted to threonine. Therefore, all of these compounds in yeast extract potentially can be converted to glycine and acetaldehyde. According to a report from BD biosciences [3], the amount of aspartic acid in yeast extract ranges from 4.8 to 5.9%; the amount of lysine ranges from 3.8 to 4.9% and the amount of threonine ranges from 1.6 to 1.8%. Based on our medium composition (4.5 g/l yeast extract), these compounds can be converted to acetaldehyde in the range of 0.170 mmol and 0.209 mmol, which are more than the amount of ethanol that *adhE* deletion strains produced. To test this hypothesis, we grew the DelE strain in medium with additional threonine (2 g/l) or lysine (2 g/l); however, we did not see any additional ethanol production.

The second option is the reaction catalyzed by 2-deoxy-D-ribose-5-phosphate acetaldehyde-lyase (EC 4.1.2.4). This enzyme is putatively encoded by Teth_0647. The reaction is shown as below:

2-Deoxy-D-ribose-5-phosphate → D-Glyceraldehyde-3-phosphate + acetaldehyde

2-Deoxy-D-ribose-5-phosphate is involved in the pentose phosphate pathway. Yeast extract contains carbohydrate in the range of 6–16%, but the exact percentage of C5 sugars is unknown. If we assume that all carbohydrates in yeast extract are 2-deoxy-5-ribose, we can estimate the upper limit for ethanol yield to be 0.269 mmol.

Fig. 3 Phylogenetic analysis of multiple alcohol dehydrogenases in ▶ *T. ethanolicus* JW200 (Teth), *T. saccharolyticum* (Tsac), *Thermoanaerobacter* sp. X514 (Teth514), *T. Brockii* (TheBr), *T. mathranii* (Tmath), *T. kivui* (Tkv), *T. tengcongensis* (Tte) and *C. thermocellum* DSM1313 (Clo1313). AdhA, AdhB and AdhE in these organisms are labeled by gene number. Distance indicates number of substitutions per site

The third option is the reaction catalyzed by acetate: ferredoxin oxidoreductase (AOR). This reaction is shown as below:

Acetate + 2 ferredoxin (red) + 2 H^+ → acetaldehyde + 2 ferredoxin (ox) + acetaldehyde + H_2O

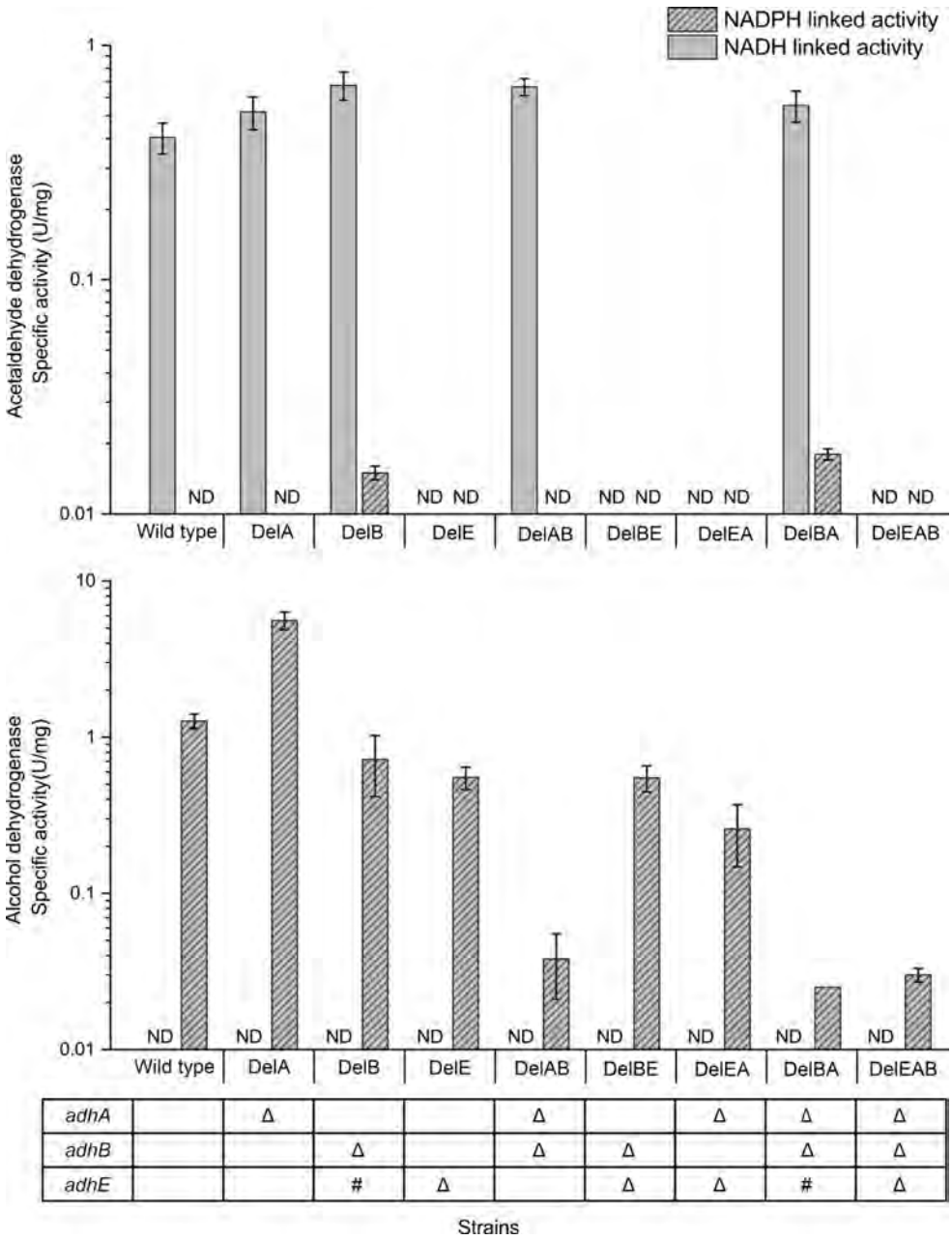
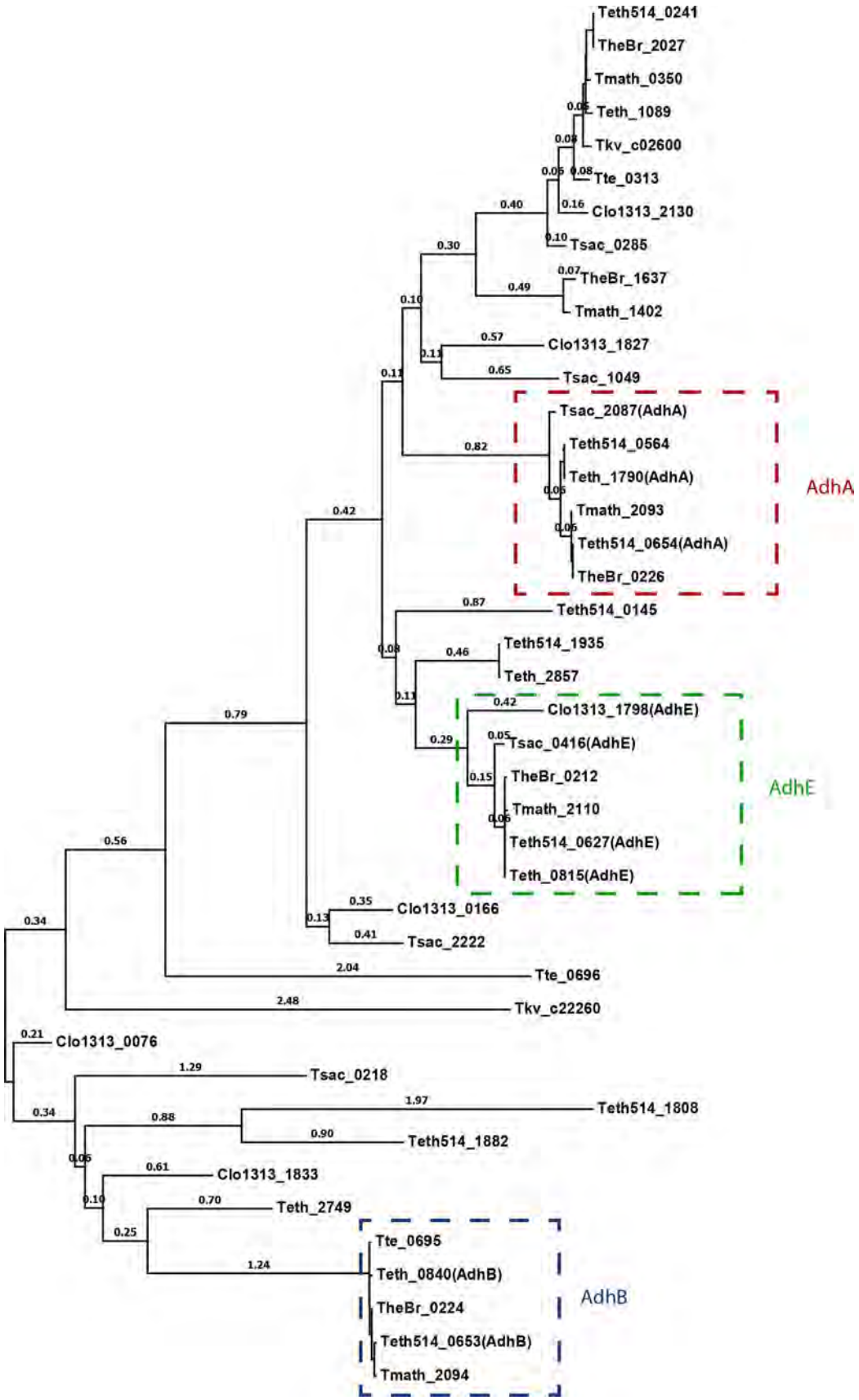


Fig. 2 Acetaldehyde dehydrogenase and alcohol dehydrogenase activity in wild type and mutants. The table below figures displays the genotype of the corresponding mutants. *Closed triangle* indicates that the gene is deleted in the mutant. *Hash* represents the mutant *adhE*, which contains a SNP (resulting in the G448D mutation in the amino acid sequence) and six base pair deletion


Teth514_0241
TheBr_2027
Tmath_0350
Teth_1089
Tkv_c02600
Tte_0313
Clo1313_2130
Tsac_0285
TheBr_1637
Tmath_1402
Clo1313_1827
Tsac_1049
Tsac_2087(AdhA)
Teth514_0564
Teth_1790(AdhA)
Tmath_2093
Teth514_0654(AdhA)
TheBr_0226
AdhA
Teth514_0145
Teth514_1935
Teth_2857
Clo1313_1798(AdhE)
Tsac_0416(AdhE)
TheBr_0212
Tmath_2110
Teth514_0627(AdhE)
Teth_0815(AdhE)
AdhE
Clo1313_0166
Tsac_2222
Tte_0696
Tkv_c22260
Clo1313_0076
Tsac_0218
Teth514_1808
Teth514_1882
Clo1313_1833
Teth_2749
Tte_0695
Teth_0840(AdhB)
TheBr_0224
Teth514_0653(AdhB)
Tmath_2094
AdhB

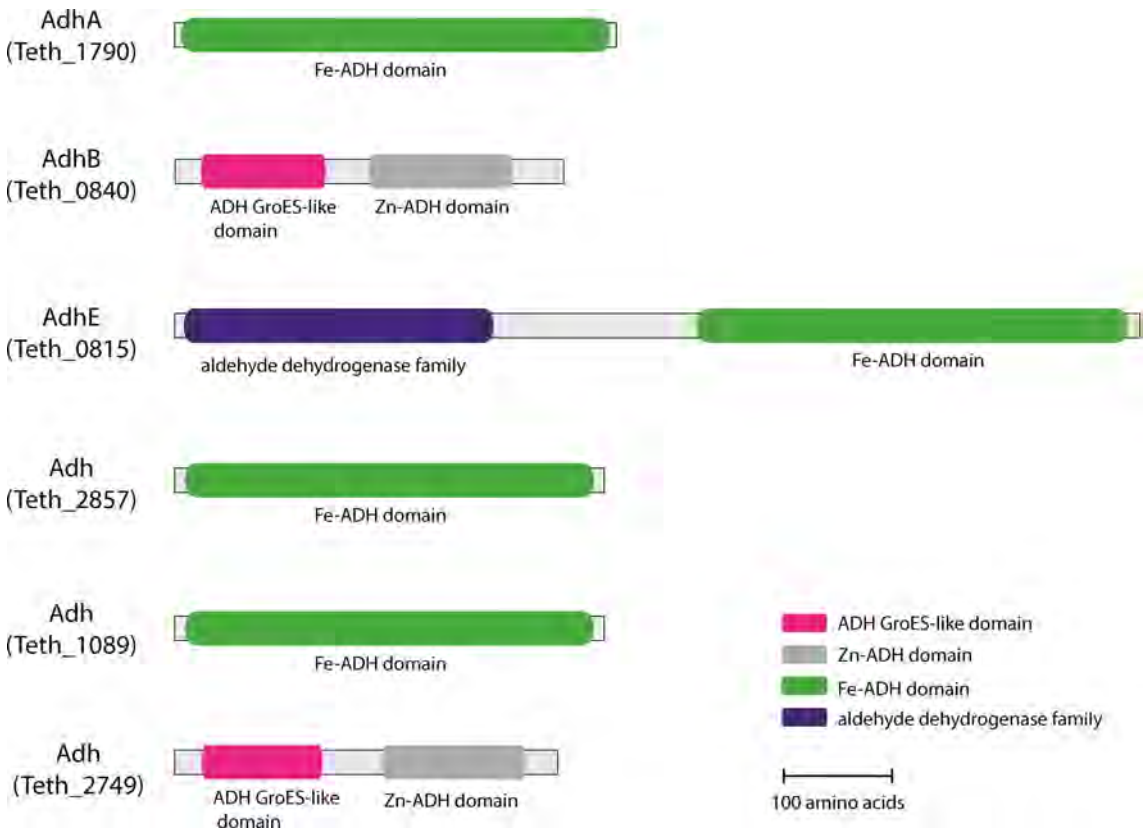


Fig. 4 Protein domain analysis of alcohol dehydrogenases in *T. ethanolicus* JW200. Protein domains were determined by Pfam. Gene numbers labeled in the parenthesis are the gene encoding corresponding enzymes. *Colors* indicate different domain families

This reaction has been found in hyperthermophilic archaea, such as *Pyrococcus furiosus* [5] and *Thermococcus* strain ES-1 [19], and has been used for the production of ethanol in *P. furiosus* [2]. Although we did not find a putative *aor* gene in *T. ethanolicus*, it is possible that this conversion could be mediated by a novel enzyme.

From the fermentation results, there is another interesting phenomenon that we observed. Although DelEA and DelBE had similar low ethanol yields due to the absence of *adhE*, their acetate and lactate yields were quite different. The lactate/acetate ratio for DelEA was 1.28, while the value for DelBE was 0.74 (Table 2). Previously, Wang et al. found that the 2-ADH encoded by Tte_0695 from *T. tengcongensis*, which is 97% identical to AdhB in *T. ethanolicus*, was present in two forms, pyruvate ferredoxin oxidoreductase (Pfor)-bound and Pfor free, in *Thermoanaerobacter tengcongensis* [43]. They further showed that AdhB was inhibited by additional Pfor. One possible explanation for our results is that AdhB has an inhibitory effect on Pfor, and thus strains where AdhB is present produce more lactate.

Phylogenetic tree and protein domain analysis of ADHs in thermophiles

To understand the relationship between the various ADH enzymes we are studying, we performed a phylogenetic analysis on all annotated ADH enzymes from *T. ethanolicus* JW200, *T. pseudoethanolicus* 39E, *Thermoanaerobacter* sp. X514, *T. saccharolyticum* JW/SL-YS485, and *C. thermocellum* DSM1313 (Fig. 3). The resulting phylogenetic tree indicates that AdhA, AdhB and AdhE form distinct clusters.

To understand why AdhA and AdhE are closely related, we analyzed their protein domain structure using the Pfam database (Fig. 4). This indicated AdhA and AdhE have similarities in their ADH domains (Fe-ADH, according to Pfam), whereas AdhB has a GroES domain and Zinc-ADH domain, neither of which are present in the other two proteins. Although AdhE has distinct ALDH and ADH domains, AdhB only has the ADH domain, which supports our hypothesis that the small amount of ALDH activity observed by others for this enzyme [31] represents a side activity of the ADH domain. The AdhB in *T. ethanolicus*

has 97% identity, at the amino acid level, with NADP-dependent alcohol dehydrogenase from *T. brockii*, and this enzyme has not been reported to be bifunctional [6, 25]. Therefore, although purified AdhB has been previously reported as a bifunctional alcohol and aldehyde dehydrogenase enzyme, its ALDH activity (if it exists) is too low to have any physiological relevance.

Conclusions

In this study, we deleted *adhA*, *adhB* and *adhE* in *T. ethanolicus* JW200 separately and in combination to study their roles during ethanol formation. Although AdhB and AdhE were identified as bifunctional alcohol dehydrogenases, AdhB was primarily responsible for acetaldehyde reduction and AdhE was responsible for acetyl-CoA reduction. Unlike AdhE, which has separate domains for catalyzing acetaldehyde reduction and acetyl-CoA reduction, AdhB only has one domain, and it is annotated as an ADH. The acetyl-CoA reduction activity previously reported for AdhB may be a side activity of the ADH domain. Neither of these bifunctional alcohol dehydrogenases was sufficient for ethanol production at high yield. The physiological roles of AdhA and AdhB appear to be similar: both provide ADH activity to the cell. The fact that single deletions of *adhA* or *adhB* actually improve ethanol production remains an interesting mystery for future investigation.

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