Expression of *adhA* from different organisms in *Clostridium thermocellum*

Tianyong Zheng\textsuperscript{1,2,†}, Jingxuan Cui\textsuperscript{1,2,†}, Hye Ri Bae\textsuperscript{2,3}, Lee R. Lynd\textsuperscript{1,2,3,*} and Daniel G. Olson\textsuperscript{2,3,*}

**Abstract**

**Background**: *Clostridium thermocellum* is a cellulolytic anaerobic thermophile that is a promising candidate for consolidated bioprocessing of lignocellulosic biomass into biofuels such as ethanol. It was previously shown that expressing *Thermoanaerobacterium saccharolyticum* *adhA* in *C. thermocellum* increases ethanol yield. In this study, we investigated expression of *adhA* genes from different organisms in *Clostridium thermocellum*.

**Methods**: Based on sequence identity to *T. saccharolyticum* *adhA*, we chose *adhA* genes from 10 other organisms: *Clostridium botulinum*, *Methanocaldococcus bathoardescens*, *Thermoanaerobacterium ethanolicus*, *Thermoanaerobacter mathranii*, *Thermococcus* strain AN1, *Thermoanaerobacterium thermosaccharolyticum*, *Caldicellulosiruptor saccharolyticus*, *Fervidobacterium nodosum*, *Marinitoga piezophila*, and *Thermotoga petrophila*. All 11 *adhA* genes (including *T. saccharolyticum* *adhA*) were expressed in *C. thermocellum* and fermentation end products were analyzed.

**Results**: All 11 *adhA* genes increased *C. thermocellum* ethanol yield compared to the empty-vector control. *C. botulinum* and *T. ethanolicus* *adhA* genes generated significantly higher ethanol yield than *T. saccharolyticum* *adhA*.

**Conclusion**: Our results indicated that expressing *adhA* is an effective method of increasing ethanol yield in wild-type *C. thermocellum*, and that this appears to be a general property of *adhA* genes.

**Keywords**: Consolidating bioprocessing, *Clostridium thermocellum*, Alcohol dehydrogenase, *adhA*, Biofuel, Ethanol

---

**Background**

*Clostridium thermocellum* is a cellulolytic anaerobic thermophile that is considered to be a promising candidate for consolidated bioprocessing of lignocellulosic biomass, into biofuels such as ethanol, due to its native ability to solubilize lignocellulose [1]. A key limitation of this organism is that it produces ethanol only at low yield (20% of the theoretical maximum) [2]. Strategies to increase ethanol yield in *C. thermocellum* include deleting the pathways for acetic acid, lactic acid, and hydrogen production [3–6], and introducing heterologous genes from ethanol production pathways in other organisms [2, 7], such as *Thermoanaerobacterium saccharolyticum*. Recently, it was shown that AdhA plays an important role in ethanol production in strains of *T. saccharolyticum* engineered for homoethanol production [8]. This enzyme was subsequently expressed in *C. thermocellum* and shown to increase ethanol yield and titer by 40% [3]. In this study, we chose *adhA* genes from 10 additional organisms, expressed them in *C. thermocellum* and observed the effect on ethanol production.

**Methods**

**Plasmid and strain construction**

Plasmids used for *adhA* expression in *C. thermocellum* are listed in Table 1. Plasmids were constructed based on the *C. thermocellum* expression plasmid pDGO144 as previously described [9]. The Clo1313\_2638 promoter [9] and *adhA* gene were cloned into the HindIII site of pDGO144 using standard molecular biology techniques. The correct reading frame and sequence of each *adhA* gene in the resulting plasmids in Table 1 were confirmed by Sanger Sequencing (GENEWIZ). Complex medium CT\_UD [10] was used to culture wild-type *C. thermocellum*.
Plasmids expressing \textit{adhA} genes were transformed into wild-type \textit{C. thermocellum} using the transformation protocol as previously described [10]. Selection was carried out using thiamphenicol at a final concentration of 6 µg/ml. Single colonies were picked and re-inoculated into CTFÜD medium containing 6 µg/ml thiamphenicol; cultures were saved for further analysis. The presence of \textit{adhA} genes in the cultures was confirmed by PCR. Primers used for the confirmation are Fwd: GACGAAAAAGCCGATGAAG, Rev: CCTTTTTTAAAAGTCCCG. The size of the PCR product was used to confirm \textit{adhA} insertion: the PCR product of the empty vector is 178 bp, and the PCR product containing the \textit{adhA} gene insertion is ~1400 bp (with slight variation due to differences in lengths of the \textit{adhA} genes).

Fermentations and end-product analysis

For fermentation end-product analysis, strains were transferred three times in defined MTC-5 medium [11] with 4.7 g/l cellobiose at 1% inoculum (v/v). End-product measurements were taken on the 3rd transfer. Cultures were grown in Corning™ Falcon™ 15 ml Conical Centrifuge Tubes and incubated anaerobically without shaking at 55 °C for 72 h. Upon harvesting, cultures were prepared as previously described for HPLC (High-Pressure Liquid Chromatography) analysis [8]. Ethanol yield was calculated as the percentage of theoretical yield based on the amount of ethanol produced and substrate consumed:

$$\text{Yield ethanol (\% maximum theoretical)} = \frac{\text{Amount of ethanol produced (mM)}}{4 \times \text{Amount of cellobiose consumed (mM)}} \times 100$$

Carbon balance was calculated based on the fermentation products measured as previously described [12]; Carbon balance (\% ) = \frac{[\text{Acetate}]+[\text{Ethanol}]+[\text{Lactate}](\text{mM})}{4 \times (\text{cellobiose consumed (mM)})}.

Phylogenetic analysis

The amino acid sequences of different AdhA proteins were aligned using CLC Main Workbench 7.7.3, and a phylogenetic tree was created using the Neighbor Joining algorithm. Distance is expressed as substitutions per 100 amino acids; multiple substitutions at the same site were corrected for using the Kimura method. Bootstrap analysis was performed with 1000 replicates.

Results and discussion

\textit{adhA} genes from different organisms

Sequences with homology to the \textit{T. saccharolyticum} AdhA were searched using the BLAST (Basic Local Alignment Search Tool) algorithm [13]. AdhA sequences from different organisms were chosen based on protein sequence identity to \textit{T. saccharolyticum} AdhA, with an identity range of 57–90% (Table 1).

---

### Table 1 Strains and plasmids used in this work

| Strain ID  | Plasmid ID     | Source of \textit{adhA} on the plasmid | Source organism abbreviation | Sequence identity to \textit{Tsac} AdhA | Source organism optimal growth temperature (°C) | GenBank accession number |
|------------|----------------|----------------------------------------|------------------------------|------------------------------------------|------------------------------------------------|--------------------------|
| LL1525     | pCBcth1        | \textit{Thermoanaerobacter mathanii}   | Tmat                         | 86%                                      | 70–75 [17]                                       | MG026506                |
| LL1526     | pCBcth2        | \textit{Thermoanaerobacterium ethanolicus} | Teth                         | 88%                                      | 70 [18]                                         | MG026510                |
| LL1527     | pCBcth3        | \textit{Clostridium botulinum}         | Cbot                         | 62%                                      | 37 [19]                                         | MG026514                |
| LL1528     | pCBcth4        | \textit{Thermococcus strain AN1}       | Ther                         | 65%                                      | 75–80 [20]                                       | MG026513                |
| LL1529     | pCBcth7        | \textit{Thermatoga petrophila}         | Tpet                         | 60%                                      | 80 [21]                                         | MG026511                |
| LL1530     | pCBcth8        | \textit{Methanocaldococcus barkeri}    | Mbak                         | 57%                                      | 82 [22]                                         | MG026508                |
| LL1531     | pCBcth9        | \textit{Marinobacter pizziophila}      | Mpie                         | 61%                                      | 65 [23]                                         | MG026515                |
| LL1532     | pCBcth12       | \textit{Thermoanaerobacterium thermosaccharolyticum} | Tthe                        | 90%                                      | 68 [24]                                         | MG026507                |
| LL1533     | pCBcth13       | \textit{Ferriavidobacterium nodosum}   | Fnod                         | 67%                                      | 65–70 [25]                                       | MG026509                |
| LL1534     | pCBcth14       | \textit{Caldicellulosiruptor saccharolyticus} | Csac                       | 76%                                      | 70 [26]                                         | MG026512                |
| LL1535     | pDGO144 [9]*   | NA                                     | NA                           | NA                                       | 55                                              | MG026515                |
| LL1536     | pCBcth17       | \textit{Thermoanaerobacterium saccharolyticum} | Tsac                       | 100%                                     | 60 [27]                                         | MG026516                |

NA not applicable

* The empty vector pDGO144 is also known as pCBcth15
Most of the selected organisms were thermophilic bacteria with an optimal growth temperature greater than 50 °C as presented in Table 1. *Clostridium botulinum*, a mesophilic bacterium that grows at 37 °C, was also chosen with the intention of exploring the heat stability of AdhA. A phylogenetic tree of AdhA proteins used in this study is presented in Fig. 1.

Fermentation behavior of *C. thermocellum* strains expressing different *adhA* genes

The 11 *adhA* genes described above, including *T. saccharolyticum adhA*, were cloned into expression plasmid pDGO144 and expressed in wild-type *C. thermocellum*. Fermentation results for all of the strains are presented in Table 2. Wild-type *C. thermocellum*

![Phylogenetic tree of the AdhA proteins and ethanol yield for 11 *C. thermocellum* strains expressing *adhA* genes from different organisms.](image)

**Table 2** Fermentation end products of *C. thermocellum* strains expressing different *adhA* genes

| Strain ID | Source of *adhA* | Ethanol mM | Acetate mM | Formate mM | Lactate mM | Malate mM | Ethanol yield (% maximum theoretical) | Carbon balance (%) |
|-----------|-----------------|------------|------------|------------|------------|-----------|-------------------------------------|-------------------|
| LL1527    | Cbot            | 32.16 ± 0.57 | 6.73 ± 0.90 | 3.51 ± 0.43 | 0.25 ± 0.04 | 0.56 ± 0.40 | 59                                    | 71                |
| LL1526    | Teth            | 26.27 ± 1.00 | 9.48 ± 1.64 | 8.78 ± 1.85 | 0.33 ± 0.01 | 0.39 ± 0.12 | 49                                    | 65                |
| LL1525    | Tmat            | 24.33 ± 2.50 | 10.07 ± 1.47 | 9.59 ± 1.11 | 0.51 ± 0.21 | 0.44 ± 0.18 | 45                                    | 63                |
| LL1529    | Tpet            | 24.07 ± 0.68 | 12.81 ± 0.74 | 4.82 ± 0.91 | 0.87 ± 0.12 | 0.50 ± 0.08 | 45                                    | 68                |
| LL1530    | Mbat            | 23.23 ± 1.56 | 12.30 ± 1.56 | 7.75 ± 1.99 | 0.94 ± 0.19 | 0.55 ± 0.10 | 43                                    | 66                |
| LL1536    | Tsac            | 23.23 ± 0.62 | 9.76 ± 0.67 | 7.53 ± 0.43 | 0.83 ± 0.00 | 0.65 ± 0.39 | 43                                    | 61                |
| LL1528    | Ther            | 23.21 ± 0.58 | 10.58 ± 0.43 | 8.22 ± 0.41 | 0.71 ± 0.06 | 0.42 ± 0.01 | 42                                    | 63                |
| LL1532    | Tthe            | 21.09 ± 0.37 | 12.95 ± 1.04 | 8.43 ± 1.53 | 0.63 ± 0.06 | 0.51 ± 0.13 | 39                                    | 63                |
| LL1534    | Csac            | 20.29 ± 0.35 | 12.93 ± 0.64 | 7.56 ± 0.46 | 0.74 ± 0.14 | 0.45 ± 0.03 | 38                                    | 62                |
| LL1531    | Mpie            | 20.26 ± 0.60 | 15.27 ± 0.27 | 9.08 ± 0.72 | 0.54 ± 0.06 | 0.63 ± 0.07 | 38                                    | 65                |
| LL1533    | Fnod            | 19.65 ± 1.71 | 14.21 ± 0.57 | 7.14 ± 1.88 | 1.32 ± 0.52 | 0.85 ± 0.12 | 36                                    | 64                |
| LL1535    | NA              | 15.67 ± 0.22 | 16.52 ± 0.94 | 3.04 ± 0.47 | 3.01 ± 1.17 | 0.98 ± 0.11 | 29                                    | 64                |

Data shown here were based on triplicate experiments. Cultures were grown in MTC-5 medium with 4.7 g/l (13.8 mM) initial cellobiose at 55 °C for 72 h. All cultures completely consumed all of the cellobiose initially present in the medium. Thiamphenicol was added at 6 μg/ml for plasmid maintenance. Error is given as one standard deviation, *n* = 3. Rows are ordered by ethanol yield in descending order.
haboring the empty pDGO144 plasmid was used as a negative control strain. Ethanol yield was calculated based on the amount of ethanol produced from the amount of cellobiose consumed. Two-tailed unpaired T tests were performed on the ethanol yields of the strains with three biological replicates to assess statistical significance. To evaluate the effect of expressing adhA genes in *C. thermocellum*, ethanol yield for each strain was compared to the empty vector negative control. The strain expressing *T. saccharolyticum adhA*, LL1536, had significantly higher ethanol yield than the empty vector control (*p* < 0.0001), agreeing with previous results [2]. The other 10 strains expressing adhA genes all had significantly higher ethanol yield compared to the empty-vector control strain (*p* < 0.05). When compared to the positive control that expressed *T. saccharolyticum adhA* (LL1536), two strains exhibited significantly higher ethanol yield: Strain LL1527 expressing *C. botulinum adhA* (*p* = 0.0001) and strain LL1526 expressing *T. ethanolicus adhA* (*p* = 0.0353) (Fig. 1). The top two AdhAs in terms of increasing ethanol yield appeared to be evolutionarily distant from each other: *C. botulinum* and *T. ethanolicus*, and we did not observe any correlation between sequence similarity and effect on ethanol production. In general, most of the additional ethanol production came at the expense of acetate production (Table 2). This is consistent with other reports indicating that there appears to be an oversupply of NADPH in *C. thermocellum* [14, 15], and that this can be used to divert C2 flux (i.e., acetyl-CoA) to ethanol in the presence of an NADPH-linked ADH enzyme [8, 9, 16]. Lactate and malate were minor fermentation products. Carbon balances were calculated based on the fermentation end products measured in this study, and they were generally 65–75% closed. The remaining 25–35% of the substrate carbon is likely present in biomass or un-measured fermentation products such as amino acids.

**Conclusions**

Our results indicate that expressing adhA is an effective method of increasing ethanol yield in wild-type *C. thermocellum*, and that this appears to be a general property of adhA genes, rather than a property specific to the adhA gene from *T. saccharolyticum*. Although most of the adhAs studied in this work are from thermophiles, the largest increase in ethanol production came from the adhA gene from *C. botulinum*, a mesophile with an optimal growth temperature of 37 °C.

**Authors’ contributions**

TZ, and DGO conceived the study. HB built the plasmids and strains in this study, and performed preliminary fermentation experiments under supervision of TZ. JC carried out fermentation studies, performed phylogenetic analysis and generated all tables and figures. TZ and JC drafted the manuscript, together with DGO and LRL, who also supervised this study.

**Author details**

1. Department of Biological Sciences, Dartmouth College, Hanover, NH 03755, USA. 2. Bioenergy Science Center, Oak Ridge National Laboratory, Oak Ridge, TN 37830, USA. 3. Thayer School of Engineering, Dartmouth College, 14 Engineering Drive, Hanover, NH 03755, USA.

**Acknowledgements**

This work is supported by the BioEnergy Science Center (BESC), a US Department of Energy (DOE) Bioenergy Research Center supported by the Office of Biological and Environmental Research in the DOE Office of Science. Notice: this manuscript has been authored by Dartmouth College under Contract No. DE-AC05-00OR22725 with US Department of Energy. The US Government and the publisher, by accepting the article for publication, acknowledges that the US Government retains a non-exclusive, paid-up, irrevocable worldwide license to publish or reproduce the published form of this manuscript or allow others to do so, for US Government purposes.

**Competing interests**

Lee R. Lynd is a founder of the Enchi Corporation, which has a financial interest in *Clostridium thermocellum*.

**Publisher’s Note**

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

**Received: 7 August 2017 Accepted: 19 October 2017**

**Published online: 30 November 2017**

**References**

1. Olson DG, McBride JE, Joe Shaw A, Lynd LR. Recent progress in consolidated bioprocessing. Curr Opin Biotechnol. 2012;23:396–405. doi:10.1016/j.copbio.2011.11.026.
2. Hon S, Olson DG, Holwerda BK, Lanahan AA, Murphy SJ, Maloney MJ, et al. The ethanol pathway from *Thermoanaerobacterium saccharolyticum* improves ethanol production in *Clostridium thermocellum*. Metab Eng. 2017;42:175–84. doi:10.1016/j.ymben.2017.06.011.
3. Argyros DA, Tripathi SA, Barrett TF, Rogers SR, Feinberg LF, Olson DG, et al. High ethanol titers from cellulose by using metabolically engineered thermophilic, anaerobic microbes. Appl Environ Microbiol. 2011;77:8288–94.
4. Rydzak T, Lynd LR, Guss AM. Elimination of formate production in *Clostridium thermocellum*. J Ind Microbiol Biotechnol. 2015;42:1263–72.
5. Biswas R, Wilson CM, Zheng T, Giannone RJ, Dawn M. Elimination of hydrogenase active site assembly blocks H2 production and increases ethanol yield in *Clostridium thermocellum*. Biotechnol Biofuels. 2015;8:20.
6. Papanek B, Biswas R, Rydzak T, Guss AM. Elimination of metabolic pathways to all traditional fermentation products increases ethanol yields in *Clostridium thermocellum*. Metab Eng. 2015;32:49–54. doi:10.1016/j.ymben.2015.09.002.
7. Background EA, Benzman M, Russo A, Hochman S, Weinhouse H, Bisswanger H, et al. Redirecting carbon flux through exogenous pyruvate kinase to achieve high ethanol yields in *Clostridium thermocellum*. Metab Eng. 2013;15:151–8. doi:10.1016/j.ymben.2012.11.006.
8. Zheng T, Olson DG, Murphy SJ-L, Shao X, Tian L, Lynd LR. Both adhE and a separate NADPH-dependent dehydrogenase gene, adhA, are necessary for high ethanol production in *Thermoanaerobacterium saccharolyticum*. J Bacteriol. 2017;199:1–10.
9. Hon S, Lanahan AA, Tian L, Giannone RJ, Hettrich RL, Olson DG, et al. Development of a plasmid-based expression system in *Clostridium thermocellum* and its use to screen heterologous expression of bifunctional alcohol dehydrogenases (adhE). Metab Eng Commun. 2016;3:120–9.
10. Olson DG, Lynd LR. Transformation of *Clostridium thermocellum* by electroporation. Methods Enzymol. 2012;510:317–30.
11. Zhang YH, Lynd LR. Quantification of cell and cellulase mass concentrations during anaerobic cellulose fermentation: development of an enzyme-linked immunosorbent assay-based method with application to Clostridium thermocellum batch cultures. Anal Chem. 2003;75:219–27.
12. Ellis LD, Holwerda EK, Hogsett D, Rogers S, Shao X, Tschapinski T, et al. Closing the carbon balance for fermentation by Clostridium thermocellum (ATCC 27405). Bioreour Technol. 2012;103:293–9. doi:10.1016/j.biortech.2011.09.128.
13. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215:403–10.
14. van der Veen D, Lo J, Brown SD, Johnson CM, Tschaplinski TJ, Martin M, et al. Characterization of Clostridium thermocellum strains with disrupted fermentation end-product pathways. J Ind Microbiol Biotechnol. 2013;40:725–34.
15. Olson DG, Hörl M, Fuhrer T, Cui J, Zhou J, Maloney ML, et al. Glycolysis without pyruvate kinase in Clostridium thermocellum. Metab Eng. 2016;2016(39):169–80.
16. Zheng T, Olsen DG, Tian L, Bambol YJ, Himmel ME, Lo J, et al. Cofactor specificity of the bifunctional alcohol and aldehyde dehydrogenase (AdhE) in wild-type and mutants of Clostridium thermocellum and Thermoanaerobacterium saccharolyticum. J Bacteriol. 2015;197:2610–9. doi:10.1128/JB.00232-15.
17. Klinke H, Thomsen A, Ahring B. Potential inhibitors from wet oxidation of wheat straw and their effect on growth and ethanol production by Thermoanaerobacter mathranii. Appl Microbiol Biotechnol. 2001;57:631–8.
18. Pei J, Zhou Q, Jiang Y, Le Y, Li H, Shao W, et al. Thermoanaerobacter spp. control ethanol pathway via transcriptional regulation and versatility of key enzymes. Metab Eng. 2010;12:420–8. doi:10.1016/j.ymben.2010.06.001.
19. Tanner FW, Oglesby EW. Influence of temperature on growth and toxin production by Clostridium botulinum. Food Res. 1936;1:481–94.
20. Li D, Stevenson KJ. Alcohol dehydrogenase from Thermococcus strain AN1. Methods Enzymol. 2001;331:201–7. doi:10.1016/S0076-6879(01)31058-3.
21. Takahata Y, Nishijima M, Hoaki T, Maruyama T. Thermotoga petrophila sp. nov. and Thermotoga naphthophila sp. nov., two hyperthermophilic bacteria from the Kubiki oil reservoir in Niigata, Japan. Int J Syst Evol Microbiol. 2001;51:1901–9.
22. Stewart LC, Jung JH, Kim YT, Kwon SW, Park CS, Holden JF. Methanocaldococcus bethoarencens sp. Nov., a hyperthermophilic methanogen isolated from a volcanically active deep-sea hydrothermal vent. Int J Syst Evol Microbiol. 2015;65:1280–3.
23. Alain K, Marteinnsson VT, Miroshnickenko ML, Onch-Osmolovskaya E, Prieur D, Birrien J-L. Marinimonas piezophilic sp. nov., a rod-shaped, thermopiezophilic bacterium isolated under high hydrostatic pressure from a deep-sea hydrothermal vent. Int J Syst Evol Microbiol. 2002;52:1331–9.
24. Hoster F, Danie R, Gottschalk G. Isolation of a new Thermotoga bacterium thermacidophilus strain (FH1) producing a thermostable dextranase. J Gen Appl Microbiol. 2001;47:187–92.
25. Patel BKC, Morgan HW, Daniel RM. Fervidobacterium nodosum gen. nov. and spec. nov., a new thermoorganotrophic, caldoactive, anaerobic bacterium. Arch Microbiol. 1985;141:63–9.
26. Willquist K, Van Niel EW. Growth and hydrogen production characteristics of Coldicellulosiruptor saccharolyticus on chemically defined minimal media. Int J Hydrogen Energy. 2012;37:4925–9. doi:10.1016/j.ijhydene.2011.12.055.
27. Mai V, Lorenz WW, Wiegel J. Transformation of Thermotoga bacterium sp. strain JAW/SY5385 with plasmid pKM1 conferring kanamycin resistance. FEMS Microbiol Lett. 1997;148:163–7.