RESEARCH

Expression of adhA from different organisms in Clostridium thermocellum

Tianyong Zheng1,2†, Jingxuan Cui1,2†, Hye Ri Bae2,3, Lee R. Lynd1,2,3* and Daniel G. Olson2,3*

Abstract

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. 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 bethoardescens, Thermoanaerobacterium ethanolicus, Thermoanaerobacter mathranii, Thermococcus strain AN1, Thermoanaerobacterium thermosaccharolyticum, Caldicellulosiruptor saccharolyticus, Fervidobacterium nodosum, Mariniloga 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 CTFÜD [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 CTFUD 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: CCTTTTTTAAAAGTCAATCCCG. 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: 
\[
\text{Yield ethanol (\% maximum theoretical)} = \left(\frac{\text{Amount of ethanol produced (mM)}}{4 \times \text{Amount of cellobiose consumed (mM)}}\right) 
\]
Carbon balance was calculated based on the fermentation products measured as previously described [12]:
\[
\text{Carbon balance (\%) } = \left(\frac{\text{[Acetate]} + \text{[Ethanol]} + \text{[Lactate]} (\text{mM})}{4 \times \text{[cellobiose consumed]} (\text{mM})}\right) 
\]

**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).
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 expressing the empty vector pDGO144 (LL1535) was used as negative control (indicated in red), and the strain expressing T. saccharolyticum adhA was used as positive control (indicated in green). All of the other 10 strains were shown as the experimental group (indicated in gray). Ethanol yields of experimental group strains were compared to the positive control using a two-tailed unpaired t test, and p values are reported where significant.

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

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

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.

Fig. 1  Phylogenetic tree of the AdhA proteins and ethanol yield for 11 C. thermocellum strains expressing adhA genes from different organisms. a The distance-based phylogenetic tree generated from the alignments of the AdhA proteins. Ethanol yield for all of the adhA-expressing strains. Strains were cultured in MTC-5 medium containing 4.7 g/l cellobiose and 6 μg/ml thiamphenicol at 55 °C for 72 h. The maximum theoretical yield is 4 mol of ethanol per mole of cellobiose consumed. Data were collected from triplicate experiments. Error bars represent one standard deviation. Wild-type C. thermocellum expressing the empty vector pDGO144 (LL1535) was used as negative control (indicated in red), and the strain expressing T. saccharolyticum adhA was used as positive control (indicated in green). All of the other 10 strains were shown as the experimental group (indicated in gray). Ethanol yields of experimental group strains were compared to the positive control using a two-tailed unpaired t test, and p values are reported where significant.
harboring the empty pDG0144 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 Biology 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**