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# Development of a plasmid-based expression system in *Clostridium thermocellum* and its use to screen heterologous expression of bifunctional alcohol dehydrogenases (*adhE*s)

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ABSTRACT

*Clostridium thermocellum* is a promising candidate for ethanol production from cellulosic biomass, but requires metabolic engineering to improve ethanol yield. A key gene in the ethanol production pathway is the bifunctional aldehyde and alcohol dehydrogenase, *adhE*. To explore the effects of overexpressing wild-type, mutant, and exogenous *adhE*s, we developed a new expression plasmid, pDGO144, that exhibited improved transformation efficiency and better gene expression than its predecessor, pDGO-66. This new expression plasmid will allow for many other metabolic engineering and basic research efforts in *C. thermocellum*. As proof of concept, we used this plasmid to express 12 different *adhE* genes (both wild type and mutant) from several organisms. Ethanol production varied between clones immediately after transformation, but tended to converge to a single value after several rounds of serial transfer. The previously described mutant *C. thermocellum* D494G *adhE* gave the best ethanol production, which is consistent with previously published results.



## 1. Introduction

*Clostridium thermocellum* is a good candidate for producing biofuels from cellulosic biomass via consolidated bioprocessing (Olson et al., 2012). This microorganism is among the most effective described at solubilizing lignocellulose (Lynd et al., 2002), and ferments glucose and glucan oligomers to organic acids, hydrogen, and ethanol. In recent years, there have been attempts (Argyros et al., 2011; Biswas et al., 2015, 2014; Deng et al., 2013; Papanek et al., 2015) at engineering *C. thermocellum* to produce ethanol as the sole product at high yield; these attempts thus far have fallen short of the high yields achieved by conventional ethanol producers such as yeast and *Zymomonas*.

Of the existing and reported genetic engineering efforts in *C. thermocellum*, most have taken the approach of gene deletions (Argyros et al., 2011; Biswas et al., 2015; Olson et al., 2010; Papanek et al., 2015; Rydzak et al., 2015; Tripathi et al., 2010; van der Veen et al., 2013). There have been a few reports of gene expression, or over expression, in *C. thermocellum* (Deng et al., 2013; Lo et al., 2015; Olson et al., 2013; Zheng et al., 2015), but methodologies are in general less well developed than for gene deletion. One example related to metabolic engineering is the expression of the *Thermoanaerobacterium saccharolyticum* pyruvate kinase in *C. thermocellum* (Deng et al., 2013). Another example is the complementing of *adhE* activity in *C. thermocellum adhE* deletion strain (Lo et al., 2015; Zheng et al., 2015). In these cases, gene expression was achieved via targeted recombination of the gene of interest onto the chromosome, a process that takes several weeks under ideal conditions (Olson and Lynd, 2012a).

Plasmid-based gene expression, on the other hand, can be performed in a single step, and therefore lends itself to higher throughput metabolic engineering applications and thus is especially relevant during screening processes. Related prior work includes an attempt to complement the *cipA* deletion in *C. thermocellum*, and resulted in partial (~33% of wild type) restoration of Avicel solubilization (Olson et al., 2013). Efforts to identify native *C. thermocellum* promoters for use in expressing genes encountered issues with obtaining consistent and reliable results with reporter enzyme activities (Olson et al., 2015).

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Here, we report improvements to a *C. thermocellum* expression plasmid, and use this improved plasmid to screen a variety of different *adhE*s for improved ethanol production in the *C. thermocellum adhE* deletion strain, LL1111.

## 2. Materials and methods

### 2.1. Plasmid and strain construction

Table 1 lists the strains and plasmids used or generated in this study; Table S1 lists the primers used in this study. Plasmids were constructed via the isothermal assembly method (Gibson, 2011), using a commercial kit sold by New England Biolabs (Gibson Assembly® Master Mix, product catalog number E2611). DNA purification was performed using commercially available kits from Qiagen (Qiagen catalog number 27,106) or Zymo Research (Zymo Research catalog numbers D4002 and D4006). Transformation of *C. thermocellum* was performed using previously described methods (Olson and Lynd, 2012a); all plasmid DNA intended for transforming into *C. thermocellum* was propagated and purified from *Escherichia coli* BL21 derivative strains (New England Biolabs catalog number C2566) to ensure proper methylation of plasmid DNA (Guss et al., 2012).

### 2.2. Re-designing the expression plasmid

Fig. 1 and S1 shows the features of the various expression plasmids and the intermediates. We first removed the *Pvu*II cloning site on our older expression plasmid, pDGO-66, in favor of a multiple cloning site (MCS), and inserted this MCS to the intergenic region between replication initiator gene *repB* and the thiamphenicol resistance gene, *cat* (Olson and Lynd, 2012b), thus placing the gene of interest between two genes that are essential for plasmid selection. We also eliminated the *gapDH* promoter from the plasmid to allow us the flexibility to use different promoters. The resulting plasmid was named pDGO125. A single-strand origin of replication (SSO) (Boe et al., 1989) was also added upstream of the double-strand origin of replication (DSO) in pDGO125, as there was no canonical SSO in plasmid pDGO-66; the resulting plasmid was named pDGO126. We later identified a promoter region upstream of the *cat* gene that we had disrupted with the MCS in plasmids pDGO125 and pDGO126; we thus moved the MCS to be upstream of the cat promoter region in both plasmids to generate pDGO125cat and pDGO126cat. Lastly, a 27 bp "insulator" sequence was introduced into plasmids pDGO125cat and pDGO126cat between the MCS and the *cat* promoter region, resulting in plasmids pDGO143 and pDGO144, respectively. All *adhE* expression plasmids used the Clo1313_2638 promoter (Olson et al., 2015) to drive expression of the *adhE* gene. Both the promoter and gene were cloned into the HindIII site at the MCS in plasmid pDGO144.

### 2.3. Determining the segregational and structural stability of plasmids

Plasmids were transformed into *C. thermocellum* strain LL1004 (wild type), colonies were picked, and the presence of the plasmid was verified by PCR with primers XSH0210 and XSH0211. To determine plasmid structural stability after transformation into *C. thermocellum*, plasmid DNA was isolated from transformants and analyzed by PCR and restriction digestion. To determine segregational stability, cultures of *C. thermocellum* strain LL1004 bearing the respective plasmids were grown with or without thiamphenicol selection, and the fraction of plasmid-containing colonies was determined by dilution plating, with and without thiamphenicol selection. Plasmid DNA from *C. thermocellum* was prepared using the Qiagen DNA miniprep kit, with the added step of incubating the harvested and re-suspended cells with Epicentre Ready-Lyse™ lysozyme solution (Epicentre catalog number R1804m) at 37 °C for 30 min in buffer P1, before proceeding with the rest of the miniprep protocol, following the instructions of the manufacturer.

### 2.4. Media and growth conditions

All chemicals were of molecular grade, and were obtained from either Sigma Aldrich or Fisher Scientific, unless otherwise specified. *C. thermocellum* strains were grown in anaerobic chambers (Coy Laboratory Products, Grass Lakes, MI, USA) at 55 °C, with the hydrogen concentrations in the chamber maintained at greater than 1.5%. Two media formulations were used, with both containing 5 g/L cellobiose (Sigma C7252) as the primary carbon source: complex medium CTFÜD (Olson and Lynd, 2012a) with initial pH of 7.0 (pH measured at room temperature) was used for growing competent *C. thermocellum* cells for transformation, as well as for recovery post-electroporation and initial plasmid tests. Defined medium MTC (Ozkan et al., 2001; Zhang and Lynd, 2003) with initial pH of 7.4 at room temperature was used to determine ethanol production from the various *adhE*s. Where needed, thiamphenicol dissolved in dimethyl sulfoxide (DMSO) was added to the cultures to a final concentration of 6 μg/ml. When switching strains from CTFÜD medium to MTC medium, the strains were transferred 3 times at a 1:100 dilution each time to remove any yeast extract carried over from the CTFÜD medium.

### 2.5. Biochemical assays

Cultures for the ethanol and cellobiose assays were inoculated with 2% inoculum, and then grown anaerobically at 55 °C for 72 h. Cells were pelleted by centrifugation (5 min at $>20{,}000\,g$), and the supernatant was used in the assays. The concentration of ethanol in the cultures was determined via ADH enzyme assay in the acetaldehyde and NADH-producing direction (Bisswanger, 2011). The reaction had the following component concentrations: 67 mM sodium pyrophosphate, 20 mM glycine, 1 mM semicarbazide, 8.3 mM $NAD^+$, and 0.1 U/ml alcohol dehydrogenase enzyme (Sigma A3263); 20 μL of sample was used in a 200 μL reaction volume. The reactions were followed on a microplate reader by monitoring the increase in absorbance at 340 nm (i.e. NADH accumulation) and comparing the results against known standards.

Cellobiose assays were adapted from glucose determination assays (Bisswanger, 2011) in that a beta-glucosidase (Novozymes 188, formerly sold by Sigma as product C6105) was included in the reaction mixture. The reaction was followed on a microplate reader by monitoring the increase in absorbance at 340 nm (i.e. NADPH accumulation). Reaction rates were determined from a linear region of the absorbance curve; standard curves were generated using solutions with known cellobiose concentrations.

### 2.6. Measuring adhE expression

*adhE* expression was measured via reverse transcription quantitative PCR (RT-qPCR). Strains were cultured in 5 ml MTC-5 defined medium, and harvested in log-phase (OD600 0.6–0.8); 0.6 ml aliquots of the cell cultures were immediately treated with RNA protect Bacteria Reagent (Qiagen catalog number 76,506) and stored at −80 °C until time for RNA purification. RNA purification, cDNA synthesis, and qPCR were performed as previously described (Zhou et al., 2015); the primers used for qPCR are described in Table S1. *adhE* expression in each strain was normalized against