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Engineering the Metabolic Profile of *Clostridium cellulolyticum* with Genomic DNA Libraries

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Abstract: *Clostridium cellulolyticum* H10 (ATCC 35319) has the ability to ferment cellulosic substrates into ethanol and weak acids. The growth and alcohol production rates of the wild-type organism are low and, therefore, targets of metabolic engineering. A genomic DNA expression library was produced by a novel application of degenerate oligonucleotide primed PCR (DOP-PCR) and was serially enriched in *C. cellulolyticum* grown on cellobiose in effort to produce fast-growing and productive strains. The DNA library produced from DOP-PCR contained gene-sized DNA fragments from the *C. cellulolyticum* genome and from the metagenome of a stream bank soil sample. The resulting enrichment yielded a conserved phage structural protein fragment (part of *Ccel_2823*) from the *C. cellulolyticum* genome that, when overexpressed alone, enabled the organism to increase the ethanol yield by 250% compared to the plasmid control strain. The engineered strain showed a reduced production of lactate and a 250% increased yield of secreted pyruvate. Significant changes in growth rate were not seen in this engineered strain, and it is possible that the enriched protein fragment may be combined with the existing rational metabolic engineering strategies to yield further high-performing cellulolytic strains.

Keywords: *Clostridium cellulolyticum*; cellulosome; ethanol; DNA library; consolidated bioprocessing

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1. Introduction

Lignocellulose has been used as a fermentation feed stock for value-added products such as: hydrogen (H_2) [1]; antibiotics [2]; isobutanol [3]; acetone, butanol, and ethanol (ABE) [4]; and several of the critical chemical targets identified by the US Department of Energy [5]. The effective incorporation of lignocellulose into traditional bioprocessing fermentations has been predicated on the advancement of consolidated bioprocessing (CBP), an engineering approach in which microorganisms simultaneously digest lignocellulose and ferment liberated simple sugars [6–8].

The Gram positive anaerobe *Clostridium cellulolyticum* H10 ATCC 35319 (recently renamed *Ruminiclostridium cellulolyticum* H10) is of interest to CBP due to its ability to breakdown and consume crystalline cellulose under mesophilic conditions [9–14]. *C. cellulolyticum* hydrolyzes cellulose through the use of a cellulosome, and ethanol is a major fermentation product. Additionally, *C. cellulolyticum* consumes five-carbon sugars [15]. The industrial potential of this organism is hampered by a slow growth rate, which also results in the secretion of the valuable central carbon metabolite pyruvate as a byproduct. The generation (doubling) time of wild-type *C. cellulolyticum* is approximately 4 h on soluble cellobiose and 8–13 h on whole crystalline cellulose [16,17], while the alcohol production rate has been reported as 1.51 mmol $gDCW^{-1}$ h^{-1} (~7.0 mg ethanol L^{-1} hr^{-1}) in batch [18] and 1.27 mmol $gDCW^{-1}$ hr^{-1} (9.4 mg ethanol L^{-1} h^{-1}) in continuous production [19]. In comparison, the desired characteristics for industrial microbial fermentation include an alcohol tolerance greater than 40 g/L and an ethanol productivity greater than 1 g L^{-1} h^{-1} [20].

C. cellulolyticum has proven amenable to genetic engineering [21,22], and the focus of this research is to alleviate the metabolic bottleneck(s) that result in slow growth and

improve acid and alcohol tolerance through the use of combinatorial metabolic engineering by enriching genomic DNA libraries. Part of the reason for the relatively poor fermentative capabilities of *C. cellulolyticum* is believed to result from its genetic adaptation to growth in low-nutrient environments. During growth, glucose-1-phosphate and glucose-6-phosphate accumulate in *C. cellulolyticum* due to the unregulated uptake of the cellulose disaccharide, cellobiose [23]. *C. cellulolyticum* was found to respond to stress by the intracellular accumulation (and eventual secretion) of pyruvate and extended lactate production at the expense of acetate and ethanol [24]. To combat this, the targeted metabolic engineering of *C. cellulolyticum* has been used to increase the gene expression of the pyruvate-consuming enzymes pyruvate decarboxylase and alcohol dehydrogenase. The resulting phenotype was characterized by a (i) 150% increase in the cellulose consumption rate; (ii) 180% increase in biomass; (iii) 48% reduction in lactate concentrations; and (iv) 100% increase in ethanol titers [25]. One of the first trials of gene knock-out in *C. cellulolyticum* resulted in a strain capable of producing 8.5-fold as much ethanol from cellulose by silencing the genes for both lactate and malate dehydrogenases. This increase in ethanol occurred at the expense of lactate and acetate production but produced a culture with a slower growth rate [26]. The focus of the research presented herein is to improve the fermentation performance of *C. cellulolyticum* using a combinatorial metabolic engineering approach with genomic DNA libraries. The original hypothesis was that the genomic DNA library would more effectively alleviate metabolic bottlenecks that lead to pyruvate accumulation/secretion and a slow growth rate.

When a metabolic network is not well understood or when multiple genes control a phenotype synergistically, a combinatorial metabolic engineering stategy can be quite effective. This strategy requires a large mutant pool and enrichment for increased fitness under the selected culture conditions, usually in the form of an increased growth rate or tolerance to a growth inhibitor. Mutants developing solvent tolerance survive when challenged at increasing solvent concentrations, out-competing non-tolerant strains [27,28]. When no stressors are present, the cultures select for traits that produce higher growth rates or the greater utilization of a substrate in order to out-compete neighboring cells for available nutrients. Combinatorial DNA libraries were generated with chemical mutagens [29], biological mutagens [30], or the overexpression of genomic DNA fragments [31–33]. In another approach, Blouzard et al. [34] adapted the phage Tn1545 transposon to generate random genome insertion mutations in *C. cellulolyticum.* In addition, the multi-scalar analysis of library enrichments (SCALEs) technology uses combinatorial libraries along with DNA microarrays to identify DNA library fragments that become enriched (i.e., contribute to cellular fitness) during the adaptation process. Borden et al. [35,36] fractionalized the genomic DNA from *C. acetobutylicum* ATCC 824 and repackaged it into expression vectors. Then, the library was serially enriched to isolate DNA fragments conferring greater tolerance to alcohols and acids. A 16S rRNA promoter region DNA fragment was identified that increased the 1-butanol tolerance by 81%.

The traditional methods for the DNA overexpression library construction involve genomic DNA shearing, end polishing, and blunt-end ligation. To improve efficiency, we developed a novel method to generate genomic DNA libraries from multiple sources using the degenerate oligonucleotide primed PCR (DOP-PCR) amplification of nanogram quantities of DNA [37]. Here, this method was used to generate multiple genomic DNA libraries for enrichment in *C. cellulolyticum*. Genomic DNA libraries were produced from the following sources in this research: (i) *C. cellulolyticum* genomic DNA and (ii) metagenomic DNA extracted from a stream bank soil sample. While our hypothesis that the genomic DNA library would address metabolic bottlenecks and improve the growth rate of the organism ultimately proved incorrect in this research, library enrichment experiments yielded cultures with massively re-ordered metabolism and dramatically improved ethanol productivity and tolerance.

2. Materials and Methods

2.1. Strains

High-efficiency *E. coli* NEB 10-beta competent cells were obtained from New England Biolabs (Ipswitch, MA, USA) and used for library construction. *Clostridium cellulolyticum* (H10) ATCC 35319 (recently renamed *Ruminiclostridium cellulolyticum* H10) (accession CP001348.1) was obtained from the American Type Culture Collection (ATCC). All plasmids and strains are listed in Table 1.

2.2. Media and Cultivation

E. coli NEB 10-beta were grown in lysogeny broth (LB) at 37 °C and shaken at 225 rpm. Selective media contained 100 mg/L ampicillin where appropriate. *C. cellulolyticum* was grown in liquid GS-2 medium [38] (containing resazurin for oxygen detection) with 6 g/L cellobiose (Sigma-Aldrich; St. Louis, MO, USA) as the carbon source. The cellobiose solution and a mixture of Mg^{2+}, Ca^{2+}, and Fe^{3+} salts (as previously defined in [38]) were autoclaved separately at 10× concentration. Medium components, except L-cysteine, were mixed, and the pH was adjusted to 7.2. The medium was then heated until resazurin was activated, as indicated by a light pink color, and autoclaved. L-cysteine solution (20% *w/v*) was autoclaved separately and 0.5% (*v/v*) added inside a Bactron anaerobic chamber (Shel Lab; Cornelius, OR, USA). The anaerobic chamber supported a *C. cellulolyticum* culture growth and was maintained at an atmosphere of $N_2/CO_2/H_2$ (90/5/5%). Culture media were allowed to de-gas for 24 h before use. *C. cellulolyticum* was cultivated at 34 °C, and erythromycin was added as the selective reagent from a 1000× concentrated ethanol solution to 10 mg/L. Agar was added at 1.5% (*w/v*) to make solid GS-2 media plates.

2.3. DNA Manipulation

All molecular biology enzymes were obtained from New England Biolabs, unless noted otherwise. All PCR primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA). Details pertaining to all strains, plasmids, and primer sequences used in this study are given in Table 1. Plasmid pSOSlink was created from plasmid pSOS95 [39] by digestion with BamHI and EcoRI restriction enzymes, followed by ligation with T4 DNA ligase to the similarly digested multiple cloning cassette, MCS1. Plasmid pSOSGate was constructed by ligating the blunt-ended T4 polynucleotide kinase phosphorylated Gateway Cloning Cassette (Life Technologies; Grand Island, NY, USA) into EcoRV digested and Antarctic phosphatase-treated pSOSlink. The plasmid was transformed into *ccdb* Survival 2 T1™ cells (Life Technologies) selected for on 50 mg/L chloramphenicol media.

Clostridium cellulolyticum genomic DNA was harvested based on a published protocol [40] as follows. An overnight culture of 50 mL of *C. cellulolyticum* was harvested at 5000 rpm and 4 °C for 10 min in an Eppendorf 5810R centrifuge. Cells were washed twice with 10 mL solution containing EDTA (0.1 M), Tris-HCL (0.05 M), and KCl (0.5 M). The washed pellet was then digested in 4 mL of solution containing NaCl (0.1 M), EDTA (0.05 M), egg-white lysozyme (Sigma-Aldrich) (10 mg/µL), and RNase (Qiagen; Valencia, CA, USA) (40 µg/mL) for 30 min at 37 °C. Next, 300 µL of 20% SDS was added and the DNA was extracted with Tris-buffered phenol/chloroform/isoamyl alcohol (25/24/1) twice followed by a single extraction with chloroform. The DNA was precipitated with 1 volume isopropanol and 0.2 volumes of 3 M sodium acetate. The washed and dried DNA was dissolved in TE buffer overnight.

2.4. Whole-Genome Amplification by DOP-PCR

DOP-PCR DNA library construction was performed using oligonucleotide primers containing degenerate (equal probability of A/T/G/C bases) regions to allow for partial and mismatched annealing with random segments of genomic DNA. The DOP1 [37] primer (5′-TAG ACA ATG GGG CAT NNN NNN NNN ATG-3′, where N has an equal probability of being A/T/G/C) was used in a thermocycling reaction with genomic DNA and *Taq* polymerase according to previously published protocols [41,42] with modifications for

long-length inserts from bacterial genomes [37,43]. The DNA was size-separated on a 1% agarose gel and fragments above 1 kb were excised and purified in a Thermo Scientific GeneJET Gel Extraction Kit and diluted to a concentration of 50 ng/µL.

Table 1. List of cell strains, plasmids, and DNA oligonucleotides used in this research.

Strain	Genotype	Reference
Clostridium cellulolyticum (H10) ATCC 35319	Wild-type	[9]
Escherichia coli NEB 10-beta	Δ*(ara-leu) 7697 araD139 fhuA* Δ*lacX74 galK16 galE15 e14-* ϕ*80*d*lacZ*Δ*M15 recA1 relA1 endA1 nupG rpsL* (Str^R) *rph spoT1* Δ*(mrr-hsdRMS-mcrBC)*	New England Biolabs
Escherichia coli NEB 5-alpha	*fhuA2* Δ*(argF-lacZ)U169 phoA glnV44 Φ80* Δ*(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	New England Biolabs
Escherichia coli Ccdb survival 2 T1^{TM}	F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*Δ139 Δ(*ara-leu*)7697*gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G *fhu*A::*IS2*	Life Technologies
Plasmids		
Psos95	Em^r, Amp^r, *thl* promoter, ColE1 ORI, pIM13 ORI	[39]
PsosLink	Em^r, Amp^r, *thl* promoter	This research
PsosGate	Em^r, Amp^r, Cm^r, *ccdb* Gateway® Cassette	This research
pCR8/GW/TOPO TA	Linearized, Spn^r	This research
Primers		
DOP1	TAGACAATGGGGCATNNNNNNNNNATG	[37]
MCS1	TTGGGATCCCTAACTAACTAGACACTAAGTCGATAT CGACATAGTGTCTAGATAGATAGGGCGCCTTG	This research
pSOS_libF	TACGGGGTAACAGATAAACCATT	This research
pSOS_libR	GATAGATAGGGCGCCACTTA	This research
Gateway_F	ATCACAAGTTTGTACAAAAAAGC	[37]
Gateway_R	ATCACCACTTTGTACAAGAAAGC	[37]

2.5. Genomic DNA Library Construction

Linearized vector pCR^{TM}8/GW/TOPO® TA (Invitrogen) was combined with 0.5 µL of the DNA mix following manufacturer's directions and incubated for 1 h at 25 °C before transforming 2 µL into 50 µL of Clonetech® Stellar Competent Cells according to manufacturer's directions. Cells were pooled from 20 individual 50 µL transformations to generate approximately 40,000 colonies and cultured in 200 mL of LB with 50 mg/L spectinomycin to OD_{600} of 0.6 before collecting for midipreps (Gerard Biotech; Oxford, OH, USA) and creating frozen stocks (stored at −80 °C).

Plasmid DNA in pCR8 was crossed with a pSOSGate by combining 150 ng of plasmid with 150 ng of the pooled pCR8 library DNA using the Invitrogen Gateway® LR Clonase® II kit and incubating overnight at 25 °C. Aliquots of 1 µL transformation mix were used to transform ten 50 µL vials of *E. coli* NEB 5-alpha competent cells, and transformants were pooled once again, generating 100,000 mutants in LB media with ampicillin (100 mg/L) selection. Cells were grown to a late log phase and DNA was harvested using the alkaline lysis procedure [44].

2.6. Metagenomic DNA Library Construction

Metagenomic DNA from a stream bank soil sample on the Virginia Tech campus in Blacksburg, VA (USA), was collected as previously described [37]. The extracted genomic DNA was amplified by DOP-PCR and cloned into pCR^{TM}8/GW/TOPO® TA linearized plasmid to generate 80,000 mutants as described in the above methods. The plasmid library was recombined with plasmid pSOSGate using the Gateway® LR Clonase® II reaction mix and transformed into *E. coli* NEB 5-alpha to generate 120,000 mutants.

2.7. DNA Library Sequencing and Analysis

To characterize the DNA libraries, Illumina MiSeq next-generation sequencing was performed by the Virginia Bioinformatics Institute. DNA libraries were prepared for sequencing using the Apollo 324 with IntegenX reagents. DOP-PCR amplified and size-selected DNA was sheared to 350 bp in a Covaris M220 ultrasonicator and resulting fragments were read in 150 bp paired-end mode with a 5% PhiX spike. The trimming of the primed reads harboring 5′ primer sequences was performed with the FASTX toolkit with the following parameters: "Mismatches = 2" and "Frameshift = 1". The alignment of sequencing reads to the native genome was performed with Bowtie2 against the *C. cellulolyticum* genome (NCBI CP001348.1) and further analyzed with Qualimap v2.0 [45].

Sequence reads from the metagenomic soil DNA was parsed to remove trailing 5′ barcode and adapter regions from the DOP1 primer and was uploaded to the MG-RAST webserver [37,46]. Paired reads were combined and filtered for low quality but not duplicates or contaminating DNA in order to gauge a sample bias from the amplified library. The resulting analysis used BLASTX and BLASTN to compare the uploaded data to multiple nucleotide and protein databases. The results of the analysis are publicly available under accession number mgm4583655.3 on the Argonne National Labs Metagenomic-RAST server.

2.8. Electro-Transformation

A total of 25 µg of plasmid DNA was purified using PCR purification columns (ThermoFisher Scientific) and methylated with 20 U MspI methyltransferase in a 200 µL reaction volume with 32 mM S-adenosyl methionine and 20 µL MspI reaction buffer overnight at 37 °C. The reaction was purified and concentrated in a PCR product purification column to 200 ng/µL before electroporation into *C. cellulolyticum* using an established protocol [47]. Following the incubation with 10 mg/mL glycine, 50 mL of *C. cellulolyticum* culture (OD_{600} = 0.60) was washed twice and suspended in 1.5 mL of 270 mM sucrose and 5 mM $NaPO_4$ (pH 7.4). A volume of 500 µL of cells was combined with 1–2 µg of DNA and electroporated in a BioRad Gene Pulser® at 1000 V cm^{-1}, 25 µF, and 750 Ω with a time constant of 8.5–9.5 ms. Cells were immediately suspended in GS-2 medium with cellobiose and incubated overnight. After outgrowth, the representative plates were made on a solid GS-2 medium with 10 mg/L erythromycin and the remaining cells were transferred to liquid GS-2 medium with 10 mg/L erythromycin until an OD_{600} of 0.4 was reached. Then, frozen stocks were made. All transformed *C. cellulolyticum* were pooled for the outgrowth library. The pooling of 100 electro-transformed samples of the methylated library fragments in pSOSgate indicated that approximately 2000 mutants existed within the culture. The electroporation of the metagenomic DNA from the stream bank soil sample was performed to generate an additional 2000 colonies as measured by plating on GS-2 solid medium plates with 20 mg/L erythromycin.

2.9. Outgrowth and Enrichment

C. cellulolyticum cultures transformed with the *C. cellulolyticum* and metagenomic DNA libraries were grown in the liquid GS-2 medium (with erythromycin) as separate cultures. Both cultures, along with a separate control culture containing an empty pSOSlink plasmid, were grown to mid-log phase (OD 0.6) and diluted 1:1000 into static flasks containing fresh liquid GS-2 medium with erythromycin in an anaerobic environmental chamber at 34 °C. A fresh dilution was made every 24 h and both cultures were plated out after 5 re-inoculations. Individual colonies were screened and sequenced. Four highly abundant mutants on the plates were chosen for further testing. Two contained fragments from the *C. cellulolyticum* genome (fragments named "H10_phage" and "H10_Bk") and two contained fragments from the metagenomic outgrowth ("ENV_M1" and "ENV_F1"). The insert-bearing plasmids were isolated and retransformed into the unchallenged *C. cellulolyticum* cultures for further evaluation.

The cultures containing the enriched DNA fragments mentioned above and the control strain bearing the empty pSOSlink plasmid were inoculated in triplicate into 50 mL of GS-2

medium with erythromycin to a normalized OD_{600} of 0.025. Samples were taken every 12 h for three days. Samples were measured for OD_{600} and metabolites, as discussed below.

2.10. Metabolite Analysis

Concentrations of extracellular ethanol, cellobiose, lactate, and acetate were measured with a Bio-Rad Aminex® HPX-87H 300 × 7.8 mm column on a Shimadzu HPLC system with an RID-10A refractive index detector using an isocratic mobile phase of 5 mM sulfuric acid maintained at a constant flow rate of 0.5 mL/min. Whole fermentation samples were filtered through a 0.2 µm filter prior to injection. The sample injection volume was 15 µL and the retention times and concentrations of the peaks were determined from pure standard solutions.

3. Results

3.1. DNA Library Construction by DOP-PCR

The DOP-PCR amplification of genomic DNA is illustrated in Figure 1. As shown in Figure 1A, a DOP-PCR primer consists of three parts: (i) a leader sequence, (ii) a degenerate sequence, and (iii) a linker sequence. The DOP-PCR primer used in this study (DOP1) is shown in Figure 1A. The purpose of the leader sequence of this primer was to stabilize the primer and guide it to amplify the coding regions of the genome. The degenerate region provided the randomized nature of the DOP-PCR primer (equal probability of A/T/G/C bases) that allowed it to bind nearly indiscriminately throughout genomic (or metagenomic) DNA. The linker sequence served to minimize the formation of hairpins and other secondary structures in the degenerate region. These prevent certain degenerate sequence combinations from amplifying genomic DNA and bias the resulting library, in our experience. When applied to genomic DNA in PCR, DOP-PCR primers produced an array of DNA products with different sizes (Figure 1B). This size range was engineered by adjusting the melting temperature and extension time parameters of the PCR. The genomic library produced from the *C. cellulolyticum* genomic DNA and the DOP1 primer is marked by the asterisk in Figure 2B. PCR was performed with the *Taq* polymerase to produce A-tailed library products, but this step may also be performed with a proofreading polymerase and subsequent A-tailing step. TA-Assembly was found to be efficient to package the library and transfer to an engineered expression vector through commercial recombination (Figure 1C). In our case, the expression vector contained the thiolase (*thl*) promoter, a robust and commonly used promoter in clostridia [36,39,40]. Finally, the packaged library was sequenced and aligned with the *C. cellulolyticum* genome to determine the coverage and bias of the genomic library. In addition to using *C. cellulolyticum* genomic DNA, a library was also made from metagenomic DNA in prior research [37] and used in strain engineering here.

In our case, creating a genomic library from *C. cellulolyticum* genomic DNA and the DOP1 primer produced a pool of library fragments that slightly under-represented the reference *C. cellulolyticum* genome. The next-generation sequencing of the initial DNA library indicated the 76% base-by-base coverage of the reference genome at a 1× read depth and 26% of the genome represented at a 5× read depth. These results are shown in Figure 1D. More specific genome coverage data are included in Supplementary File S1. The DNA library had an aligned GC content of 39.9%, compared to 37% for the *C. cellulolyticum* reference genome. The metagenomic DNA library fragments from the soil sample were matched to the targets on the MG-RAST server with a maximum e-value cutoff of 10^{-5} and a minimum identity of 60%. This library had a GC content of 53 ± 10%, represented 4773 organisms, and contained 569,220 identifiable protein-coding regions. These data are publicly available on the MG-RAST server (ID: mgm4583655.3), and the phyla represented in the library are shown in Supplementary File S2. The metagenomic library has also been described in other research [37] when used in a different application. The details of both libraries are summarized in Table 2.

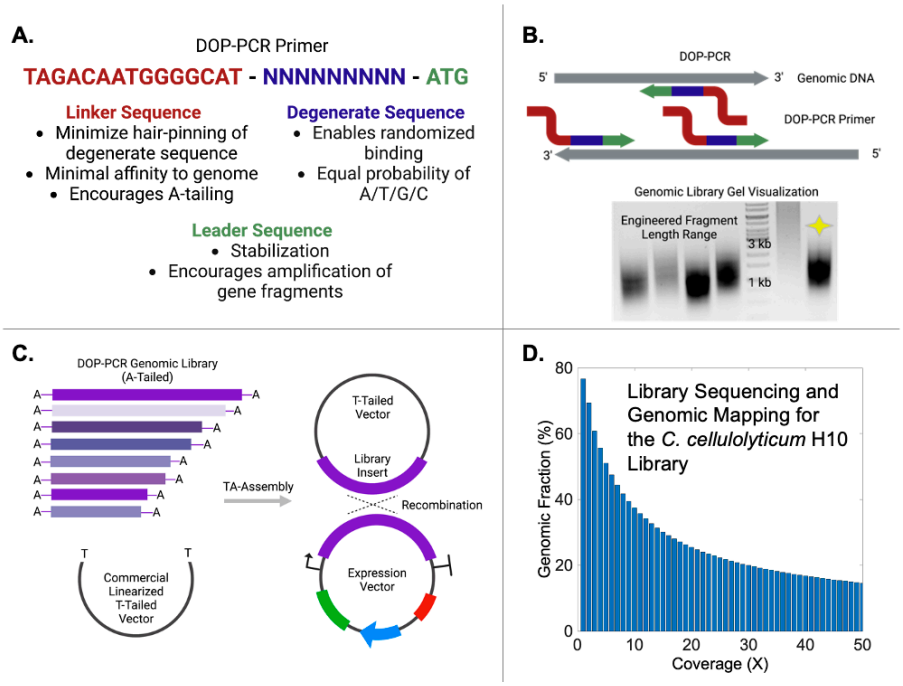

Figure 1. An overview of DOP-PCR. (**A**) The distinct sequences of a DOP-PCR primer. (**B**) Illustration of the DOP-PCR primer binding and gel visualization of engineered libraries. The *C. cellulolyticum* library is marked by asterisk. (**C**) The assembly of DNA library products into an expression vector. (**D**) Coverage of the DOP-PCR DNA library constructed from *C. cellulolyticum* genomic DNA against the reference genome (NC_011898.1). Coverage refers to the depth or number of times an individual location in the reference is found in the aligned sequencing data. A genomic fraction of 100% at 1× coverage would indicate that every base in the reference is represented at least once in the DNA library.

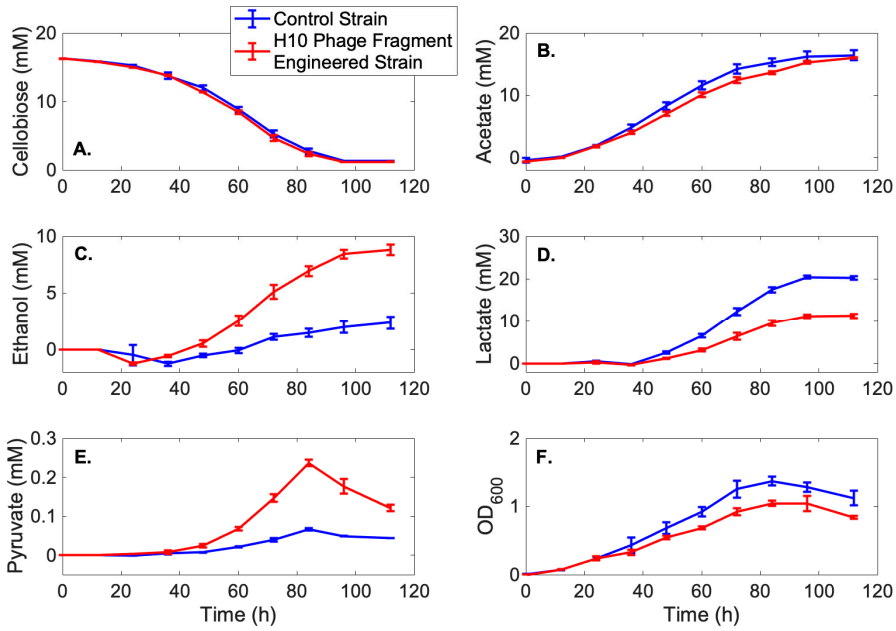

Figure 2. Batch fermentation of *C. cellulolyticum* plasmid control strain (blue) and an engineered strain containing the H10_Phage DNA fragment (red). The following metabolic profiles are shown: (**A**) cellobiose consumption, (**B**) acetate production, (**C**) ethanol production, (**D**) lactate production, (**E**) pyruvate production and re-utilization, and (**F**) growth (OD_{600}). Each point represents an average of three biological replicates, and error bars represent one standard deviation.

Table 2. Sequencing results of the DOP-PCR amplified genomic and soil sample metagenomic DNA used for library construction and enrichment. Protein coding features include the total annotated genes in the references if available. The soil metagenomic DNA library has also been described elsewhere [37].

DNA Library	Number of Reads	Total Hits (%)	GC Content	Alpha Diversity	Protein Coding Features (%)	Organisms Featured
C. cellulolyticum	3,419,032	3,347,393 (97.3)	39.9%	N/A	3345 (93.7%)	1
Soil metagenome	2,226,424	1,922,706 (86.4)	53 ± 10%	490.8	569,220 (N/A)	4773

DNA fragments from both libraries were ligated into the pCR8™/GW/TOPO® TA plasmid and over 40,000 *E. coli* colonies were produced upon transformation to generate frozen stocks and 100,000–120,000 clones in the shuttle vector, pSOSGate. The limiting transformation step was electroporation into *C. cellulolyticum*. This procedure achieved less than 2000 colonies identifiable by plating on solid GS-2 medium. Despite the methylation of the plasmid DNA, the transformation of over 200 µg of plasmid DNA, and the glycine incubation procedure [47], our transformation yields were initially below ten mutant colonies per µg of DNA. We relied on the direct liquid inoculation of the transformed cultures without plating to bypass the organism's known low plating efficiency. This procedure was published elsewhere [48], and it was found that low plating efficiency may actually underrepresent our actual library size by a factor of 10^5. However, we remain unsure of the accuracy of this statement and identify this as a potential limitation of the methodology used in this research.

After ten re-inoculations of the library outgrowth culture, DNA fragments that impart the largest fitness advantage (or least fitness burden) were observed to dominate the culture. Even though the transformed DNA library was limited in size, the DNA fragments conferring a growth advantage were present in the DNA library. Enrichment was nearly uniform, as only two unique DNA fragments could be detected in a sample of the *C. cellulolyticum*-enriched library (n = 10 colonies tested) and two within the environmental metagenomic DNA library (n = 18 colonies tested).

3.2. Genome Alignment of Enriched DNA Fragments

The isolated DNA fragments from the enrichment experiments are listed in Table 3. The DNA fragment "H10_BK" contains the region spanning two β-ketoacyl synthase genes in a polyketide synthesis cluster in the *C. cellulolyticum* genome, including most of a polyketide synthase dehydrogenase *domain* within the gene *Ccel_0859*. The DNA fragment "H10_phage" is a 359 bp section of the N-terminal phage minor structural protein (encoded by *Ccel_2823*) found within the *C. cellulolyticum* genome in a region rich in phage-related proteins, possibly inserted during viral recombination events in the organism's evolutionary history [49]. The environmental metagenomic library yielded the DNA fragments "ENV_F1" and "ENV_M1" (Table 3). The former codes for a 1.1 kb fragment which BLAST aligns partially with both the NADH:Flavin oxioreductase gene and the cytochrome C-type protein of *Hydrogenophaga* sp. PBC. The ENV_M1 fragment has no sequence alignment; however, it contains translational reading frames that best match three separate proteins of *Pedosphaera pavula*: a prevent-host-death family protein, an identified hypothetical protein, and a portion of the ubiquinol-cytochrome C reductase.

3.3. Metabolic Profiles

The plasmids containing the enriched DNA fragments were isolated and re-transformed. The initial fermentation trial with the four mutant strains and a plasmid control strain (three biological replicates of each) yielded a significantly improved performance only from the strain containing the H10_Phage DNA fragment. While the other fragments (H10_BK, ENV_F1, and ENV_M1) were enriched by the culture, significant differences from the plasmid control strain were not observed when these fragments were isolated and the

culture grown with each alone (results in Supplementary File S2). For the strain containing the H10_Phage DNA fragment, the time-course metabolic profiles of the acetate, ethanol, lactate, pyruvate, cellobiose, and culture growth are shown in Figure 2 along with those for the plasmid control strain. The ethanol concentration after 118 h of fermentation is of particular interest, which was 8.8 mM for the strain expressing the H10_Phage DNA fragment and 2.4 mM for the plasmid control strain. Cellobiose consumption rates were nearly identical, and cellobiose was completely consumed by 96 h (Figure 2A). This generated a yield of ethanol produced per cellobiose consumed ($Y_{P/S}$) increase of more than 250% for the engineered strain compared to the control. In addition to similar cellobiose consumption, the acetate production was nearly identical for the engineered and control strains. The strain containing H10_Phage produced 250% more pyruvate, peaking at 84 h, and commensurate with biomass. The carbon re-distribution appears to have come from lactate, which did not accumulate past 11 mM for the mutant (a drop of approximately 50%). The culture density (OD_{600}) of the strain containing the H10_Phage fragment was almost 20% less than that of the plasmid control, and it reached a lower maximum density than the control (Figure 2F) and the culture during the enrichment process.

Table 3. List of enriched DNA library fragments after ten subcultures on cellobiose.

Sequence Identifier	Genome Origin [1]	Gene/Protein Similarity
H10_Phage	*C. cellulolyticum* CP001348.1:3379404-3379762	Ccel_2823: Phage minor structural protein
H10_BK	*C. cellulolyticum* CP001348.1:989500-990681	Ccel_0859: Beta-ketoacyl synthase Ccel_0860: Beta-ketoacyl synthase
ENV_F1	*Hydrogenophaga* sp. PBC FR720599.2	*fre:* NADH:Flavin oxioreductase/NADH oxidase; Cytochrome C-type protein
ENV_M1 [2]	*Pedosphaera pavula*	*phd:* Prevent-host-death family protein; Hypothetical protein; Ubiquinol-cytochrome C reductase

[1] As determined from the BLAST nucleotide search with the highest alignment score. [2] No nucleotide alignment possible; transcribed protein matches indicated from highest BLASTX score.

4. Discussion

The enrichment of a genomic DNA library created by DOP-PCR, high-efficiency cloning, and electro-transformation into *C. cellulolyticum* yielded DNA fragments (that were not full-length genes) associated with significantly improved ethanol and pyruvate yields. Following enrichment, the variety of genomic DNA fragments decreased substantially as mutants with the highest fitness outcompeted and outnumbered other mutants. How the enriched genomic DNA library fragments function within the cell is of interest. The mechanisms leading to fitness are likely multigenic and are not always predictable. Although we expected an increased rate of growth to be associated with fitness, this was not apparent in our study, suggesting that the enriched phenotypes acted according to other modes such as: raising localized ethanol tolerance, enabling higher plating efficiency during the final analysis of enrichment samples, or improving the export of antibiotic compounds to harm neighboring cells [2]. The plasmid pSOSGate contained the strong constitutive thiolase (*thl*) promoter from *C. acetobutylicum* [39], and resulting RNA may have acted with a regulatory role instead of being translated. Thus, the exact natures of the function of the enriched DNA library fragments have not yet been determined but remain a topic of interest for future studies.

The overexpression of the enriched H10_Phage fragment alone resulted in a metabolic re-distribution in *C. cellulolyticum*, affecting the fermentation patterns of ethanol, acetate, lactate, and pyruvate. The hydrogen production is also possible from *C. cellulolyticum* as a means of oxidizing ferredoxin to prevent pyruvate buildup by driving it towards acetyl-CoA [50]. The H10_Phage fragment contains a 359 bp fragment of the uncharacterized and non-essential phage minor structural protein, which is recognized in other clostridia.

A BLASTN search revealed *C. bornimense* (accession HG917868.1) and *Ruminiclostridium herbifermentans* (accession CP061336.1) as containing nearly identical nucleotide sequences (95.5% and 76.3%, respectively). To our knowledge, no other studies have been performed to determine the metabolic function of the H10_Phase DNA fragment. Additionally, it was not determined how this fragment impacted the global -omics of the engineered culture nor how metabolic network balancing was impacted. It is also unknown whether this DNA fragment will impact metabolism in other cell types. These remain questions of interest.

The H10_BK fragment encodes the portions of two β-ketoacyl synthase genes in the fatty acid synthase. This multi-enzyme is responsible for the elongation of fatty acids through the consumption of acetyl-CoA, possibly affecting acetyl-CoA production from pyruvate and preventing over-accumulation, similarly to the proposed effect of amino acid synthesis enzyme overexpression [51]. However, when cultured alone, the fermentation profile remains similar to the control. Interestingly, the β-ketoacyl synthase also enables antibiotic protection, though primarily against thiolactomycin [52]. The enriched environmental DNA fragments both contain a likely cytochrome C type functional group, which typically serves in the electron transport chain. These proteins interact with a large variety of substrates but are typically down-regulated upon exposure to reducing-power-limited substrates such as methanol [53], limiting their utility in fermentation. The ENV_M1 fragment also contains part of the prevent-host-death (Phd) protein, which counters the death-on-curing protein responsible for plasmid maintenance [54].

While the focus of this study was to isolate a single DNA fragment capable of improving the growth and the metabolic profile of *C. cellulolyticum*, the resulting metagenome of the enriched culture is also worthy of future studies. The stability of the enriched population and cell–cell interactions were unaddressed in this study. However, it is certainly possible for an enriched and cooperative metagenome to produce characteristics different from each of the individual strains constructed in this study.

While larger libraries would allow for a more comprehensive screen, cloning and transformation in *C. cellulolyticum* remain difficult processes. An improved electro-transformation protocol using a modified electroporation device was reported to achieve up to 10^4 CFU/μg plasmid DNA [55]. However, we were not able to replicate this level of success. The optimization of the transformation procedure of the close relative *C. thermocullum* included many factors not examined herein, including controlling the temperature of the electroporation cuvette, a 0 °C outgrowth period with recovery media, field strengths approaching 25 KV/cm, isoniacin pre-treatment, or methylation sensitivity of *E. coli* from which plasmid DNA was isolated [56]. The expression of the organism's native genome could be circumvented entirely using a recently developed transposon-based mutagenesis system, where portions of the genome are swapped by a Tn1545 transposable element located on a transformation plasmid. The propagation of the cell and plasmid result in the accumulation of mutations greater than the initial number of transformed cells [34]. This method, however, requires extensive sequencing to characterize the mutations and is limited to rearranging existing DNA, not expressing foreign genes.

In summary, we demonstrated another application for combinatorial metabolic engineering by genomic DNA library enrichment prepared by DOP-PCR [37]. Although limited in size, the library was enriched for genes coding for uncharacterized phage proteins, oxidoreductases, and cytochrome C type proteins, which may have the ability to catalyze multiple reactions within the cell. Without needing to perform an exhaustive characterization of all library mutants, the enrichment strategy identified four gene fragments that increased cell fitness when expressed on a multi-copy plasmid within *C. cellulolyticum*. The mutant containing the H10_Phage fragment showed a 250% increase in the ethanol yield from cellobiose, decreased acetate and lactate production, and higher maximum accumulation of pyruvate than a control-bearing strain while grown on cellobiose. This was in contrast to our original hypothesis that genomic DNA library enrichment would alleviate the metabolic bottleneck leading to pyruvate secretion, resulting in increased growth. It is believed that the H10_Phage fragment was enriched instead because of its

ability to secrete and tolerate higher levels of ethanol, which may have been toxic to other mutants. It is also likely that the other enriched DNA fragments conferred an increased ethanol tolerance and plating efficiency. Thus, even though a faster-growing strain of *C. cellulolyticum* was not produced in this research, a strain with a 250% increased yield of ethanol and pyruvate production from the cellobiose utilization was engineered.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9070605/s1, Supplemental File S1; Supplemental File S2.

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