INVESTIGATING TRANSCRIPTOMIC RESPONSES TO BIOFUEL STRESS IN *CLOSTRIDIUM ACETOBUTYLICUM*: TRANSCRIPTOME ASSEMBLY AND GENOME ANNOTATION OF A MODEL FERMENTATIVE BACTERIUM.

by

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Ad maiorem dei gloriam.

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ABSTRACT

Chapter 1

METHODS

1.1 Culture

Wild type *Clostridium acetobutylicum* ATCC 824 was cultured anaerobically in 4L New Brunswick Scientific BioFlo 310 bioreactors at 37 °C, pH ¿= 5.0, 200 mL min^{-1} N_2 and 200rpm agitation in a defined *Clostridia* growth medium, as described previously[1]. When the cultures were grown to A_{600}=1, the N_2 flow rate was decreased to 50 mL min^{-1} and cultures were either stressed to a final concentration of 60 mM *n*-butanol, 40 mM potassium butyrate, or left unstressed. 15 mL samples were acquired at 15, 75, 150, and 270 minutes after treatment and OD synchronization. Samples were centrifuged at 8,000rpm, 4 °C for 20 minutes. After discarding the supernatant, cell pellets were then immediately frozen at −85 °C.

1.2 RNA preparation

RNA was extracted by first washing the cell pellets in 1mL of RNase-free SET buffer (25% sucrose, 50 mM EDTA [pH 8.0], 50 mM Tris-HCl [pH 8.0]) before resuspending cells in a 220 mL solution of RNase-free SET buffer containing 4.55 U mL^{-1} proteinase K and 20 mg mL^{-1} lysozyme and incubating for 6 minutes. Resuspended cells were vortexed with 40mg of RNase-free glass beads (≤106 µm) at maximum speed and room temperature for 4 minutes. Each sample was mixed immediately with 1 mL of ice-cold QIAzol (Qiagen, Valencia, CA, USA) and then 200 µL of ice-cold chloroform, mixing well with each addition. After a 3 minute room temperature incubation, samples were centrifuged at 11,000rpm and 4 °C for 15 minutes. The aqueous phase was then mixed with 1.3 mL of ice-cold ethanol before transferring to a miRNeasy Mini

spin-column (Qiagen, Valencia, CA, USA) and centrifuging at 11,000rpm and 4 °C for 15 seconds.

Next, 700 µL of RWT buffer was added to the column, before centrifuging at 11,000rpm and 4 °C for 15 seconds, discarding the collection tube and transferring the column to a fresh collection tube. The column was washed twice with 500 µL of RPE buffer before centrifuging at 11,000rpm and 4 degreeCelsius for 15 seconds each. The membrane was then dried with an additional centrifugation step at 11,000rpm and 4 degreeCelsius for 1 minute. The RNA was eluted twice by incubating with 50 µL of nuclease-free water for 1 minute and eluting for 1 minute at 11,000rpm and 4 degreeCelsius.

After quantification on a Nanodrop ND-1000, samples were then precipitated in 0.3M sodium acetate and 75% ethanol overnight, centrifuged at 14,000 rpm for 30 minutes, washed twice with 400 µL ice-cold 70% ethanol, and rehydrated in 50 µL RNase-free water. Next, samples were treated with the Turbo DNA-free kit (Ambion, Austin, TX, USA). 5 µL of 10X Turbo DNase buffer and 1 µL of Turbo DNase ($2\text{U}\mu\text{L}^{-1}$) were added to each sample before incubating at 37 degreeCelsius for 30 minutes. Next, 5 µL of DNase inactivation reagent were added to each sample, mixing occasionally for 5 minutes. The samples were then centrifuged at 10,000rpm and 4 degreeCelsius for 90 seconds, precipitating the DNase. The samples were moved to fresh 1.5 µL tubes.

Samples were then precipitated, washed twice more with 70% ethanol, and resuspended in 20 µL of nuclease-free water, requantified, and aliquoted for quality analysis with the BioAnalyzer platform (Agilent, Wilmington, DE, USA), and 10 µg aliquots in 10 µL samples were stored at −85 °C.

1.3 RNA enrichment, RNA-seq library preparation, and Sequencing

Ribosomal RNA was removed with the MicrobExpress kit (Ambion, Austin, TX, USA) according to their protocol. Briefly, beads were prepared by taking 50 µL for each sample, washing with an equal volume (50 µL) of water capturing for 5 minutes on a MagnaSphere (Promega, Madison, WI, USA) magnetic stand and aspirating.

Subsequently, the beads were resuspended in an equal volume (50 µL each) of binding buffer and capturing as above. The beads were then resuspended in an equal volume (50 µL each) of binding buffer and warmed to 37 °C. Next, 200 µL of binding buffer was added to each 10 µg RNA aliquot with 4 µL of capture oligo mix. The mixture was warmed to 70 °C for 10 minutes, then cooled to 37 °C for 15 minutes. Next, the rRNA was captured by mixing 50 µL of beads with each sample, incubating for 15 minutes at 37 °C, and capturing as above. The enriched RNA was transferred to a fresh 1.5 mL tube. The beads were then washed with 100 µL of pre-warmed (37 °C) wash solution, incubating on the magnetic stand for 5 minutes, and adding the wash solution to the enriched RNA. The samples were then ethanol precipitated at 20 °C overnight with 35 µL of 3 M Sodium Acetate, 5 mg mL^{-1} Glycogen, and 1175 µL of chilled 100% ethanol. The samples were washed twice with 70% ethanol and resuspended in 25 µL. The samples were enriched further by repeating the MicrobExpress treatment. Small 10-100 ng aliquots were analyzed at each step with the BioAnalyzer to monitor enrichment.

Selected samples were enriched further with Terminator 5'-phosphate dependent exonuclease kit (Epicentre, Madison, WI, USA). Terminator Exonuclease 1 µL (1U µL^{-1}) was added with 2 µL 10X Buffer A to each RNA sample. The reaction was run in a thermocycler for 60 minutes at 30 °C. The reaction was terminated with the addition of 1 µL of 100 mM EDTA and .... Tris HCl at pH 8.0. The samples were then purified by ethanol precipitation (0.3 M Sodium Acetate and 75% ethanol) with two 70% ethanol washes, as above. Enriched RNA was quantified as above and assessed for quality with the BioAnalyzer platform (Agilent, Wilmington, DE, USA). High quality samples were used to prepare RNA-seq libraries with the ScriptSeq v2 library preparation kit and indexed PCR primers (Epicentre, Madison, WI, USA). Briefly, 1 µL of fragmentation solution and 2 µL of cDNA synthesis primer was added to 50 ng of RNA and the solution was fragmented for 5 minutes at 85 °C in a ..... thermocycler. To each reaction, 0.5 mM of Dithiothreitol, 3 µL of cDNA synthesis premix, 0.5 µL StarScript Reverse Transcriptase. is added to each sample and run with the following cycle: 5

minutes at 25 °C, 20 minutes at 42 °C. After cooling each reaction to 37 °C, 1 µL of finishing solution was added, incubating for 10 minutes. The RNA is degraded by fragmenting further for 3 minutes at 95 °C, cooling to 25 °C. The first strand cDNA is di-tagged by adding 7.5 µL of terminal tagging premix and 0.5 µL of DNA polymerase. The terminal tagging reaction is run at 25 °C for 15 minutes and 95 °C for 3 minutes. The di-tagget cDNA is then purified with the AMPure XP bead system (Beckmann Coulter, Brea, CA, USA). First, the library is mixed with 45 µL of homogenous bead mixture. After thorough mixing, each solution is transferred to a 1.5 mL tube and the library is captured with the magnetic stand and the supernatant aspirated. Each library is then washed twice with 200 µL of 80% ethanol. After resuspending in 24.5 µL of nuclease-free water, the beads are captured and each library is transferred to a new 200 µL microfuge tube. Adapters are added to the di-tagged cDNA during PCR by adding 25 µL FailSafe Premix E, 1 µL forward primer, 1 µL of ScriptSeq v2 indexed reverse PCR primer, 0.5 µL of FailSafe Polymerase. The PCR conditions are as follows: cycles of 30 seconds of 95 °C, 30 seconds of 55 °C, and 3 minutes of 68 °C. After 12 cycles, the reaction terminates with a 7 minute incubation at 68 °C before purifying the library with the AMPure system, as above. Libraries were multiplexed and sequenced for 76 cycles over two lanes of an Illumina HiSeq 2500 at the University of Delaware Sequencing and Genotyping Center (Newark, DE, USA).

1.4 Sequencing Statistics, Alignments

Paired-end sequencing resulted in ···· pairs of 76 bp reads which are deposited in the Sequence Read Archive (·····). Summary statistics for the libraries are shown in table/appendix (···). The basic bioinformatic processing pipeline is described on Github. In brief, the fastq headers are briefly pre-processed for downstream applications. Then, adaptors are removed from the reads with Trimmomatic[2]super. Base quality is adjusted by trimming to the minimum base quality of 20. Before aligning to the *Clostridium acetobutylicum* ATCC 824 genome, the data is subjected to *in silico* ribosomal RNA removal. The reads are aligned to the rRNA sequences with

Bowtie 2.1.0[3]. The unmapped reads are then aligned to the genome and megaplasmid sequences (NC˙003030.1 and NC˙001988.2). The alignment files were then cleaned, sorted, indexed, and validated with SAMtools[4] and Picard[5].

1.5 Transcriptome Assembly

Reference and *de novo* assembly was done with Trinity[6]. Fastq files were modified by appending the second column of the fastq Casava 1.8+ header to the first before processing and alignment. Next, the resulting alignment files were merged and sorted before appending the pair information (/1 or /2) according to the Trinity documentation. To assess the assemblies, I have been contributing to a transcriptome assembly assessment software project: Transrate. This software assesses transcriptome assemblies by calculating coverage statistics and assessing the assemblies agreement with the reference proteome. I have made several additions to this software. Specifically, unpaired reads and strand specific alignment were integrated into the coverage/alignment statistics. Additionally, singleton reads were produced from the alignment process and were then further assessed for possible sources of contamination. Finally, the assembly itself was aligned to the reference genome, assuring the validity of the assembly and the identity of the assembled transcripts.

1.6 Coverage and Differential Expression

Coverage vectors for each strand were calculated with BEDtools[7]. Coverage vectors for each transcript were then acquired with a custom Ruby script. Summarization and visualization of these data was performed in R[8]. Next, transcriptional start sites were identified with TSSi[9] after extracting (200? 300?) bp windows surrounding the beginning of the assembled transcript with a custom Ruby script. Coverage vectors from each transcript were visualized with these results in R. Read counts per transcript were quantified with HTSeq[10]. Differential expression analyses followed the conservative approach of DESeq2[11],[12]. Calculations and visualizations were done in R with various packages for heatmaps, principal components analysis, clustering, and

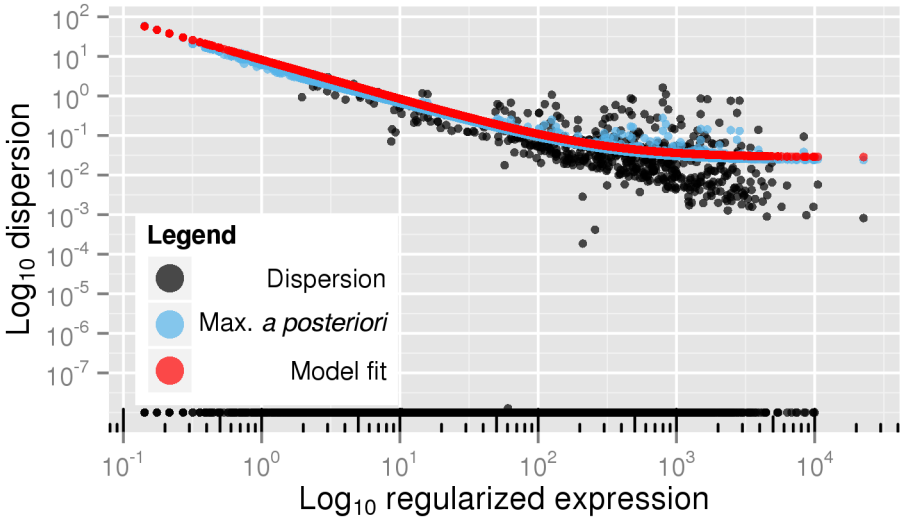
advanced graphics[13]. Data were also processed manually for visualization in Circos graphs[14].

1.7 Molecular Methods

Fold changes were confirmed for randomly selected genes by qRT-PCR. Transcriptional start sites were verified by 5′RACE for randomly selected genes. Small RNAs were confirmed by RT-PCR and Northern blot.

Chapter 2

RESULTS



Chapter 3

DISCUSSION

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CONCLUSIONS

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