CRISPRi-mediated Repression of *ldhA* Increases Ethanol Production in MG1655 E. Coli.

Haley Higginbotham, Kylie Gallagher, Noreen Lyell, Leslie McClain, Becky Meyer, Daniel Stein, Wilson Gomarga, Sarah Dohadwala, Pranav Lalgudi

Abstract

Concerns about the environmental impacts and sustainability of human activities are motivating increasing reliance on biofuels. The biofuel ethanol has become the largest biotechnological commodity and so there is great economic incentive to develop and improve production methods. Ethanol is naturally produced by E. Coli in anaerobic fermentation, but only in low concentrations, as it is but one of numerous potential metabolic products. Due to the high demand for ethanol, it is important to gain a better understanding of how modifying the expression of specific genes can alter the balance between these competing pathways to affect levels of ethanol production. In this project, we demonstrate a strategy for increasing ethanol production by utilizing a CRISPRi system targeting the expression of lactate dehydrogenase A (ldhA), an enzyme involved in the production of the alternative fermentation product D-lactate. Here we show that the repression of *ldhA* via a CRISPRi system is sufficient to increase ethanol production in MG1655 E. Coli cells, and that a promoter-targeting system may be more effective than one targeting the coding sequence of *ldhA*. These results suggest that *ldhA* repression is an effective means for upregulating ethanol production, and provide a strategy for improving commercial output, thereby further enabling a transition from fossil fuels to more sustainable and environmentally-friendly alternatives.

Introduction

In efforts to reduce environmental harm, fossil fuels are increasingly being replaced with more sustainable alternatives such as biodiesel and ethanol. To illustrate, between 2001 and 2011 ethanol increased from 1 to 10% of gasoline consumption by volume in the US, and through its use as a biofuel, has become the largest biotechnological commodity ("Biofuels Issues and Trends"). Ethanol is naturally produced by E. Coli in anaerobic fermentation, but only in low concentrations. Through metabolic engineering though, we can take advantage of natural ethanol synthesis pathways and modify microorganisms such as E. coli to produce more ethanol, which can then be harvested for commercial use.

.

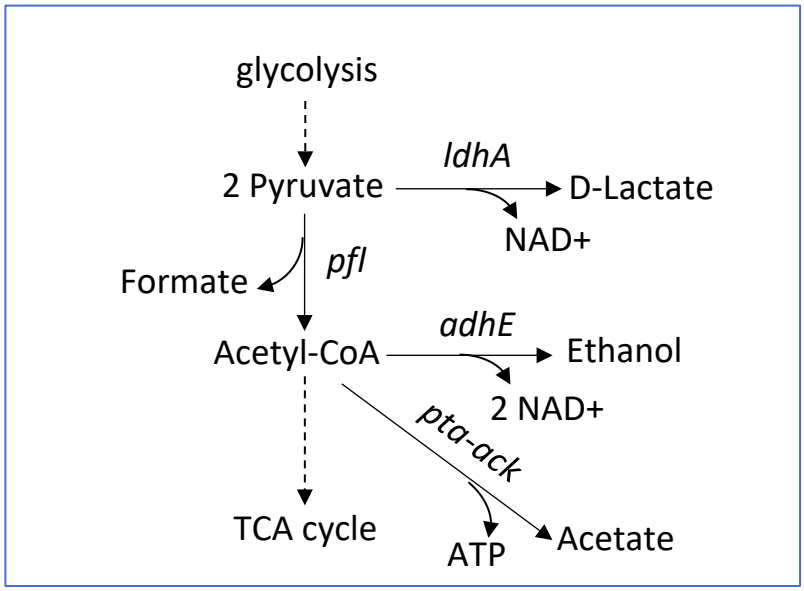


Figure 1: Mixed-acid Fermentation Pathways in E. Coli

To provide a metabolic context for this experiment, the relevant pathways in E. Coli mixed acid fermentation are shown. These pathways include the action of the enzymes ldhA, aldehyde-alcohol dehydrogenase (adhE), phosphotransacetylase-acetate kinase

(pta-ack), and pyruvate formate lyase (pfl) towards the production of end products such as D-lactate, ethanol, and acetate.

In MG1655 E. Coli, the pyruvate product of glycolysis can be converted to a formate and acetyl-CoA via the action of the enzyme pfl. In mixed acid fermentation, this acetyl-CoA can then be converted to ethanol or acetate via the action of adhE or pta-ack. Alternatively however, the enzyme ldhA can catalyze the production of D-lactate from the pyruvate. Activity of ldhA thus shunts pyruvate away from downstream pathways, including that for ethanol production. In this way, inhibition of *ldhA* may increase the levels of precursors for the ethanol pathway. Additionally, both the D-lactate and ethanol pathways regenerate NAD+, whereas acetate production releases ATP (Figure 1) (Förster and Gescher 2014). It is therefore conceivable that, when *ldhA* is repressed, the other cellular mechanisms may attempt to compensate for decreased NAD+ regeneration via the D-lactate pathway by upregulating ethanol production. Also, because the ethanol production pathway does not require O_2, it is favored in anaerobic conditions. We thus hypothesized that, especially under anaerobic conditions, ethanol production can be increased by *ldhA* repression, potentially due to higher levels of precursors and cellular stress to regenerate NAD+. In this project therefore we aimed to increase ethanol production by repressing *ldhA* via a CRISPRi system.

The development of CRISPRi techniques has been a technological breakthrough that has provided a foundation for many modern metabolic engineering efforts. CRISPRi utilizes an inactivated Cas9 protein (dCas9). Cas9 is used natively in bacterial cells to bind and cleave invading viral genomes. In CRISPRi, the dCas9 is localized to a target sequence by binding to a guide RNA (gRNA), which is designed to be complimentary and specific to the gene being targeted for repression. Unlike its active version, when the dCas9 complex binds the target

sequence it does not cleave the DNA, but its binding alone can allosterically prevent transcription. If the complex is targeted to the coding sequence, it acts through inhibition of transcriptional elongation by blocking polymerase procession. Meanwhile, complexes targeting the promoter region of the gene can act both by inhibiting elongation and initiation by blocking the binding and procession of factors of the transcription initiation complex, such as transcription factors or polymerase (Larson et al 2013). We hypothesis that both CRISPRi systems targeting the coding sequence and those targeting the promoter of *ldhA* will increase ethanol production by repressing *ldhA* in this way, but that the additional repressional mechanisms available to promoter-targeting systems may result in a slight advantage over coding sequence targeting systems. To test this, we first targeted the promoter region of *ldhA* in an attempt to increase ethanol production. Then, we compared the ethanol output under the promoter-targeting system to that of CRISPRi systems targeting the coding sequence of *ldhA*.

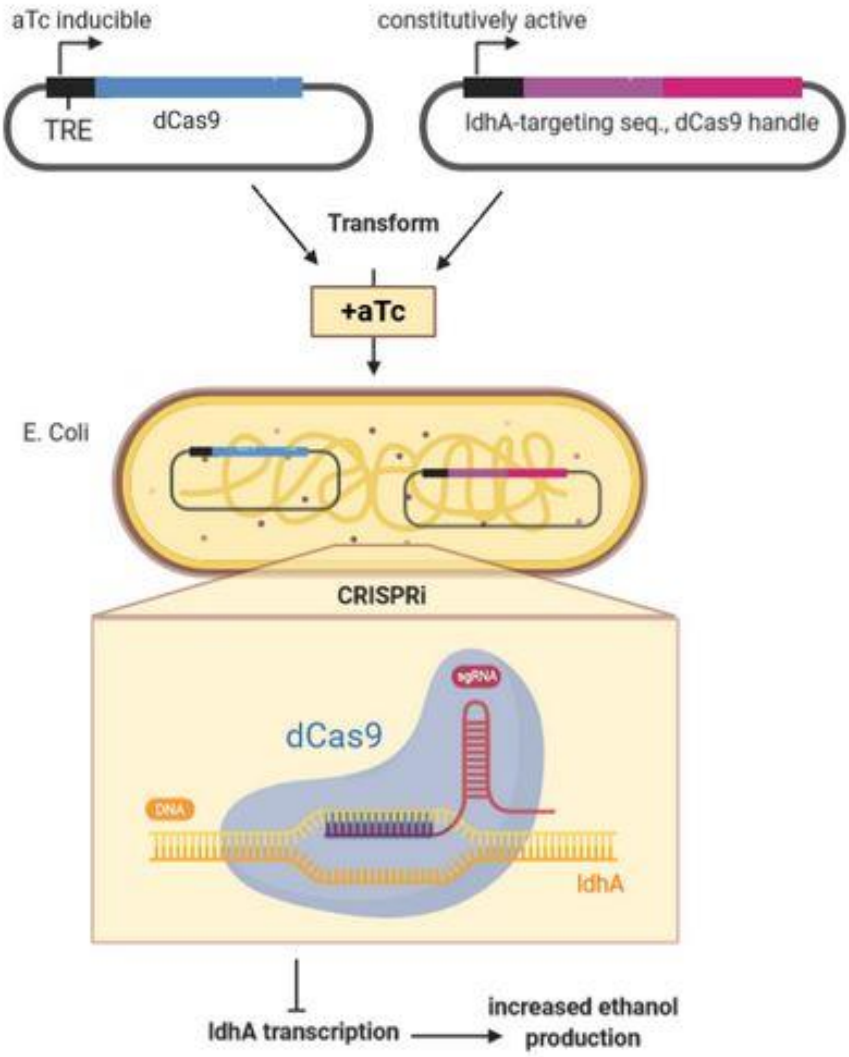


Figure 2: CRISPRi Can Be Utilized to Increase Ethanol Production Via Inhibition of *ldhA* transcription

In order to inhibit *ldhA* via CRISPRi, the E. coli must be transformed with 2 plasmids. One vector contains a dCas9 insert and aTc-inducible promoter while the other contains a constitutively active promoter and sgRNA for targeting *ldhA* with Cas9. The sgRNA consists of a *ldhA* targeting sequence and the dCas9 handle. Adding aTc then promotes expression of the dCas9 and thereby leads to the formation of the sgRNA/dCas9 complex, which binds the promoter region of the *ldhA* gene and thereby inhibits

transcription by blocking polymerase binding. The repression of *ldhA* then results in increased ethanol production by increasing precursor availability.

In order to measure the effects of *ldhA* repression on ethanol production we utilized a CRISPRi system targeting *ldhA*. The plasmids containing the dCas9 (pdCas9) and the gRNA construct (pgRNA_ldhA) were co-transformed into MG1655 E. Coli. The gRNA_ldhA insert contained a sequence complementary-to/targeting a region in *ldhA*, a dCas9 handle sequence (to bind dCas9), and a constitutively active promoter. The dCas9 however contained a tetracycline response element (TRE) in its promoter. In the cell's native state, tetracycline repressor (TetR) binds to the TRE and prevents transcription. The tetracycline homolog anhydrotetracycline (aTc) can bind TetR and induce a conformational change that leads TetR to unbind, and therefore release its repression of the gene (Rodriguez-Garcia et al, 2005). aTc was thus used to activate the CRISPRi system by inducing expression of dCas9, resulting in repression of *ldhA* expression and a consequent increase in ethanol yield (Figure 2). Overall, we demonstrate that activated CRISPRi systems targeting either the promoter or coding sequence of *ldhA* increase ethanol production, especially in anaerobic conditions, and that the *ldhA* promoter targeting system may be more effective than systems targeting the coding sequence. This suggests CRISPRi-mediated *ldhA* repression with a promoter-targeting gRNA and anaerobic culture conditions as strategies for optimizing ethanol yields.

Results

The overall goal of the experiment was to increase ethanol production in E. Coli through metabolic engineering via a CRISPRi system. The role of ldhA in driving the production of an alternate mixed acid fermentation product (D-lactate) makes it a promising candidate for

CRISPRi-mediated repression to increase ethanol production. We thus sought to create a CRISPRi system targeting the promoter of *ldhA*. This system consists of the pgRNA_ldhA and pdCas9 vectors to express the guide RNA and dCas9 respectively. We therefore first confirmed successful insertion of the gRNA and dCas9 sequences and then proceeded to culture the MG1655 cells with the system and measure the resultant ethanol production.

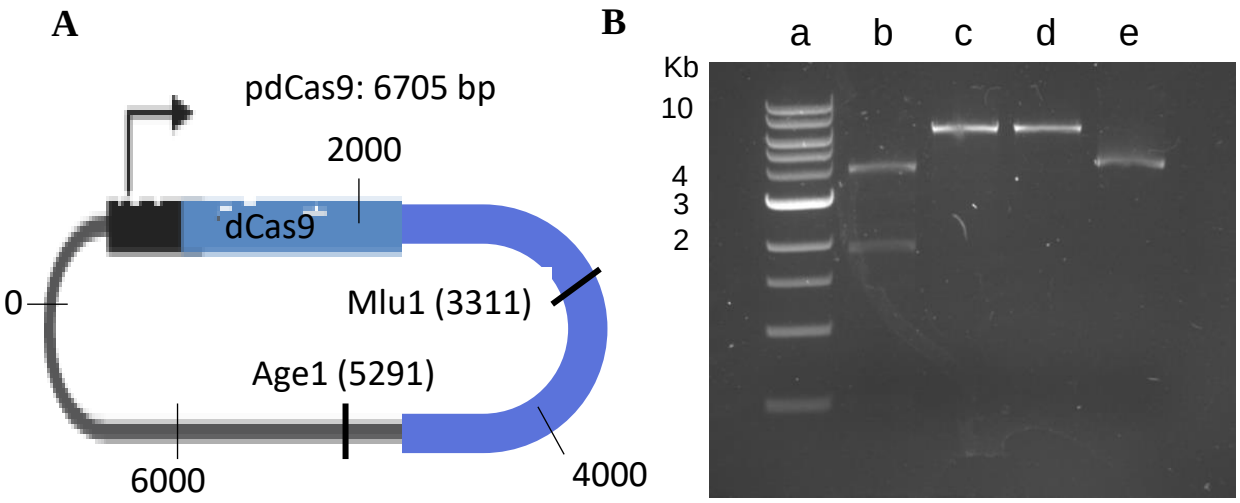


Figure 3: Diagnostic Digest Confirms dCas9 Insert

The Mlu1 and Age1 restriction sites are located at 3311 and 5291bp respectively within pdCas9 (A). The pdCas9 vector was digested with Mlu1 and Age1 then resolved on 1% agarose gel to confirm the insertion of dCas9 (B). 1 kb NEB DNA ladder (a). Double digest with Mlu1 and Age1 (b). Digest with Age1 (c). Digest with Mlu1 (d). Plasmid alone (e).

Mlu1 and Age1 Digest Confirms dCas9 Insertion:

To confirm the insertion of dCas9 in the expression vector, plasmid was digested with Mlu1 and Age1. The dCas9 insert contained a Mlu1 restriction site and the expression vector contained an Age1 site. The full plasmid was expected to be 6705 bp in length when pdCas9 was correctly inserted and so digests with only one of the two restriction enzymes (lanes c & d)

resulted in one clear band between the 6 kb and 8 kb ladder bands. A double digest with both Mlu1 and Age1 produced two bands - one near 2 kb and the other between 4 and 5 kb, which corresponds with the expected band lengths of 1980 and 4725bp. The well with uncut plasmid produced one band near 6 kb (Figure 3). The supercoiling of the uncut plasmid is theorized to have resulted in this band being slightly lower than that of the full plasmid length. Overall, the results of the digests suggested the successful insertion of dCas9 in the expression vector. The pdCas9 could therefore be transformed into cells in order to introduce dCas9 expression.

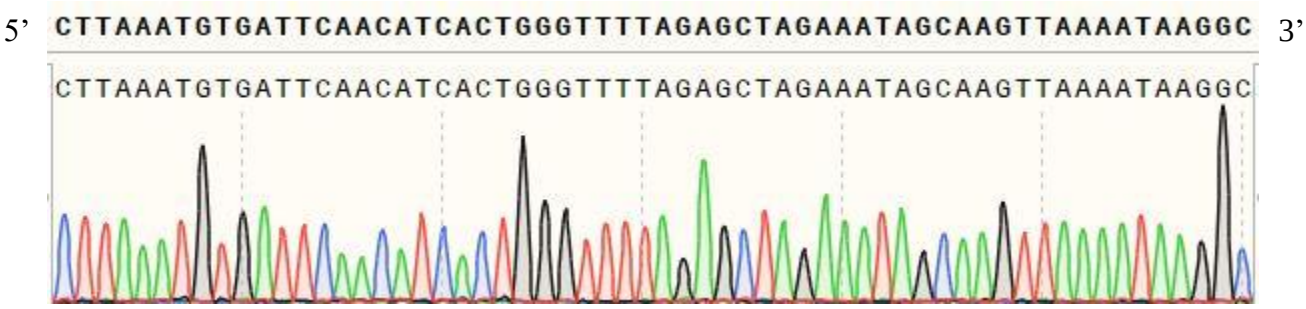


Figure 4: Sequencing of pgRNA_ldhA Confirms Insertion

The pgRNA_ldhA was sequenced to confirm incorporation of the gRNA construct. The top sequence is that of the designed gRNA while the bottom is the sequencing output and corresponds to the chromatogram.

Sequencing Confirms Insertion of gRNA_ldhA in Expression Vector

To target the dCas9 to the promoter of *ldhA* a gRNA was designed and incorporated into an expression vector via site-directed mutagenesis (SDM). The pgRNA_ldhA was sent to Genewiz for sequencing. The sequencing results revealed perfect correspondance with the designed sequence. No mismatches were observed over the 60 bp insert, thus confirming successful gRNA_ldhA insertion (Figure 4). Since the gRNA_ldhA was successfully

incorporated in the expression vector, it was then co-transformed into the MG1655 cells with the pdCas9, where the effects of this CRISPRi system on ethanol production were then measured.

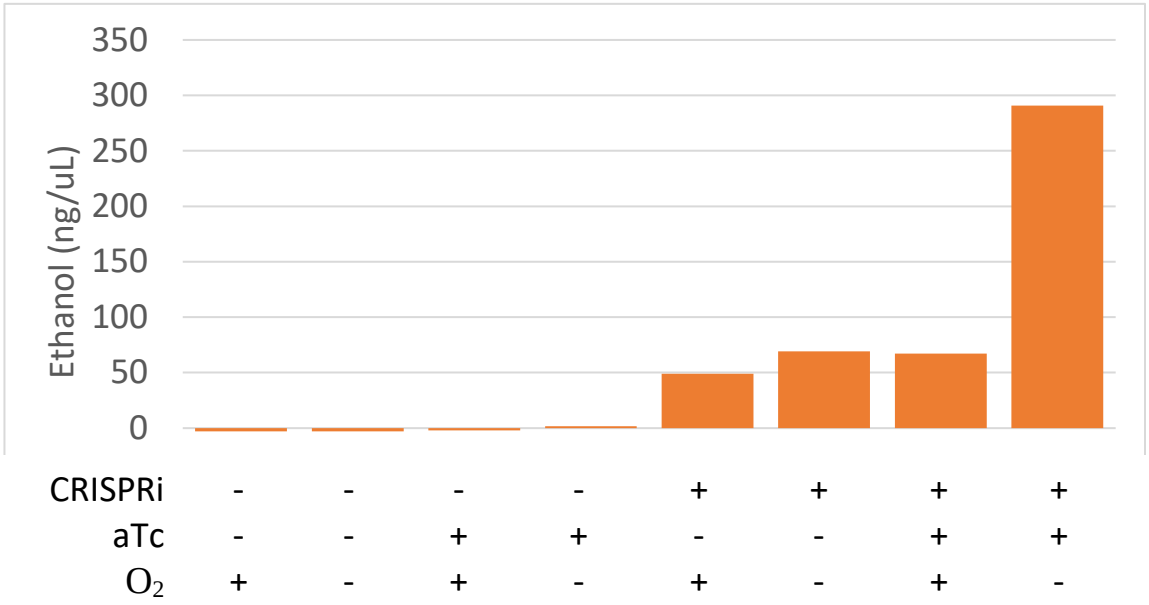


Figure 5: Ethanol Production Increases with CRISPRi System

A colorimetric assay was used to measure the levels of ethanol for each culture condition (+/- the pdCas9 and pgRNA_ldhA (CRISPRi), aTc, and O_2). A linear regression mapping absorbance to ethanol concentration was created using 5 graduated standards of known ethanol concentration, and used to approximate the ethanol concentrations in each condition. Results were background-adjusted by subtracting the result for the CRISPRi-, aTc-, O_2+ condition, and normalized by the optical density of the samples to adjust for differences in cell numbers. The molar mass of ethanol, 46.07 g/mol was used to convert to ng/uL.

Targeting the Promoter of *ldhA* with CRISPRi Increases Ethanol Yield

The results of a colorimetric assay were normalized by the optical density of each sample to account for cell number, and revealed that the MG1655 E. Coli cells that were co-transformed with this CRISPRi system produced more ethanol on average per cell than the CRISPRi- cells (Figure 5). This effect was observed even in the absence of aTc to induce activation of the CRISPR system as, the normalized ethanol concentration was 72 ng/uL higher for the CRISPRi+ than the CRISPRi culture in the O_2-, aTc- condition, and 50 ng/uL higher for the CRISPRi+ than CRISPRi- in the O_2+, aTc- condition. The anaerobic, induced (aTc+), CRISPRi+ condition produced the highest concentration, 290 ng/uL, as expected, and this was 289 ng/uL higher than the ethanol production of its CRISPRi- counterpart. Overall, the ethanol concentration was higher in the CRISPRi+ culture than for the CRISPRi- culture for every conditional pair. These results suggest the efficacy of *ldhA* repression for enhancing ethanol yield. While the promoter-targeting CRISPRi system was shown to be effective, it is possible that a gRNA targeting the coding sequence of *ldhA* may have a different effect on yield. Therefore, in order to determine the optimal CRISPRi system for increasing ethanol production via *ldhA* repression we compare the effects of promoter and coding-sequence targeting systems.

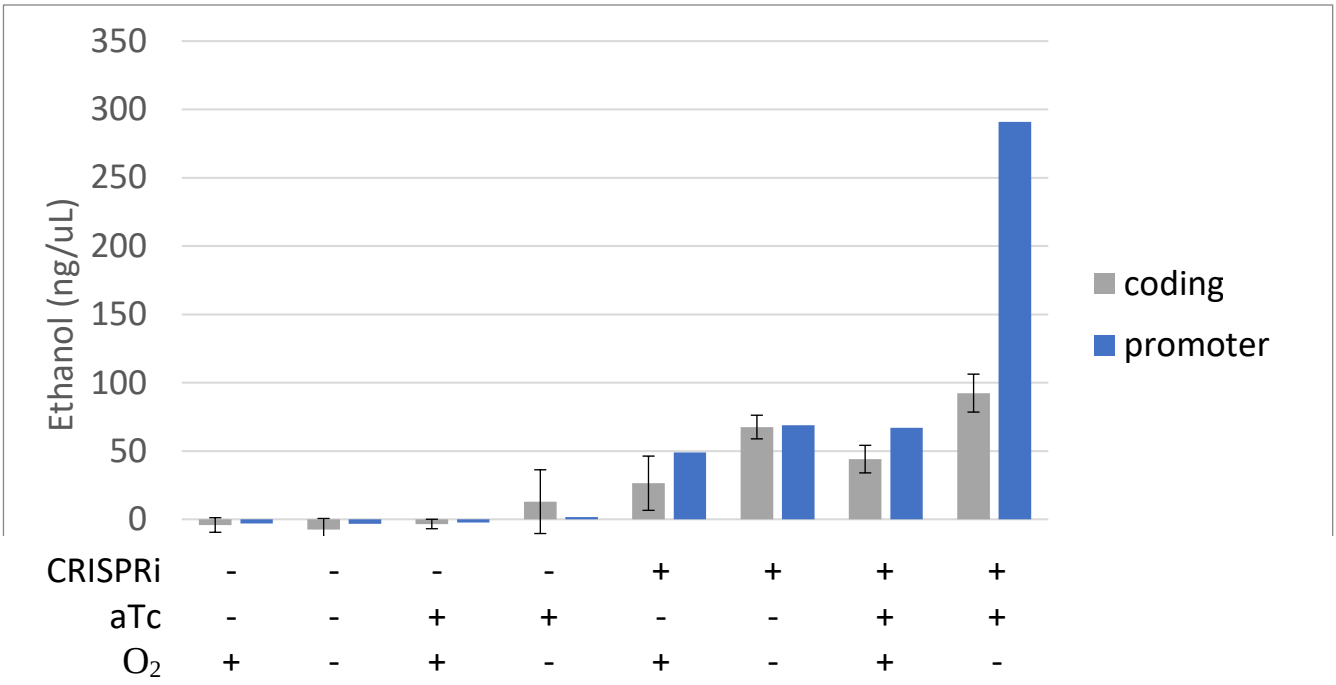


Figure 6: Promoter-Targeting Provides Greater Ethanol Production

Ethanol production in each culture condition was measured for both a CRISPRi system targeting the promoter of *ldhA* as well as systems targeting the coding sequence of *ldhA*. A colorimetric assay was used quantify the ethanol output, which was converted to ng/uL via fitting to a best fit function produced with ethanol standards of known concentration. Results were background-adjusted by subtracting the results for the CRISPRi-, aTc-, O_2+ condition, and normalized by the optical density of the sample to adjust for cell number. Results for the CRISPRi system targeting the promoter region represent individual measurements whereas those for the coding-sequence targeting system represent averages (+/- std. dev.) from 3 experiments.

Promoter of *ldhA* May Be More Effective Target Than Coding Sequence

In order to determine the optimal gRNA construct to increase ethanol production via a ldhA-targeting CRISPRi system, a promoter-targeting system was compared to coding-sequence targeting systems. Three experiments were performed with coding-sequence targeting gRNAs and so the assay output for these experiments were averaged for each condition and compared to the measured values from the single promoter-targeting experiment (Figure 6). Both the coding sequence and promoter targeting systems increased ethanol output, as higher concentrations were measured for each CRISPRi+ condition relative to its CRISPRi- counterpart for both systems. Similar trends are also seen in both systems for aTc and O_2, as an increase in ethanol production was seen even in the absence of aTc-mediation induction, and anaerobic conditions generally had higher ethanol production than aerobic conditions. The highest ethanol output was seen in the anaerobic condition with the aTc induced CRISPRi for both systems. However, the ethanol output for the promoter targeting system in this condition was ~3 fold higher than that of the coding sequence targeting system. Both systems were thus seen to be effective at increasing ethanol production, though the promoter targeting system resulted in relatively more ethanol.

Discussion

We aimed to increase the production of ethanol in E. coli by inhibiting *ldhA* via CRISPRi. A plasmid with dCas9 inserted into the desired expression vector was needed in order to produce the dCas9 that will bind the *ldhA*-targeting sgRNA, which will then bind to the promoter of *ldhA* to block the binding of transcriptional complexes. Gel electrophoresis of a digest of the dCas9 plasmid produced bands at the expected kb lengths, showing successful insertion of the dCas9. This experiment served as a control which enabled us to be reasonably confident that the dCas9 was present in the plasmid before the E. coli were transformed with this

plasmid. Similarly, the sequencing results provided confirmation that the pgRNA_ldhA plasmid carried the designed *ldhA*-targeting gRNA. Had the experiment not been successful further evidence of successful dCas9 and gRNA insertion would have been needed before proceeding to transformation.

Since the insertions were successful, we proceeded with transformation, and ultimately the measurement of ethanol production under O_2+/-, aTc +/-, and CRISPRi +/- culture conditions. The *ldhA* promoter targeting system successfully enhanced ethanol production as all CRISPRi+ conditions had higher yields than their corresponding CRISPRi- conditions. The enhancement of ethanol production was observed even in the absence of aTc, suggesting that the expression of dCas9 is not entirely repressed by TetR, and so, while aTc may lead to increased expression of dCas9, it is not necessary for expression. The increase in ethanol concentration was also higher in anaerobic conditions than aerobic conditions. This result was expected since ethanol is produced through mixed acid fermentation and is metabolically favored under anaerobic conditions. The results thus supported our hypothesis that a *ldhA* promoter targeting CRISPRi system would increase ethanol production, especially under anaerobic conditions.

We theorize that this effect is due to *ldhA* repression leading to higher levels of ethanol precursors and potentially cellular stress to regenerate NAD+. One limitation to the study however is that mechanism of the CRISPRi system was not confirmed. Future studies could measure *ldhA* mRNA levels and quantify the concentrations of precursors such as pyruvate and acetyl-CoA to confirm that the CRISPRi system represses *ldhA* expression and provide support for mechanistic theories about its effects on ethanol production.

The effects of the *ldhA* promoter targeting system and coding sequence targeting systems were found to be very similar. The results supported our hypothesis, as both systems effectively

increased ethanol production, though the promoter targeting system was more effective at doing so, particularly in the anaerobic and induced condition. We theorized that this result may be because a promoter-targeting system has more mechanisms by which it can inhibit transcription (i.e. by blocking initiation as well as elongation). The significance of these results cannot be determined however as the promoter targeting experiment was not performed in replicate and so it lacks an indicator of variability. It is possible that the higher ethanol production observed for the promoter system was due to other variables rather than an underlying biological mechanism. For instance, the experiments for the different CRISPRi systems were conducted at different times and by different individuals, potentially introducing significant variability. Further research is therefore needed to determine the significance of the results.

This study demonstrates a system to increase the yield of a high-demand commodity, ethanol. In the future, the method provided can be built upon, further refined, and combined with other strategies for modifying E. coli metabolism to increase ethanol production. By increasing our understanding of the how to optimize and engineer natural pathways, such research provides the opportunity to increase the commercial feasibility of biofuel production. The success of the biofuel industry will be critical for decreasing reliance on fossil fuels, which is strongly motivated by growing concern over the depletion of natural resources and climate change driven by greenhouse gas emissions (Koppolu and Veneela 2016).

Methods

Diagnostic Digest of pdCas9

To confirm insertion of dCas9 in the expression vector diagnostic digests were performed with the restriction enzymes Age1 and Mlu1 (NEB). There were 4 digest conditions: just Age1,

just Mlu1, no enzyme, or both enzymes. The digest mixes consisted of NEBuffer 2.0 (NEB), 125ng pdCas9 (from the Prather Lab), and 10 U of Age1 and/or 10 U of Mlu 1. Digests were then incubated at 37°C for one hour before being stored at -20 °C. To separate the DNA fragments in the four digested samples a 1% agarose gel in TAE gel electrophoresis buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA)(BioRad) was run at 125 V for 45 minutes. SYBR Safe DNA gel stain (ThermoFisher) was used to visualize the resultant bands.

SDM and gRNA Amplification and Isolation

The gRNA used was designed to target the template strand in the promoter of *ldhA* and bind to dCas9 to block formation of the transcription complex. The sequence of the gRNA was as follows: 5' - CTTAAATGTGATTCAACATCACTGGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGC - 3'

The first 25 bp are complimentary to template strand of the region +35 to +10 bp upstream of the transcription start site of *ldhA* and the remainder serves as a tag for dCas9 binding. The gRNA was incorporated into the pgRNA expression vector via site-directed mutagenesis according to the protocols in the Q5 Site Directed Mutagenesis Kit from NEB. The designed gRNA primer and universal CRISPRi reverse primer (5' - ACT AGT ATT ATA CCT AGG ACT GAG CTA GC - 3') specific to the pgRNA at 10 uM each were combined with 25 ng/uL of pgRNA plasmid DNA and combined with an equivalent volume of the Q5 Hot Start High-Fidelity 2X Master Mix. The mixture was then thermocylced under the following conditions: 30s at 98°C to denature the DNA; 25 cycles of 10s at 98°C, 30s at 55°C, then 2 min at 72°C to amplify; then 2 min at 72°C for the final extension before holding at 4°C. A KLD reaction was then performed by

combining 1:10 amplification product, 1:2 2X KLD Reaction Buffer, and 1:10 KLD Enzyme Mix and incubating for 5 min. at room temperature.

To amplify the pgRNA vector, an E. coli strain (NEB 5α cells of genotype fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17) was then transformed with the SDM reaction product. The transformation was performed with 1:10 SDM product to chemically competent NEB 5α, which were incubated on ice for 30 min., then heat shocked at 42 °C for 30 s, and incubated on ice for 5 min. Then SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4, and 20 mM glucose) was added and the cells were gently shaken at 37 °C for 1 hour. Then the cells were plated on 1.5% agar containing Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 1% NaCl) and 100 ug/mL ampicillin (to select for cells containing the pgRNA) and incubated overnight at 37 °C.

A mini-prep was performed to isolate plasmid DNA from the transformed NEB 5α. The pgRNA_ldhA clones were mini-prepped per the protocols in the QIAprep Spin Miniprep Kit (Qiagen), except elution was performed in 30 uL of H20 with pH 8.

Preparation of pgRNA for Sequencing Analysis

To confirm proper insertion of the gRNA in the pgRNA_ldhA, the plasmid was sequenced. Forward and reverse primers were used containing the sequences 5' - GGG TTA TTG TCT CAT GAG CGG ATA CAT ATT TG - 3' and 5' - CGC GGC CTT TTT ACG GTT C - 3' respectively. Each sequencing reaction mixture contained the ~53 ng/uL plasmid DNA and 1.7 uM primer in nuclease-free water. The samples were sent to Genewiz for sequencing.

Transformation of CRISPRi System Into MG1655 E. Coli Cells

E. coli MG1655 were used to examine the effects of the designed CRISPRi system on ethanol production. To use this strain, the pgRNA_ldhA plasmid was transformed with the pdCas9 plasmid into E. coli MG1655. Mini-prepped pdCas9, the pgRNA clone, and competent MG1655 were mixed in a 1:1:20 ratio and incubated on ice for 30 min. Co-transformations were incubated on a heat block at 42 °C for 45 sec. and then incubated on ice for 2 min before pre-warmed SOC media was added. The co-transformations were then incubated for 1 hour at 37 °C on a nutator before being plated on 1.5% agar containing LB broth, 100 ug/mL ampicillin (to select for cells containing pgRNA), and 25 ug/mL chloramphenicol (to select for cells containing pdCas9) and then incubated for ~18 hr. at 37°C. Transformed (CRISPRi+) MG1655 E. Coli were then transferred to tubes with LB containing the same concentrations of ampicillin and chloramphenicol to be cultured in either aerobic or anaerobic conditions with or without 2 uM aTc. CRISPRi- MG1655 E. Coli were likewise transferred, but to tubes without antibiotic, and all cultures were stored at 37°C.

Quantification of Ethanol Production

The ethanol produced per cell in each culture condition was quantified by normalizing the output of a colorimetric Ethanol Assay Kit from Sigma-Adrich by optical density. Optical density was measured with a spectrometer as the absorbance at 600 nm of 1:10 dilutions of the samples (Supplementary Figure 1). The cultures were then pelleted by centrifugation and the supernatant was then diluted 1:15 in LB. The diluted samples were then transferred into wells with an equivalent volume of Reaction Mix (23:25 Ethanol Assay Buffer, 1:25 Ethanol Enzyme Mix, and 1:25 Ethanol Probe). An equivalent volume of Reaction Mix was also added to

standards with known concentrations of ethanol (0.04, 0.08, 0.12, 0.16, 0.2 nmol/uL). The output of the colorimetric assay was measured as absorbance at 570 nm via a plate reader spectrophotometer. Linear regression was performed on the output for the ethanol standards and the resultant equation was used to determine the ethanol concentration in the samples (Supplementary Figure 2). These concentrations were background-adjusted by subtracting the result for the CRISPRi-, aTc-, O_2+ condition and then normalized by the optical density of each sample. The molecular weight of ethanol, 46.07 g/mol, was used to convert to ng/uL.

References

"Biofuels Issues and Trends." *U.S. Energy Information Administration*, Oct. 2012, www.eia.gov/biofuels/issuestrends/pdf/bit.pdf.

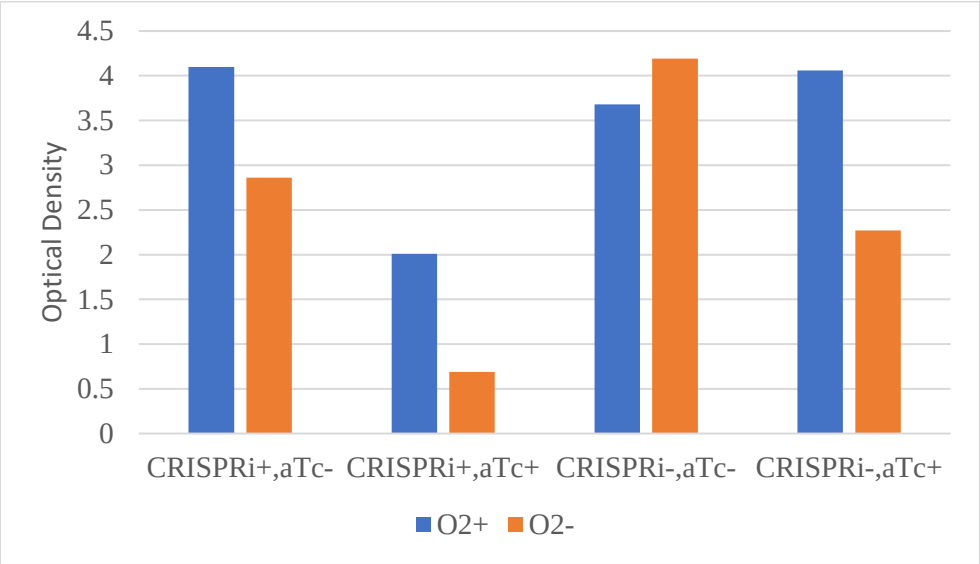
Förster, Andreas H, and Johannes Gescher. "Metabolic Engineering of Escherichia coli for Production of Mixed-Acid Fermentation End Products." *Frontiers in bioengineering and biotechnology* vol. 2 16. 23 May. 2014, doi:10.3389/fbioe.2014.00016

Koppolu, Veerendra, and Veneela Kr Vasigala. "Role of Escherichia coli in Biofuel Production." *Microbiology insights* vol. 9 29-35. 14 Jul. 2016, doi:10.4137/MBI.S10878

Larson, Matthew H et al. "CRISPR interference (CRISPRi) for sequence-specific control of gene expression." *Nature protocols* vol. 8,11 (2013): 2180-96. doi:10.1038/nprot.2013.132

Rodriguez-Garcia, A., et al. "Natural and Synthetic Tetracycline-Inducible Promoters for Use in the Antibiotic-Producing Bacteria Streptomyces." *Nucleic Acids Research*, vol. 33, no. 9, 2005, doi:10.1093/nar/gni086.

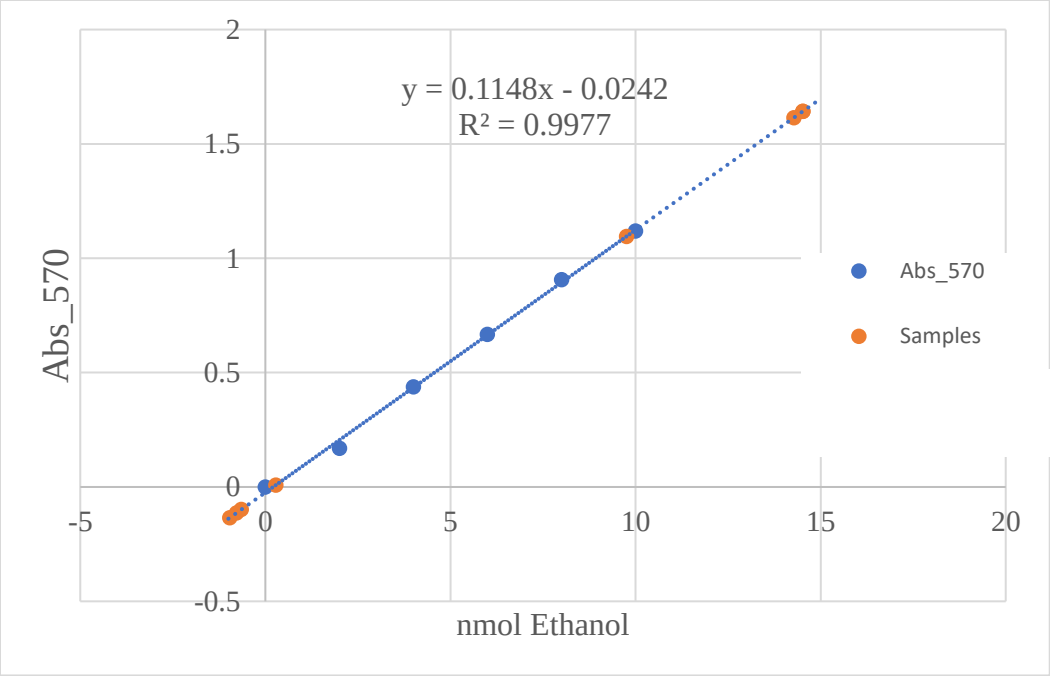
Supplementary Material



Supplementary Figure 1: Optical Density (OD) Varied Between Conditions

Optical density was measured for each culture condition so that ethanol production could be normalized by cellular concentration. A spectrometer was used to measure absorbance at 600nm as a measure of optical density for 1:10 dilutions of each culture condition.

Aerobic conditions are more favorable for cellular growth and so O_2+ conditions were expected to have higher OD than O_2- conditions. Accordingly, the OD was higher for the O_2+ condition in 3 of the 4 sets of conditions. The aTc+ conditions also had lower OD than aTc- conditions for 3 of the 4 sets. This suggests that aTc may have a slight antibiotic effect despite supposedly being an inactive version of Tc. The CRISPRi+ condition also had lower OD than CRISPRi- conditions for 3 of the 4 sets. This indicates a nonzero level of nonspecific transcriptional inhibition by the dCas9 system.



Supplementary Figure 2: Fitting Samples with Ethanol Standards

The ethanol concentration for each sample was extrapolated by fitting colorimetric assay output for the sample to the equation produced via linear best fit from the assay output for known graduated concentrations of ethanol (0.04, 0.08, 0.12, 0.16, 0.2 nmol/uL). The best fit equation produced was y = 0.1148x – 0.0242 where y represents the assay output and x is the quantity of ethanol in the sample (in nmol). The linear regression produced an R2 value of 0.9977.