An iGEM-Compliant Shuttle Vector for Clostridial Chassis Organisms

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INTRODUCTION

Concerns about energy security, fuel price volatility, and the environmental impact of fossil fuels have stimulated the interest of both the public and the scientific community in the development of renewable energy sources, including biofuels. Recent research in synthetic biology has resulted in the production of alcohols not known to be produced naturally, and on the extension of carbon chain length. These systems have been expressed in *E. coli*.

We hypothesize that the expression of the same systems in native alcohol producing organisms, such as *Clostridium acetobutylicum* or *Clostridium beijerinckii* will result in improved yield. These organisms have been optimized by the evolutionary process for solvent production. Although final solvent tolerance in *E. coli* has been shown to be competitive with native producers in some cases (1), there are other advantages for using the *Clostridia*, including the ability to degrade hemicellulose and the production of H_2. Some *Clostridia* are also able to directly ferment crude glycerol, a byproduct of biodiesel production, to 1-butanol, which is a value added feature for biodiesel plants (2).

One major problem with the *Clostridia* is the lack of tools available for genetically manipulating them.

OBJECTIVES

Our objective was to construct an iGEM standard compliant shuttle vector for *E. coli* and *Clostridia* that will allow the heterologous expression of iGEM registry parts in *Clostridia*.

PARTS

Clos ORI (Bba_K1207000)
The replication machinery from the *E. coli – C. acetobutylicum* shuttle vector pIKM1 was cloned in iGEM compliant format.

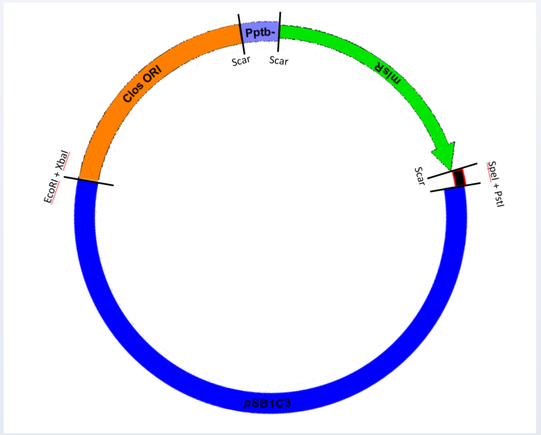
Phosphotransbutylase promoter
Pptb was ordered from GenScript as a fully synthesized gBlock. No ribosome binding site was included. Part has been verified by sequencing, but was not submitted to the registry before the deadline.

mlsR (Bba_K1207001)
The macrolide-lincosamide-streptogramin B resistance marker from the shuttle vector pIKM1 was cloned in iGEM compliant format.

Other parts obtained but not yet characterized or submitted:

AdhE from *Clostridium cellulolyticum*
AdhE from *Clostridium saccharolyticum*
Sadh from *Clostridium beijerinckii*
Adh6 from *Saccharomyces cerevisiae*

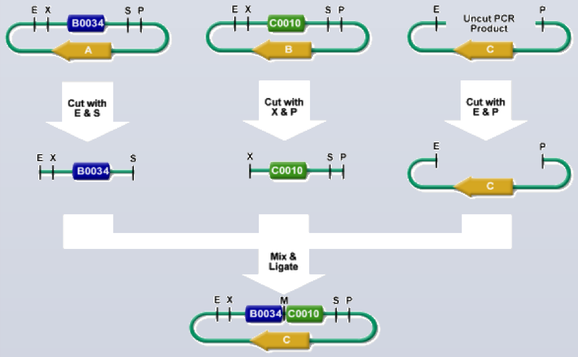
PLASMID MAP



METHODS

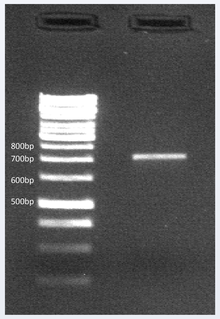
The *Clos Origin* and *mlsR* genes were cloned directly by PCR from the *E. coli – C. acetobutylicum* shuttle vector pIKM1, using primers with iGEM prefix and suffix tags. The *Pptb* promoter sequence was synthesized by GenScript. Each part was digested with EcoRI and I and ligated into an appropriate iGEM plasmid backbone for use in 3A assembly. Top10 *E. coli* were transformed with these plasmids. Libraries were grown and plasmids were extracted, digested and screened for inserts by gel electrophoresis. Plasmids containing appropriately sized inserts were sequenced with the primers VF2 and VR to confirm the insert sequence was correct. Once sequence verified, 3A assembly (method shown below) was performed in single pot reactions.

3A Assembly

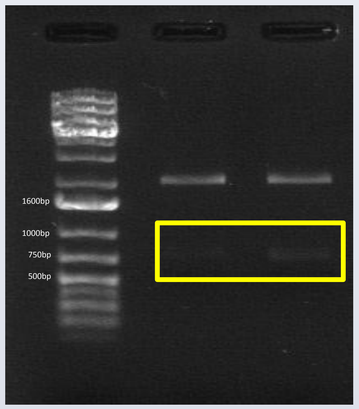


(Image Source: http://2010.igem.org/3A_Assembly)

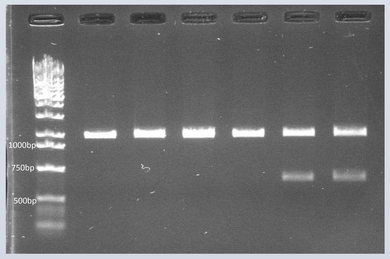
RESULTS



Gel of the *C. acetobutylicum* origin after running it through a QIAquick PCR Purification Kit. (expected size of insert is 691 bp.)



Gel of two successful 3A assemblies between the origin, P_{ptb-} and pSB1A3. (expected size of insert is 821 bp)



Gel of two successful *mlsR* colonies post digestion (expected size of insert is 735bp)

Unfortunately, we must report that we were unable to complete the construct due to issues with 3A assembly.

FUTURE WORK

First, the construct must be completed. It must then be transformed into an *E. coli* strain containing the pAN1 plasmid to methylate the plasmid and protect it from the *Cac824I* restriction system present in *C. acetobutylicum.* It must them be extracted and the *Clostridium* will be electrotransformed and grown on erythromycin media.

After verifying that the construct replicates and confers resistance to erythromycin under the ptb promoter, as expected, the external iGEM cloning sites will be removed and a spacer including the cloning sites will be added between the *Clos Ori* and *Pptb* parts. This result will be a new backbone that can be modified as with any other iGEM plasmid backbone.

A series of alcohol dehydrogenase cassettes will be tested for increased total titer and rate of production in *C. acetobutylicum*.

Synthetic pathways will also be tested in *Clostridium* to compare the production of alcohols in native alcohol producers to those results obtained using *E. coli*. Some examples of these cassettes include KIVD and Adh6, as well as BktB and Ter.

Other areas of consideration will be: increasing the solvent resistance and aerotolerance, delay of sporulation, alternative carbon and energy sources, as well as increasing the reductant pool.

REFERENCES

1. **C. R. Shen, Lan, E. I., Dekishima, Y., Baez, A., Cho, K. M., and Liao, J. C.** Driving forces enable high-titer anaerobic 1-butanol synthesis in *E. coli*. 2011. App. Environ. Microbiol. **77:** 2905-2915.
2. **T. O. Jensen, Kvist, T., Mikkelsen, M. J., Christensen, P. V., and Westermann, P.** Fermentation of crude glycerol from biodiesel production by *Clostridium pasteurianum.* 2012. J. Ind. Microbiol. **39:** 709-717.

TEAM ATTRIBUTIONS

Jeremiah Miller – Team Founder. Provided leadership and managed lab resources and finances. Also performed wetwork.
Erin Weese – Designed and isolated *Pptb*, original team member, helped get the team running.
Zachariah Herron – Wiki design and wetwork, original team member, helped get the team running.
Sam Jones – Our wetwork MVP! Most hours spent pipetting.
Justin Montgomery – Maintenance of lab notebooks and wetwork.
Michel Essien, Ian Pratt, Jayme Haynes and Holly Baginski each contributed time to wetwork.

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