Carbohydrate-recognizing regulators from *Ruminiclostridium thermocellum* work orthogonally in *Bacillus subtilis*

Submitted by

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Biomedical Sciences

To

The Honors College

Oakland University

In partial fulfillment of the

requirement to graduate from

The Honors College

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April 4, 2019

Abstract

Orthogonal regulation is important for the field of synthetic biology in order to reduce cross-talk between endogenous and recombinant regulators in engineered microbes. My goal for this project was to determine the feasibility of using a carbohydrate sensing regulatory system from the thermophilic bacterium *Ruminiclostridium thermocellum* in *Bacillus subtilis*. Both Firmicutes evolved from a common ancestor; however, this regulatory system evolved in *R. thermocellum* and not in *B. subtilis*, making it a candidate for orthogonal regulation. If successful, this system will be used to construct a biosensor microorganism, using *B. subtilis*, that will respond to plant-derived carbohydrates such as cellulose, xylan, and pectin, by producing green fluorescent protein (*gfp*). *R. thermocellum* anti-sigma and sigma (σ) factors will be cloned into a replicating *B. subtilis* plasmid using isothermal assembly, while the cognate promoter will be cloned in front of *gfp* on a non-replicating plasmid using Golden Gate assembly. We modified an existing non-replicating plasmid for use in Golden Gate cloning, including replacing the strong lactose-inducible promoter with a *lacZ* cassette flanked by BsaI sites and using site directed mutagenesis to remove a BsaI recognition site from the β-lactamase gene. Both plasmids were transformed into supercompetent *B. subtilis* SCK6. Using a spectrofluorimeter, we quantified levels of non-target expression from the *R. thermocellum* promoter in *B. subtilis*. Constructed plasmids and engineered *B. subtilis* strains will ultimately be used to construct biosensors for use in directed evolution of carbohydrate recognizing proteins.

Carbohydrate-recognizing regulators from *Clostridium thermocellum*

work orthogonally in *Bacillus subtilis*

The two main bacterial species used in this project were *Bacillus subtilis* and *Ruminiclostridium thermocellum* (formerly '*Clostridium thermocellum*'). Both are Gram-positive bacteria, indicating they have only one cell membrane, and both deposit a thick layer of peptidoglycan surrounding the cell helping to form the cell wall. These two bacteria are descended from a common ancestor; however, they are physiologically different. *Ruminiclostridium thermocellum* is a plant biomass-degrading thermophile that has evolved to utilize a unique carbohydrate sensing mechanism as a part of its lifestyle (Kahel-Raifer et al., 2010). In contrast, *Bacillus subtilis* is a Gram-positive model organism commonly engineered for recombinant gene expression (Y. Lu, Zhang, Ly, Bie, & Z. Lu, 2012). This species is rod-shaped, capable of forming spores, and frequently found in soil and the gastrointestinal tract (Earl, Losick, & Kolter, 2008). *B. subtilis* was once considered an obligate aerobe; however, it has been demonstrated that *B. subtilis* can use nitrate in place of oxygen as an electron acceptor during cellular respiration, reinforcing the notion that *B. subtilis* has the capacity to live in the gastrointestinal tract (Earl et al., 2008).

Literature Review

Ruminiclostridium thermocellum is a strongly cellulolytic bacterium, and for this reason has potential future implications for use of bacteria as a source of biofuel through plant biomass degradation (Gold & Martin, 2007). *R. thermocellum* uses a macromolecular complex of enzymes and carbohydrate binding proteins called a cellulosome, which is involved in the hydrolysis of various polysaccharides (Abdou et al., 2008; Gold & Martin, 2007). Cellulases and

other catalytic subunits are displayed on the cellulosome through interactions with a scaffolding protein, CipA (Abdou et al., 2008; Gold & Martin, 2007). Prior studies have observed that the level of cellulolytic activity and expression of those genes are regulated by the carbon source available and the growth rate, where slower growth rates lead to greater cellulosomal protein expression (Abdou et al., 2008; Gold & Martin, 2007). Regulation of genes encoding for cellulsomal enzymes in *R. thermocellum* is in response to the presence or absence of polysaccharides using multiple extracytoplasmic factor anti-σ^I/σ^I pairs (Kahel-Raifer, et al., 2010; Nataf et al., 2010). When no polysaccharide is present, the extracellular sensory domain of the anti-σ factor remains inactivated and the σ factor stays bound to the anti-σ factor (Figure 1a); however, when this sensory domain recognizes a polysaccharide, there is a conformational change in the protein structure, allowing the σ factor to dissociate from the anti-σ factor and induce cellulosomal gene expression (Figure 1b).

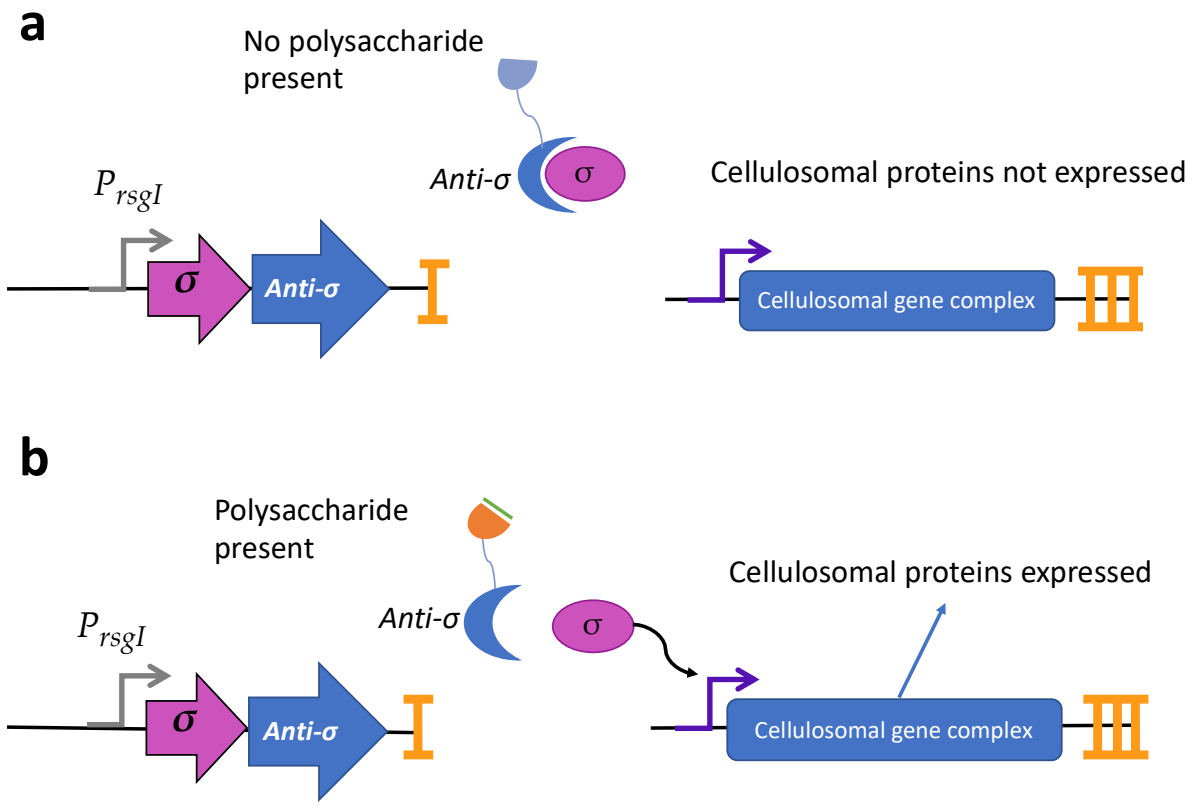

Figure 1. Regulation of genes encoding for cellulosomal enzymes in R. thermocellum

Scientists in one of the pioneering laboratories focused on cellulosomes were also the first group to discover a role for the extracytoplasmic factors (ECFs) SigI-RsgI in *R. thermocellum* (Bayer, Belaich, Shoham, & Lamed, 2004). Upon further evaluation of the RsgI-like proteins, it was found that domains of these proteins bind to carbohydrates and disrupt the interaction between σ and anti-σ factors (Bahari et al., 2011; Kahel-Raifer et al., 2010). In 2010, Nataf et al. observed expression of the bicistronic operon following induction with cellulose, cellulose and xylan, or xylan alone, using qRT-PCR (2010). They identified the σ factors σ^{I1} and σ^{I6} and mapped the recognition sites using 5'-rapid amplification of cDNA ends (RACE) (Nataf et al., 2010). From there, additional cellulosomal genes regulated by σ^{I6} could be identified, such as genes that code for xylanase enzymes (Sand et al., 2015). The search for σ^{I6} promoters expanded with the search for promoter consensus elements and genes regulated by σ^{I3} in *B.*

subtilis (Muñoz-Gutiérrez et al., 2016). Building on the *B. subtilis* system designed to probe potential σ^I promoters, Ortiz de Ora et al. examined the regulation of this system in depth by studying the role of σ^I and anti- σ^I factors on cellulosomal gene expression and the importance of the -35 promoter element as a key for specificity of the sigma factor (2018). For example, the A-tract motif is a conserved region for the binding of σ^I factors, whereas the variable domain differentiates between σ^I factors that will bind, increasing the specificity (Ortiz de Ora et al., 2018). This work also included use of the reporter gene, *gfp*, and searched for SigI1, 2, 3, 4 and 6 regulated genes (Ortiz de Ora et al., 2018). Based on the known interaction of polysaccharides with anti-σ^I factors (Kahel-Raifer, et al., 2010; Nataf et al., 2010), the released alternative σ^I factor will recruit the sole bacterial RNA polymerase for transcription, ultimately controlling the composition of enzymes found in the cellulosome (Ortiz de Ora et al., 2018).

While both *R. thermocellum* and *B. subtilis* use alternative σ^I factor(s), the *B. subtilis* genome encodes for seven extracytoplasmic (ECF) sigma factors, mostly regulated by antibiotics (Helman, 2016) plus a single *rsgI-sigI* pair (Asai et al., 2007) which is induced by heat-shock (Zuber, Drzewiecki, & Hecker, 2001). Alternative σ factors often autoregulate their own expression and in general and they are located in an operon with an associated anti-σ factor gene (Ortiz de Ora et al., 2018). Anti-σ factor proteins from extracytoplasmic function systems will consist of an extracellular sensory domain that interacts with an external signal, a transmembrane domain that associates with the cytoplasmic membrane, and an intracellular domain which binds to its cognate σ factor (Sineva, Saykina, & Ades, 2017).

Expression of *R. thermocellum* ECF regulatory proteins in *B. subtilis* is orthogonal, meaning that in nature, the genes evolved independently in *R. thermocellum* and would not

interfere with regulatory systems in *B. subtilis*. Current efforts to design toolboxes of promoters, ribosome binding sites (RBS), and protein degradation tags for *B. subtilis* have relied on endogenous promoters (Guiziou et al., 2016; Radeck et al., 2013). However, there have been recent attempts that engineering orthologous ECFs in *B. subtilis* which were successful at controlling gene expression (Pinto et al., 2019).

This project will attempt to engineer *Bacillus subtilis* so that it is able to utilize the polysaccharide sensing SigI-RsgI ECF regulators (Figure 1) that are usually found in *R. thermocellum*. This work aims to demonstrate that *B. subtilis* is able to produce both the SigI and RsgI proteins, and that this entire system will be capable of orthogonal regulation in *Bacillus subtilis*. While there has been much improvement in recent years, there is currently a lack of ability in the field of synthetic biology to engineer functional orthogonal regulation systems as the ones found in nature (Rao, 2012). Implementing a system like this into *B. subtilis* would be the first step in designing a sensor microorganism using *B. subtilis* as the platform.

Methods and Materials

Bacterial strains and growth media used

Bacterial strains. Bacterial strains used in this work are listed below in Table 1.

Table 1. Bacterial strains used to construct the sensor microbe

Species	Genotype	Source
Escherichia coli 10β	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)*	NEB
Bacillus subtilis KO7	Δ*nprE*, Δ*aprE*, Δ*epr*, Δ*mpr*, Δ*nprB,* Δ*vpr*, Δ*bpr*	BGSC
Bacillus subtilis SCK6	Em^R, *his*, *nprE18*, *aprE3*, *eglSΔ102*, *bglT*/*bglSΔEV*, *lacA*::PxylA-*comK*	(Zhang, 2011)
Bacillus subtilis Reg19	*trpC2*, *ΔmanPA*::*ermC*, PmtlA-*comK-comS*	(Rahmer, 2015)
B. subtilis MCB1	*B. subtilis* SCK6, pDR111_gfp(Sp)	This work
B. subtilis MCB2	*B. subtilis* SCK6, pMCB01	This work
B. subtilis MCB3	*B. subtilis* SCK6, pMCB02	This work

Growth medium. Media used to support growth of either *E. coli* or *B. subtilis* are listed below in Table 2.

Table 2. Growth media used

Media	Ingredients	Purpose
LB broth	Tryptone, yeast extract, NaCl	General bacterial growth
LB agar	Tryptone, yeast extract, NaCl, agar	General bacterial growth
Transformation buffer	Sorbitol, mannitol, trehalose, glycerol	Bacillus electroporation
Recovery media	Tryptone, yeast extract, NaCl, sorbitol, mannitol	Bacillus electroporation
SOC	Tryptone, yeast extract, NaCl, KCl, $MgCl_2$, $MgSO_4$, glucose	*E. coli* transformation
2xYT	Bacto tryptone, Bacto yeast extract, NaCl	General bacterial growth
ZYM-5052	Tryptone, yeast extract, 50x 505 stock, 50x M stock, 1M $MgSO_4$ stock, 1000x trace metals	Auto-induction
10x Bacillus salts	$(NH_4)_2SO_4$, K_2HPO_4*$3H_2O$, KH_2PO_4, Na^+ citrate, $MgSO_4$*$7H_2O$	Bacillus natural competence
10x Medium A base	Yeast extract, casamino acids	Bacillus natural competence
Medium A	10x medium A base, 10x Bacillus salts	Bacillus natural competence
Medium B	Medium A, $CaCl_2$, $MgCl_2$	Bacillus natural competence

Glycerol stocks

Table 3. Glycerol Stocks used in this work

Species	Plasmid
E. coli DH5α	pDR111_gfp(Sp)
E. coli DH5α	pHCMC02
E. coli 10β	pDR111_gfp(Sp)
E. coli 10β	pHCMC02
E. coli 10β	pMCB01
E. coli 10β	pMCB02
E. coli 10β	pMCB05
B. subtilis Reg19	None
B. subtilis SCK 6	None
B. subtilis SCK6	pDR111_gfp(Sp)
B. subtilis SCK6	pHCMC02
B. subtilis SCK6	pMCB01
B. subtilis SCK6	pMCB02
B. subtilis SCK6	pMCB02, pMCB05

Molecular cloning and plasmids used

Table 4. Plasmids used in this study

Plasmid	Genotype	Description	Source
pDR111_gfp(Sp)	*bla amyE'* $P_{hyperspank}$-*gfp*(*Sp*) *spec lacI 'amyE*	*B. subtilis* integration vector	(Overkamp, 2013)
pMCB01	*bla amyE'* BsaI P*lac-lacZα gfp*(Sp) BsaI *spec lacI'amyE*	Golden-Gate ready vector	This study
pMCB02	*bla amyE'* P_{sigI6}-*gfp(Sp) spec lacI' amyE*	P_{sigI6} and *gfp* expression	This study
pUC19	*bla* P*lac-lacZ*α	*E. coli* cloning vector	NEB
pHCMC02	P_{lepA} *cat bla*	*E. coli-B. subtilis* shuttle vector	(Nguyen, 2005)
pMCB05	P_{lepA} *cat bla Cthe_2120*	σ^{I6} expression	This study

Plasmid DNA isolation. Plasmid DNA was isolated from either *E. coli* or *B. subtilis* using alkaline lysis combined with silica spin columns in the Wizard mini-prep (Promega) following manufacturer's protocols. Purified plasmid DNA was eluted in a minimal volume, and vacuum concentration was used to increase the concentration of plasmid DNA when needed. Plasmid DNA was quality checked by DNA gel electrophoresis as described below, and the A260nm/A280nm ratio as determined by a NanoDrop spectrophotometer (ThermoFisher).

Gibson Assembly. Isothermal assembly was first detailed by Daniel Gibson and colleagues in 2009 in a paper titled "Enzymatic assembly of DNA molecules up to several hundred kilobases." This method involves a single reaction for DNA assembly which can combine molecules as large as 583 kb and clone joined products in *E. coli* as large as 300 kb (Gibson et al., 2009). The NEBuilder software suite was utilized to design the construction of

plasmids using Gibson assembly. Equimolar amounts of vector and insert(s) are combined in a PCR tube with Gibson Assembly Master Mix from NEB and de-ionized water to bring the reaction volume to 20 µL (Gibson et al., 2009). The reaction mix is then incubated at 50°C for one hour before being transformed into competent cells (Gibson et al., 2009).

Golden Gate cloning. The construction of vector pMCB02 was performed using Golden Gate cloning (Engler, Kandzia, Marillonnet, & El-shemy, 2008). This method of cloning was developed to use restriction enzymes that cut outside of their recognition sites, allowing for "scar-less" cloning that doesn't include the recognition site of the restriction enzyme used after cloning. Golden Gate assembly takes place in a one-tube reaction and allows for site-specific recombination of DNA (Engler et al., 2008). There are other methods of recombination where it is possible to add restriction sites in introns, which are spliced out of the gene and not expressed; however, this only works for eukaryotes, since prokaryotes do not contain introns within their DNA and have no method for splicing this unwanted DNA out (Engler et al., 2008). Golden Gate assembly makes use of type IIS restriction enzymes which cut DNA outside of recognition site, creating overhangs of DNA, allowing two fragments to be joined to form a product without the original restriction site (Engler et al., 2008). The McClean method of Golden Gate Assembly was the protocol followed in this work. This protocol requires the creation of a 10 µL solution containing 20 femto moles of plasmid, 5 units of BsaI, 1,500 units of T7 ligase, 1 µL of 10X buffer for T4 ligase with 10 mM ATP, and DI water to bring to 10 µL. T4 ligase buffer is used because it has a preservative which prevents the degradation of T7 at high temperatures. In the thermocycler the solution is alternated between 42°C and 16°C for 5 minutes each for 30 cycles. The solution is then brought to 42°C for 30 minutes, followed by an additional 30 minutes at

60°C. Finally, the solution is brought to 80°C for 10 minutes. The DNA product is then ready for transformation into *E. coli*.

Blue-White Colony Screening. After Golden Gate assembly, colonies were screened through blue-white screening for pMCB02 where the P_{sigI6} promoter region replaced *lacZ*. *E. coli* cells transformed with the Golden Gate assembly product were plated on media containing the appropriate selective antibiotic as well as X-gal/IPTG Firozeh media from Growcells. After overnight incubation, cells that appear blue demonstrate the presence of β-galactosidase, meaning that the *lacZ* cassette was not excised during Golden Gate. White colonies, on the other hand, are assumed to have the DNA insert of interest because of the inability to hydrolyze X-gal.

Amplification of DNA for molecular cloning

Polymerase chain reaction. Polymerase chain reaction (PCR) is a method of amplifying DNA segments by using the original DNA segment of interest, forward and reverse primers which bind to it, nucleotides (dNTPs), a buffer solution, and a polymerase enzyme. For this work Q5 polymerase (NEB Labs) was used for amplification following the manufacturer's recommendations (0.02 U/μL Q5 polymerase, 1x Q5 buffer, 0.5μM forward and reverse primers, 0.2 mM dNTPs). Each reaction used a series of temperature changes which allow for the DNA strands to separate (98 to 95˚C), the primers to bind (variable), and Q5 polymerase to extend (72 ˚C) the new segment using the nucleotides in the reaction mix (98˚C, 30s) followed by 25 cycles of 95˚C, 10s; variable, 30s; 72˚C, variable time; and a final extension at 72˚C and 2 min. Annealing temperatures were calculated using the Tm calculator from NEB (https://tmcalculator.neb.com) and the extension time was determined based upon the length of the DNA segment (30s per 1kb).

PCR amplicon purification. Purification of the DNA segments amplified by PCR was performed using the Wizard® SV Gel and PCR Clean-Up System protocol and kit from Promega. This is an important step to remove the buffers and enzymes present in a PCR reaction and leave only the DNA product behind for future use when cloning.

Agarose Gel Electrophoresis. Gel electrophoresis works by applying an electrical field along which negatively charged DNA molecules move toward a positive electric pole through an agarose gel (Sambrook & Russell, 2006). The distance traveled by the band of DNA is dependent upon the size of the DNA molecule. Longer DNA sequences have a greater molecular weight and do not travel as far through the gel as shorter, lighter DNA molecules. One of the wells in the gel is filled with a DNA ladder which displays bands of known lengths which can be used in comparison to a DNA sequence of unknown nucleotide length in order to estimate the number of nucleotides in the DNA molecule in the sample of interest.

Table 5. Oligonucleotides used for Gibson Assembly of pMCB01		
Oligonucleotide Name	Nucleotide Sequence (5'-3')	Purpose
Frag-BA-fwd	CCGCGAGAGCCACGCTCACCGGCTCC	BA fragment amplification
Frag-BA-rev	TTGCGTTGGGTCTCTTAGTCGACAGCTAGCTGATTAACTAATAAGGAGGACAAAC	BA fragment amplification
Frag-DC-fwd	CCGCATAGGGTCTCCTCGAGCACGTTCTTGC	DC fragment amplification
Frag-DC-rev	AGCGTGGCTCTCGCGGTATCATTGCAG	DC fragment amplification
lacZalpha w prom FWD	tagctgtcgactaaGAGACCcaacgcaattaatgtgagttagc	*lacZ* fragment amplification
NEW lacZ w prom R	GAACGTTGCTCGAGGAGACCCTATGCGGCATCAGAGCAGA	*lacZ* fragment amplification
Internal-DC-fwd	GAATTTACACGCTGACGCTG	DC2 fragment amplification
Internal-DC-rev	CAGCGTCAGCGTGTAAATTC	DC1 fragment amplification
Internal-BA-fwd	CCAGCCAGCCAGACGCAGACGC	BA2 fragment amplification
Internal-BA-rev	GCGTCTGCGTCTGGCTGGCTGG	BA1 fragment amplification

Table 6. Oligonucleotides used for pMCB02 construction

Oligonucleotide Name	Nucleotide Sequence (5'-3')	Purpose
sigI6-rsgI6	acaatgCGACATAAAAccattccggtataCGAAtcgatataaga	Promoter
sigI3-rsgI3	tatgaacCCCTCAAAAaaatcatttggtgCGTAcaagtattgaa	Promoter
sigI1-rsgI1	taatATACACAAAaaaagcagatgtataCGAAgtaatctactg	Promoter
RC PsigI6 R	agctacaatgCgacatAAAaccattccggtataCGaAtcgatataaGa	Anneal oligos for Golden Gate
RC PsigI6 F	actatCttatatcgaTtCGtataccggaatggtTTTatgtcGcattgt	Anneal oligos for Golden Gate
RC PsigI3 R	agcttatgaaCccctcAAAaaaatcatttggtgCGtAcaagtattgaa	Anneal oligos for Golden Gate
RC PsigI3 F	actattcaatacttgTaCGcaccaaatgattttTTTgagggGttcata	Anneal oligos for Golden Gate
RC PsigI1 R	agcttaatatacacaaAAAaagcagatgtataCGaAgtaatctactG	Anneal oligos for Golden Gate
RC PsigI1 F	actaCagtagattacTtCGtatacatctgcttTTTttgtgtatatta	Anneal oligos for Golden Gate
New PsigI6 R	tcgaACAATGCGACATAAAACCATTCCGGTATACGAATCGATATAAGA	Anneal oligos for Golden Gate
New PsigI3 R	tcgaAGCTTATGAACCCCTCAAAAAAATCATTTGGTGCGTACAAGTATTGAA	Anneal oligos for Golden Gate
New PsigI1 R	tcgaTAATATACACAAAAAAAGCAGATGTATACGAAGTAATCTACTG	Anneal oligos for Golden Gate

Table 7. Primers used for colony PCR of pMCB02-04

Primer Name	Nucleotide Sequence (5'-3')	Purpose
pMCB03-F	TTTTGAGGGGTTCATAtcgagca	Colony PCR for P_{sigI3}
pMCB03-R	tctcttgccagtcacgttacg	Colony PCR for P_{sigI3}
pMCB02-F	CCGGAATGGTTTTATGTCGCA	Colony PCR for P_{sigI6}
pMCB02-R	tcttgccagtcacgttacgt	Colony PCR for P_{sigI6} and P_{sigI1}
pMCB04-F	tgtcgactaCAGTAGATTACTTCGT	Colony PCR for P_{sigI1}

Table 8. Primers used for pHCMC02 construction

Primer Name	Nucleotide Sequence (5'-3')	Purpose
2120_fwd	atagtaggagtgaggatcctctagaGTGGATTGGCATTTTCAAGG	2120 amplification
2120_rev	ctcattaggcgggctgccccgggCGCAAATCCACCTCCTTTTC	2120 amplification
2119_fwd	atagtaggagtgaggatcctctagaGTGATTGTAGGAAAAGTTCTTG	2119 amplification
2119_rev	ctcattaggcgggctgccccgggTCAGGGAATTCTGTAAGTAG	2119 amplification
2119-2120_fwd	atagtaggagtgaggatcctctagaGTGGATTGGCATTTTCAAG	2119-2120 amplification
2119-2120_rev	ctcattaggcgggctgccccgggTCAGGGAATTCTGTAAGTAG	2119-2120 amplification
2119-F	gctagtgtattttgcgtttaatagtaggagtgaggatcctGTGATTGTAGGAAAAGTTCTT	40 nt overlap for GA
2119-R	cgtgacgtgaaaaaagcccgctcattaggcgggctgccccTCAGGGAATTCTGTAAGTAG	40 nt overlap for GA
2120-F	gctagtgtattttgcgtttaatagtaggagtgaggatcctGTGGATTGGCATTTTCAAGG	40 nt overlap for GA
2120-R	cgtgacgtgaaaaaagcccgctcattaggcgggctgccccTCACCGCAAATCCACCTCCTTTTC	40 nt overlap for GA
2119-2120-F	gctagtgtattttgcgtttaatagtaggagtgaggatcctGTGGATTGGCATTTTCAAG	40 nt overlap for GA
2119-2120-R	cgtgacgtgaaaaaagcccgctcattaggcgggctgccccTCAGGGAATTCTGTAAGTAG	40 nt overlap for GA

Table 9. Primers used for pHCMC02 construct sequencing		
Primer Name	Nucleotide Sequence (5'-3')	Purpose
pHCMC02_3524_F	ACCGTAACCAATACTGCCCAGTC	2120 sequencing
pHCMC02_3937_R	CAAATTCTTTCCGAGCTTCGTCC	2120 sequencing
2119_4271_F	AAAGTGCCGTGCCTGAAGTG	2119 sequencing
2119_4710_R	GGCTGTACCAAATCCAAAGGCATG	2119 sequencing
2119-2120_4174_F	GAGGTGGATTTGCGGTGATTGTAG	2119-2120 sequencing
2119-2120_5468_R	GGCTGTACCAAATCCAAAGGCATG	2119-2120 sequencing

Bacterial Transformation

***B. subtilis* transformation methods.** Transformation of *B. subtilis* was tested using three methods: natural competence, electroporation or induced competence. For electroporation, 1 mL of a 12-hour *B. subtilis* K07 culture was subcultured into 40 mL fresh LB containing 0.5 mol L^{-1}. This culture was grown at 37°C until reaching the logarithmic phase when the cells were harvested and washed with transformation buffer (to be stored at -80°C as electrocompetent cells (Lu et al., 2012). Prior to electroporation, 60 µL of competent cells was incubated with 1 µL of plasmid in a chilled electroporation cuvette (1 mm gap). Cells were then electroporated at 2200 V, 25 µF, 200 Ω (Bio Rad Xcell), after which 1 mL of recovery medium was added to the cells. Cells were recovered for 3 hours at 37°C and shaking at 250 rpm before plating on LB with the appropriate selective antibiotic.

B. subtilis natural competence was also attempted for transformation, following a protocol developed by Yasbin, Wilson, and Young (1975). In this method, colonies of *B. subtilis* K07 were inoculated into 20 mL of Medium A (see above) to adjust the OD_{650} to 0.1-0.2 in a total volume of 20 mL. The culture was incubated for 90 minutes at 37˚C and 350 rpm past the point which the culture left the log phase of growth (OD_{650} 0.4 - 0.6). 50 µL of this culture was subcultured into 450 µL of pre-warmed Media B which was then incubated for an additional 90 minutes at 37°C and 350 rpm. For transformation, 1 µg of pDR111_gfp(Sp) and pHCMC02 were added to an aliquot of cells, after which the cells were incubated for an additional 30 minutes and 100 µL of culture was spread onto LB agar plates with antibiotics.

The final method of *B. subtilis* transformation tested was using the supercompetent strains SCK6 (1A976) and Reg19 (1A1276). *B. subtilis* Reg 19 was developed by Rahmer, Heravi and Altenbuchner (2015). Transformation begins with the inoculation of a single colony of this strain into 5 mL LB containing 1 µg mL^{-1} erythromycin for an overnight culture. In the morning, the OD_{600} is read, it should be around 1.7. This culture is added to increase the OD_{600} of 10 mL LB to 0.1 before being incubated for an additional 90 minutes at 37°C and 200 rpm. At this point, 0.5% (w/v) mannitol is added to the culture and the incubation continues for 90 more minutes. The cells are then washed in LB and diluted to an OD_{600} of 0.5. Then 3 µG of the pHCMC02 and pDR111_gfp(Sp) plasmids are added to 1 mL aliquots of this diluted culture and allowed to grow for another hour at 37°C before being plated on selective LB agar.

On the other hand, the transformation of *B. subtilis* SCK6, developed by Zhang and Zhang begins with the inoculation of one colony into 3 mL of LB plus 1 µg mL^{-1} EM which is incubated overnight for about 12 hours at 37°C and 200 rpm (2011). The culture is then diluted to an OD_{600} of 1.0 in LB containing 1% (w/v) xylose before being incubated for 2 more hours. At

this point, 1 µg of DNA is added to 100 µL of cells which are incubated for a final 90 minutes at 37˚C and 200 rpm before plating on selective media. Both supercompetent *B. subtilis* strains were able to be transformed effectively; however, SCK6 appeared to have more transformants for both pDR111_gfp(Sp) and pHCMC02 plasmids and was therefore the strain used in future transformations of *B. subtilis*. *B. subtilis* transformants were selected on LB agar plus chloramphenicol (CM) (10 µg mL^{-1}) or spectinomycin (SP) (100 µg mL^{-1}) for plasmids derived from pHCMC02 or pDR111_gfp(Sp), respectively.

Chemical competent transformation of *E. coli*. The heat-shock method of *E. coli* 10β transformation used in this work is found in the Gibson Assembly® Master Mix Instruction Manual from NEB. Chemically competent 10β cells were thawed on ice and 50 µL was aliquoted into a 1.5 mL microcentrifuge tube before 2 µL of the DNA product was added to the cells. This mixture was placed on ice for an additional 30 minutes, then the tubes were heat-shocked at 42°C for 30 seconds. The tubes were placed on ice for two minutes, then 950 µL of room temperature SOC media was added and the tubes were incubated at 37°C and 250 rpm for at least 1 hour before 100µL of cells were plated on pre-warmed agar plates. This transformation protocol was primarily used to transform the products from Gibson Assembly into *E. coli* 10β. *E. coli* transformants were selected on LB agar plus ampicillin (100 µg mL^{-1}) for both plasmids pHCMC02 and pDR111_gfp(Sp) and their derivatives.

Confirmation of transformants

Colony PCR. Both *B. subtilis* and *E. coli* transformants that were selected on the appropriate antibiotic were first screened for the correct insert using colony PCR. Colony PCR is a method of testing a colony for the presence of a DNA sequence of interest (Woodman et al, 2008). CloneID master mix, a colony, and the forward and reverse primers for the DNA

sequence are combined in a PCR reaction tube and placed in a thermal cycler. CloneID master mix reagent (Lucigen Corporation) was used per manufacturer's instructions with the corresponding oligonucleotide primers (see Table 3). Colony PCR reactions were analyzed by DNA agarose gel electrophoresis as detailed above. Positive transformants were then sequenced to confirm the correct sequence during plasmid construction.

Sanger sequencing. Purified plasmid was sent to a service laboratory (Eton Biosciences) for Sanger sequencing to confirm the nucleotide sequence of all inserts. Sequencing primers were designed using the following parameters: Length: 18-24 nucleotides, Tm: 58-62°C, %GC: 45-55 in A Plasmid Editor (ApE, v2.0.55).

Green Fluorescent Protein Production

One colony of *B. subtilis* 1A976: SCK6 transformed with pDR111_gfp(Sp) from a selective media plate containing 100 μg mL^{-1} spectinomycin was inoculated into 5 mL of ZYM-5052 auto-induction media containing the same selective antibiotic and incubated for 18 hours at 37°C and 250 rpm. One fluorescent, green colony was observed under the microscope to confirm that the gene for green fluorescent protein (*gfp*) was being expressed. One reason why the expression of *gfp* was low may have been that the cells needed more aeration while incubating. This was adjusted in subsequent trials.

Future induction of *gfp* was performed using isopropyl β-D-1-thiogalactopyranoside (IPTG) rather than ZYM-5052 media. The protocol for this method is modeled after the protocol outlined by Overkamp et al. (2013). A 5 mL overnight culture of the strain of interest and LB broth containing selective antibiotics was diluted (1:50) in 2xYT media without antibiotics. The dilution was incubated for 2 hours prior to adding IPTG, which was followed by an additional

incubation period of 3 hours before cells were harvested, washed with 1x PBS buffer, and read on a microtiter plate by a fluorimeter for fluorescence, indicating *gfp* protein levels.

Results

Optimizing *Bacillus subtilis* genetics tools

In order to heterologously express *R. thermocellum* regulatory systems, we needed to first establish a working *B. subtilis* genetics system. *Bacillus subtilis* strain KO7 (Table 1) was first used, because seven extracellular proteases had been deleted from its genome, leading to less interference when producing extracellular, recombinant proteins. I first attempted to transform *B. subtilis* using electroporation. For electroporation, higher voltage is used to move plasmid DNA into the host cell; however, if the voltage is too high, the cells could be damaged or killed (Lu et al., 2012). Another factor contributing to the success of the transformation is the stability of the plasmid and its ability to be replicated. After obtaining inconsistent results of colonies growing on selective media and difficulty transforming the vector pHCMC02 into *B. subtilis* K07, additional transformations were attempted to optimize the procedure by adding larger amounts of plasmid and electroporating at various voltages (20 kV – 25 kV). Despite these efforts, the electroporation technique was not a reliable method of transformation for future use.

Natural competence was also attempted with *B. subtilis* KO7 and the plasmids pHCMC02 and pDR111_gfp(Sp) (Yasbin et al., 1975). Transforming *B. subtilis* can be difficult because the timing of natural competence is often complicated. Similar to the electroporation method, this technique yielded inconsistent growth of transformants under antibiotic selection and was not used further as the method of *B. subtilis* transformation.

After observing inconsistent transformation results from *B. subtili*s KO7 following both electroporation (Lu et al., 2012) and natural competence transformation techniques (Yasbin et al., 1975), I tested the super-competent strains *B. subtilis* SCK6 (X. Zhang & Y. P. Zhang, 2011) and *B. subtilis* Reg19 (Rahmer et al., 2015) obtained from the Bacillus Genetics Stock Center. Transformation using vectors pHCMC02 and pDR111_gfp(Sp) were both successful in *B. subtilis* strains SCK6 and Reg19 as indicated by growth under selective pressure. This method of transformation was used for future transformations of DNA into *B. subtilis.*

Previous research has been performed to produce a stable plasmid for intracellular protein expression in *B. subtilis*, pHCMC02 (Nguyen et al., 2005). Once the vector pMCB01 (Table 4) was successfully constructed and cloned into *E. coli* 10β it needed to be transformed into *B. subtilis*. Both pMCB01 and pDR111_gfp(Sp) were transformed into *B. subtilis* 1A976: SCK6 following the transformation protocol given by X. Zhang and Y. P. Zhang (2011). Later, pMCB02 was constructed and transformed into both *E. coli* 10β and *B. subtilis* 1A976: SCK6 before being stored as glycerol stocks.

Construction of a Golden Gate-ready integrating vector for *B. subtilis*

While *B. subtilis* transformation was being optimized, I also began construction of the integrating vector that would insert the gene encoding for green fluorescent protein under control of one of the *R. thermocellum* promoter sequences (Muñoz-Gutiérrez et al., 2016; Ortiz de Ora et al., 2018). Due to the short size of the oligonucleotides encoding for the *R. thermocellum* promoters, we opted to use Golden Gate cloning (Engler et al., 2008) to integrate promoter sequences into pDR111_gfp(Sp), rather than Gibson Assembly which requires long complementary overhangs. Since pDR111_gfp(Sp) contained a recognition site for the restriction

enzyme, BsaI, used in Golden Gate cloning, I first needed to use site-directed mutagenesis to remove the BsaI site already in the vector.

In order to design and construct a Golden Gate-ready vector, we used Gibson Assembly, with three overlapping fragments. Primers were then designed using NEBuilder software, v1.0 (https://nebuilderv1.neb.com/) that would amplify the vector pDR111_gfp(Sp) in two sections (BA and DC) with overlapping homologous regions to the *lacZ* gene from pUC19 via PCR (see Figure 2). Using the designed oligonucleotide primers, fragments BA, DC, and *lacZ* were amplified *via* PCR, along with a site-directed mutation at position 4694 from pDR111_gfp(Sp) to mutate the BsaI recognition site. PCR products for each respective segment were confirmed using gel electrophoresis for their expected molecular size (Figure 3).

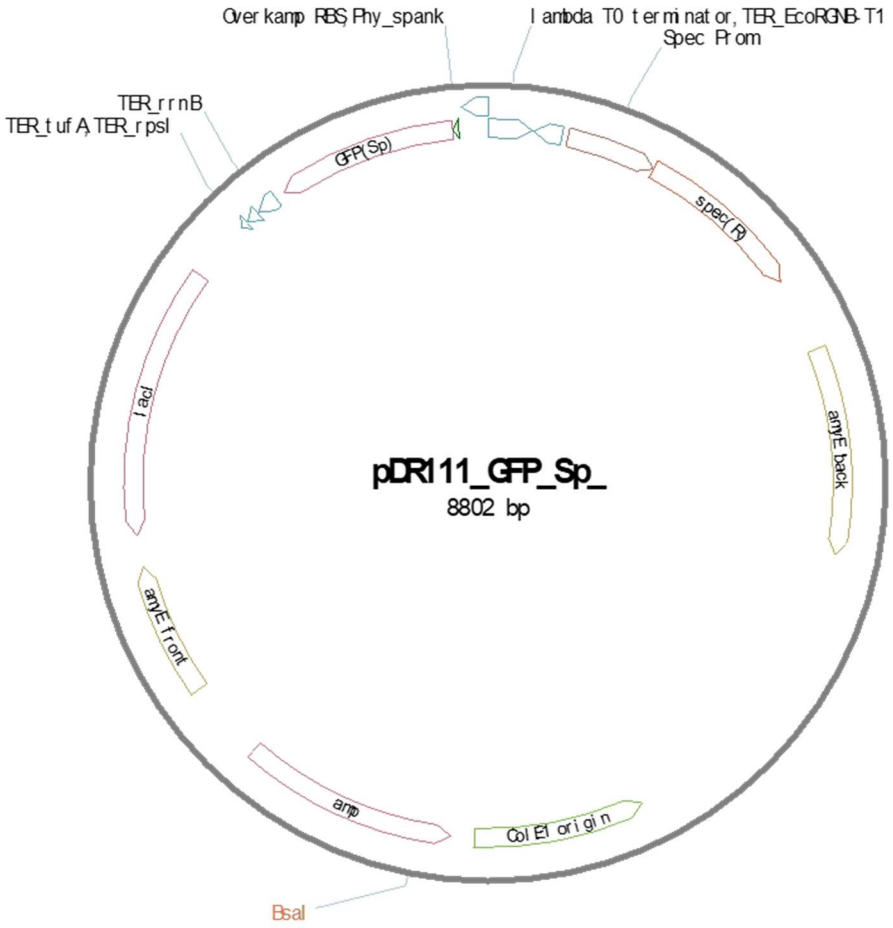

Figure 2. pDR111_gfp(Sp) vector map

Once gel electrophoresis confirmed that the fragments BA, DC, and *lacZ* were the expected size, the amplified fragments were purified to eliminate the buffer and reagents used in PCR so that only the intended DNA product remained. From there, I was able to use Gibson Assembly to anneal and ligate the new Golden Gate-ready cloning vector (pMCB01) together using the BA, DC, and *lacZ* fragments. The Gibson assembly reaction was then transformed into *E. coli* 10β cells using a heat-shock method, with transformation of water used as the negative control. Transformants were screened on LB agar ampicillin (100 µg mL^{-1}).

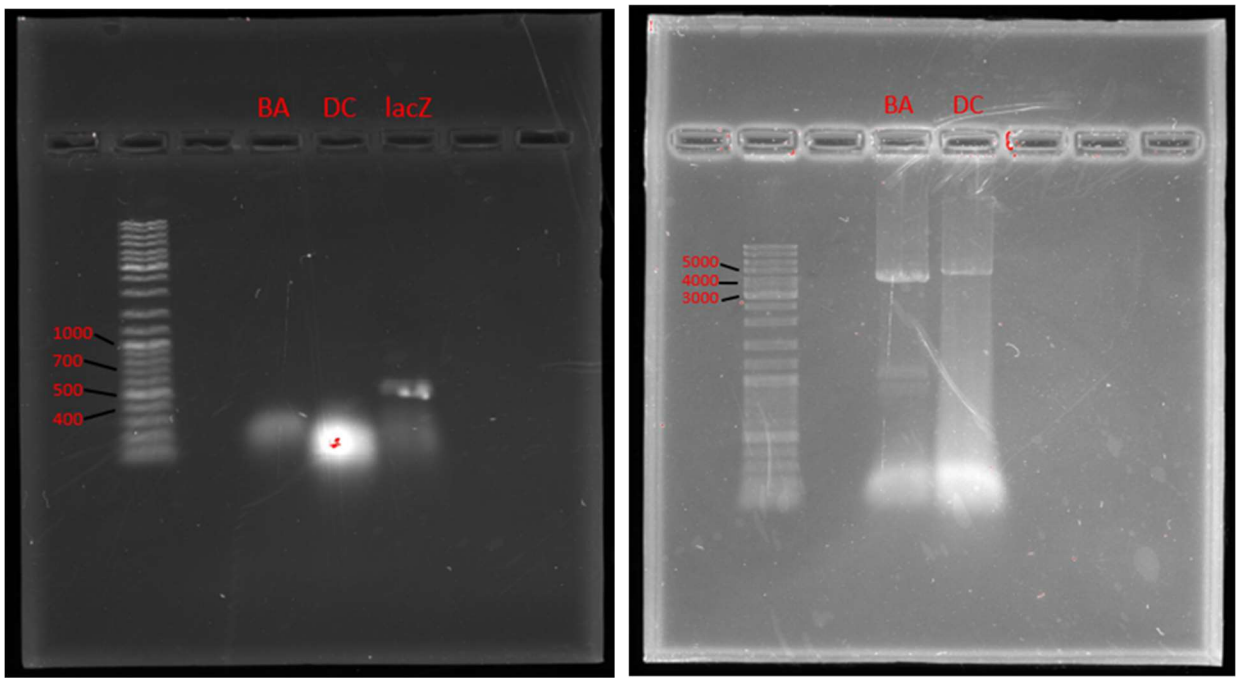

Figure 3. Gel of PCR amplified lacZ, BA and DC

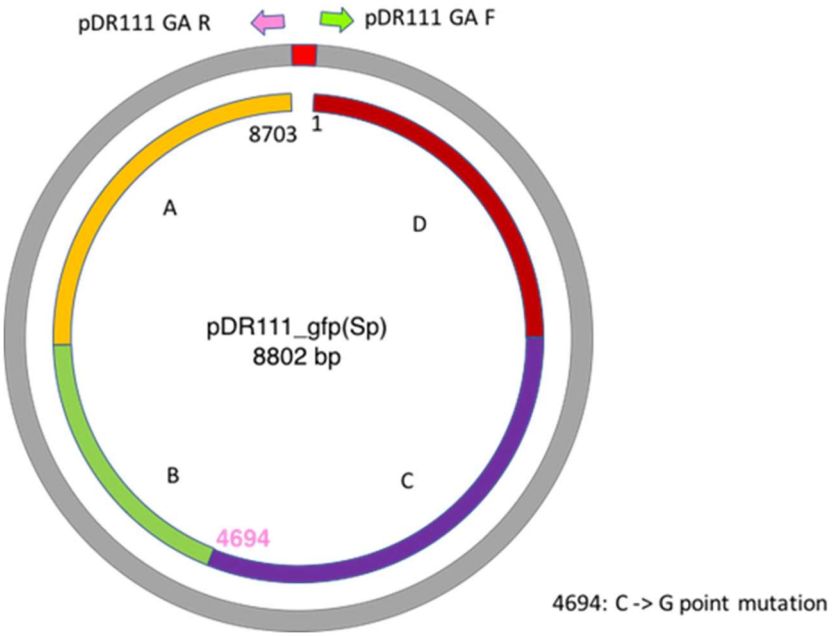

Figure 5. pDR111_gfp(Sp) fragment design

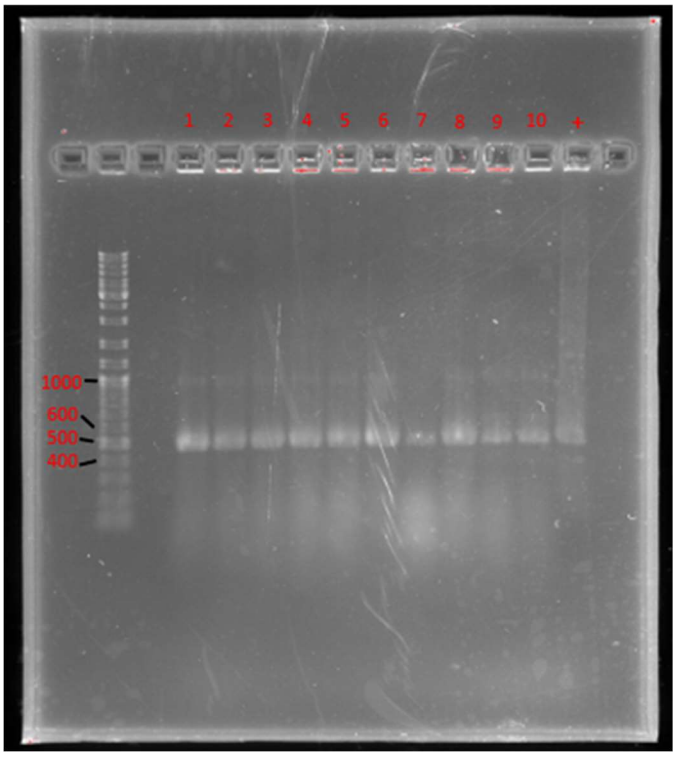

Figure 4. Colony PCR of pMCB01 using primers to check for lacZ

After transformation, 10 colonies were selected for confirmation by colony PCR using the cloning primers for the *lacZ* gene in order to check for its presence in cloning vector pMCB01. The annealing temperature used in the colony PCR reaction was 60°C which corresponds to the annealing temperature of the *lacZ* primers and the extension time used was based on the length of the *lacZ* gene insert (443 bp). Each of the colony PCR samples was run through gel electrophoresis to determine if *lacZ* was inserted into pMCB01 (Figure 5). The agarose gel displayed bands around 450 base pairs in length, which is the expected length for the *lacZ* insert, for all 10 colony PCR samples. The positive control, the PCR amplified and cleaned *lacZ* cassette, also showed a band in the same region.

Once the presence of *lacZ* was confirmed in the vector, pMCB01 plasmid was purified from each of the positive 10 colonies from colony PCR. This DNA was then used in a restriction enzyme digest as an additional method of testing for the presence of the correctly constructed vector. Each sample of pMCB01 was digested with the restriction enzyme BamHI and the original pDR111_gfp(sp) plasmid was used as a control. BamH1 was used because it is expected that there are two BamHI sites present in pMCB01, while only one BamHI site is present in pDR111_gfp(Sp), resulting in two bands observed in a gel of pMCB01 at 2.5 kb and 6.6 kb; and one band observed for the linearized pDR111_gfp(Sp) plasmid. The gel of this digest shown in Figure 6, indicates that lanes 4, 8, 9, and 10, containing colonies #4, 8, 9, and 10 appeared to show the expected bands. Colony #9 appeared to have the most prominent bands at 2.5 and 6.6

kb; therefore, cells grown from the original colony #9 were then stored at -80˚C as glycerol stocks of *E. coli* 10β + pMCB01.

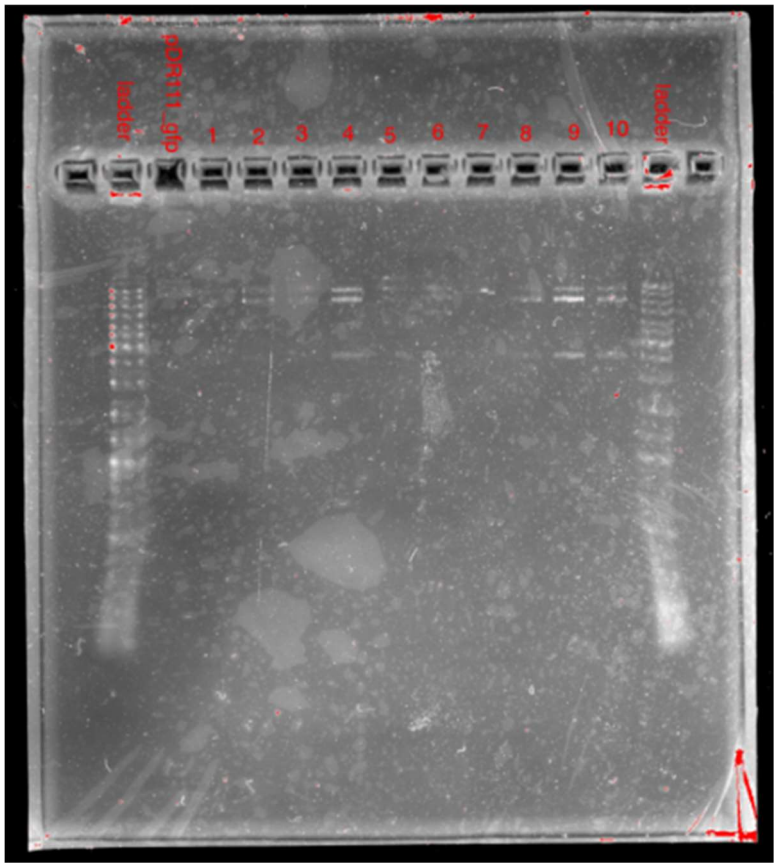

Figure 6. pMCB01+BamHI restriction enzyme digest

Insertion of P_{sigI6} upstream of *gfp* to construct pMCB02

The vector pMCB02 was constructed by annealing the oligonucleotides RC P_{sigI6} F and New P_{sigI6} R together via the IDT annealing oligonucleotides protocol. Once annealed, the P_{sigI6} oligonucleotides were combined with pMCB01, the restriction enzyme BsaI, T7 ligase, T4 ligase buffer, and DI water before being placed in the thermocycler. The McClean Golden Gate protocol was followed. Both a 3:1 and 10:1 ratio of annealed oligonucleotides to plasmid were tested.

Similar to Gibson assembly, the Golden Gate assembly products were transformed into *E. coli* 10β cells by heat-shock and plated onto selective media containing 100 µg/mL ampicillin. Additionally, 200 µL of Firozeh X-gal/IPTG solution was also added to the surface of the media about 30 minutes prior to plating the 10β cells so that transformants could be identified via blue/white colony screening. Following an overnight incubation, colonies that appear white on this media contain the insert of interest, while blue colonies do not. Nine white colonies from the both the plates of cells transformed with Golden Gate products run with a 3:1 and 10:1 ratio of annealed oligonucleotides:pMCB01 were selected for using colony PCR. Forward and reverse primers specific for pMCB02 were used to confirm the presence of P_{sigI6} in the transformants. Figure 7 is a gel image of the colony PCR products which displays bands around the expected 387 base pairs and no bands in that region for the negative controls of colony PCR products from a pMCB01 colony, purified pMCB01 plasmid, and pMCB02 primers only added to the reaction tube with clone ID buffer. Five of the positive colonies from the 3:1 Golden Gate reaction were selected and grown to create glycerol stocks of *E. coli* 10β + pMCB02 and run midi-preps of the plasmid DNA using the Promega Wizard kit and protocol. The midi-preps of pMCB02 were then sent for DNA sequencing in order to confirm the presence of P_{sigI6} via another method.

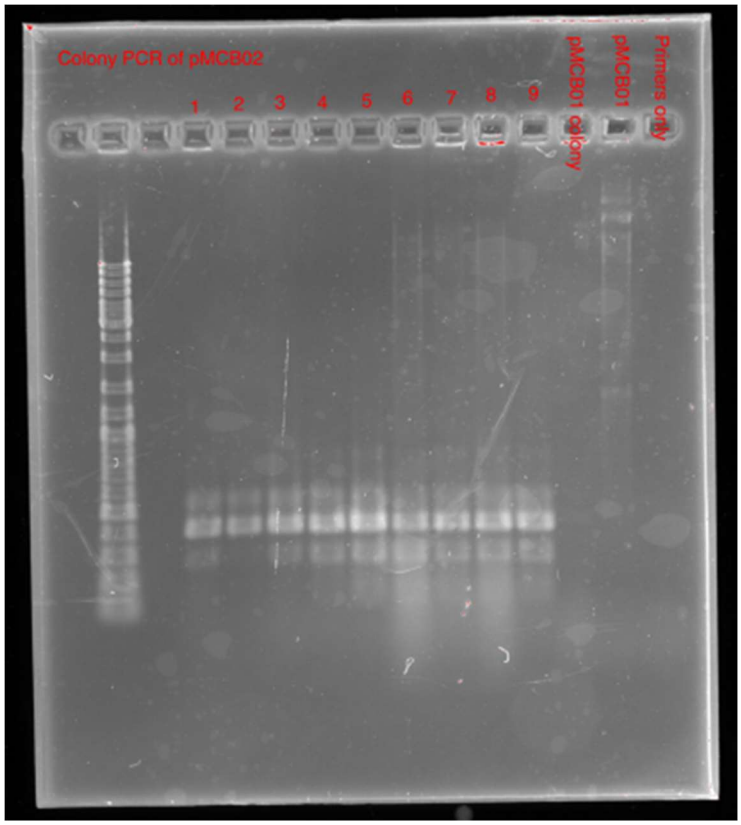

Figure 7. Colony PCR of pMCB02

Building a carbohydrate response system

The next step is the construction of pHCMC02 vector with genes from *R. thermocellum* cloned in. PCR was used to amplify genes Cthe_2119, Cthe_2120, and Cthe_2119-2120 from ATCC 27405 gDNA using unique primers for each reaction. The three PCR amplified products were checked via gel electrophoresis and appeared to be in the expected location in comparison to the gene ladder as seen in Figure 8. The 2119 segment appeared as a band near 2.3 kb, 2120 appeared as a band near 700 bp, and 2119-2120 around 3.0 kb. The three gene segments were then ready for Gibson assembly; however, pHCMC02 must first be linearized by the restriction enzymes XbaI and SmaI which cut the vector in the same place, ensuring linearization. Each Gibson assembly product is transformed into *E. coli* 10β and plated on selective media

containing 100 µg/mL ampicillin. DNA sequencing was used in an effort to confirm that Cthe_2119, Cthe_2120, and Cthe_2119-2120 were present in pHCMC02; however, the Gibson Assembly reaction appeared to be unsuccessful initially. Longer, 40 nucleotide primers for Cthe_2119, Cthe_2120, and Cthe_2119-2120 were designed with about 20 base pairs of overhangs and overlap with the gene sequence. The primers were used to amplify the genes Cthe_2119, Cthe_2120, and Cthe_2119-2120 from the previously PCR amplified genes. The agarose gel image in Figure 8 shows bands in the expected region for each gene (Cthe_2119: 2.3 kb, Cthe_2120: 0.76 kb, Cthe_2119-2120: 3.0 kb); however, there was also smearing and faint bands possibly demonstrating non-specific binding. This step needed to be optimized so that the bands are clearly defined and it can be assumed that only the desired product is in the sample.

The vector pMCB05 was constructed by using Gibson assembly to clone Cthe_2120 into pHCMC02. The Gibson product was transformed into *B. subtilis* SCK6 cells harboring pMCB02 and selected for on media containing both SP 100 ug/mL and CM 10ug/mL.

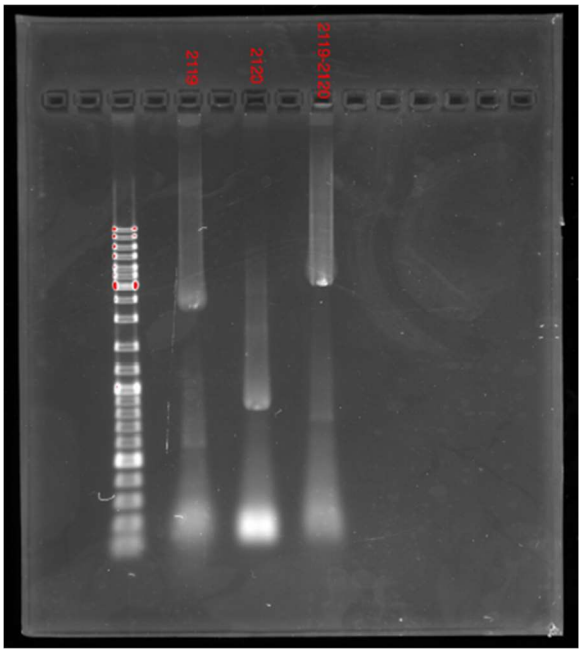

Figure 8. PCR of Cthe_2119, Cthe_2120, and Cthe_2119-2120

Induction of Green Fluorescent Protein Expression

In a preliminary test for *gfp* production in *B. subtilis*, strains containing the plasmids pMCB01, pMCB02 and pDR111_gfp(Sp) were examined using the IPTG induction methods described above. It was found that *B. subtilis* containing pDR111_gfp(Sp) expressed much higher levels of *gfp* than the strains containing pMCB01 and pMCB02 as depicted below.

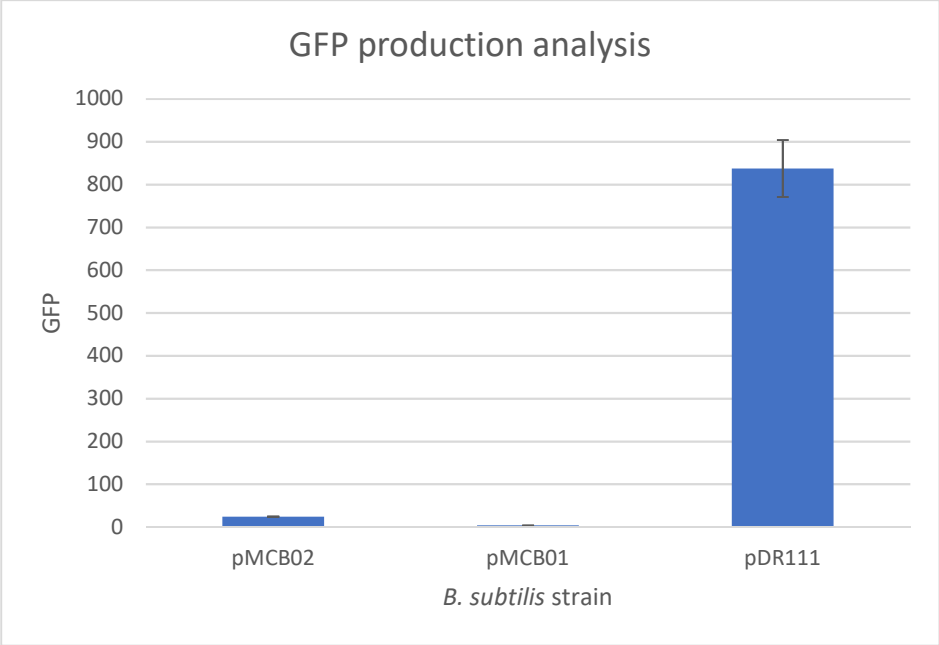

Figure 9. Fluorescence of B. subtilis *SCK6 strains after IPTG induction*

Once pMCB05 was successfully transformed into *B. subtilis* SCK6 harboring pMCB02, it was grown to be tested for *gfp* production. It was expected that these *B. subtilis* SCK6 cells would use the system outlined in Figure 11 and produce *gfp* constitutively. Grown with it were SCK6 cells with pDR111_gfp(Sp) and SCK6 cells with pMCB02 to serve as controls. IPTG was used to induce the *B. subtilis* SCK6 + pDR111_gfp(Sp) for *gfp* production at 3.5 hours post-inoculation into 2xYT media containing no antibiotics. The figure below shows that there was no difference between *B. subtilis* SCK6 strains containing pMCB02 and both pMCB02 + pMCB05. After induction with IPTG, *B. subtilis* cells with pDR111_gfp(Sp) present exhibited much more fluorescence when compared to the other samples at the same timepoint.

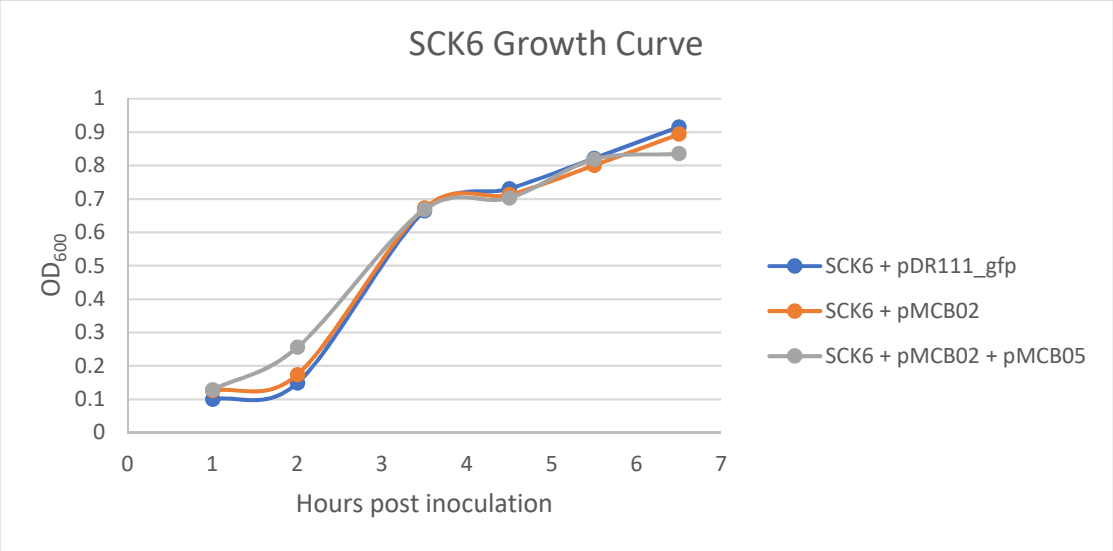

Figure 10. B. subtilis *SCK6 growth curve*

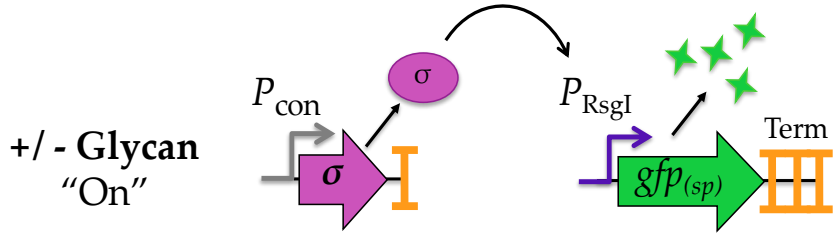

Figure 11. Expected regulation of gfp *in* B. subtilis *SCK6 with pMCB02 and pMCB05*

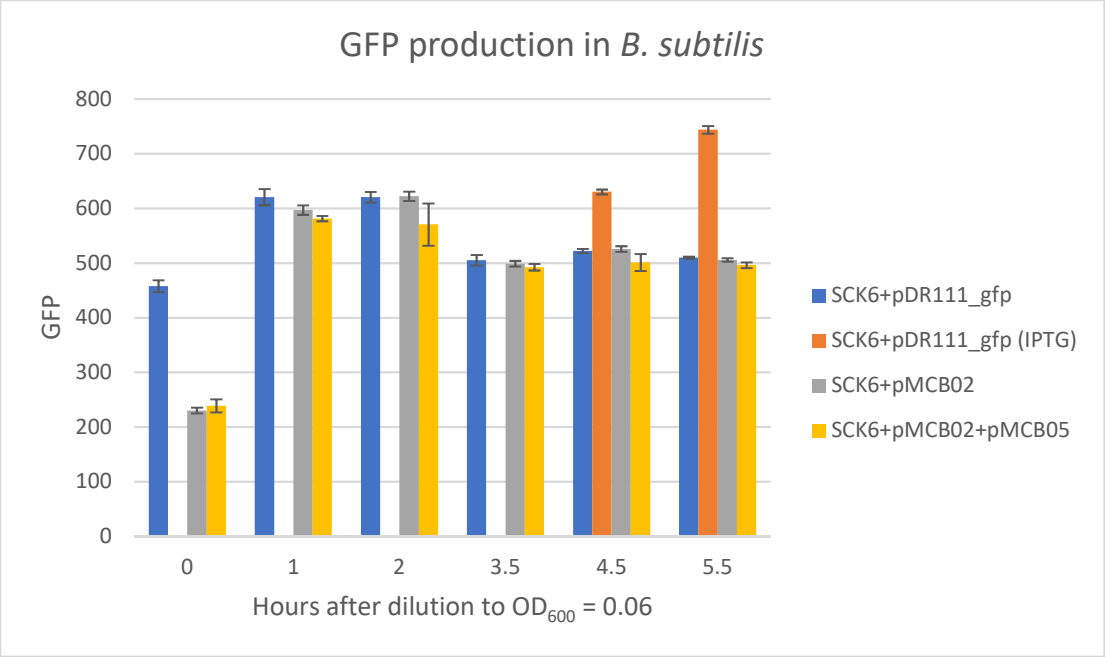

Figure 12. GFP production in B. subtilis *SCK6 strains*

Outcomes

Although significant progress has been made, additional work needs to be done in the future for this project. The construction of pMCB01, pMCB02, and pMCB05 were critical steps for the engineering of a *B. subtilis* strain capable of expressing *R. thermocellum* genes. The integration of Cthe_2120 into pHCMC02 using Gibson Assembly was successful; however, Cthe_2119 and Cthe_2119-2120 should also be integrated in the same way in the future. Once those genes are cloned into the expression vector, pHCMC02, and the associated promoter region is cloned into pMCB01, they can then be transformed into *B. subtilis* cells and tested for gene expression via levels of *gfp* production.

Conclusion

The methods utilized in this paper proved to be effective at constructing synthetic plasmids by using Gibson Assembly and Golden Gate Assembly in combination. This work also demonstrates the ability for *B. subtilis* to take in these plasmids through transformation. As

anticipated, it was also found that the *gfp* production was highest in the *B. subtilis* strain containing unaltered pDR111_gfp(Sp) when induced with IPTG and significantly lower in strains containing both pMCB01, where *lacZ* was inserted and pMCB02, where P_{sigI6} was added to pMCB01. Although I was not initially successful getting *B. subtilis* with both pMCB02 and pMCB05 in its genome to express *gfp*, future work will be performed to optimize *gfp* production as well as look into the use of alternative constitutive promoters and σ factor-promoter pairs, other than σ^{I6} and P_{sigI6}. Once *gfp* production is consistent in *B. subtilis* using this system, integration of both the σ and anti-σ factor into *B. subtilis* will be tested with the hope of repressing *gfp* expression as seen in Figure 13. Engineering *B. subtilis* to perform cellulose degradation the way *R. thermocellum* does would expand the understanding of how Clostridia recognize glycans as well as expand the possibilities of using bacteria for biofuel production.

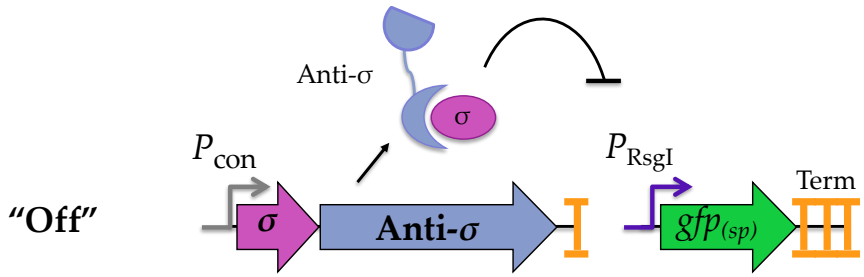

Figure 13. Expected regulation of gfp *in* B. subtilis *containing both regulators*

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