Facilitating Quorum Quenching with Autoinducer Inactivation Enzyme

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Abstract

A range of gram-negative bacterial species use diffusible autoinducer molecules such as AHL to facilitate intercellular communication. This enables bacteria to coordinate communal activities including bioluminescence, regulation of antibiotic production, or biofilm formation. However, cloning the autoinducer inactivation gene, *aiiA*, downstream of the qrr4 promoter in *E.coli* facilitates the degradation of AHL. Breaking down this quorum sensing molecule hinders bacterial communication (quorum quenching), and consequently, prevents the formation of biofilm.

Introduction

The universal signaling molecule, *N*-acyl-homoserine lactones (AHL), is used by gram-negative bacteria to communicate and coordinate activities on a population-scale (Bassler & Miller, 2001). These biological functions may include expression of virulence factor, biofilm formation, production of antibiotics, bioluminescence and much more (Park et al., 2008). This synchronized behavior in bacteria is achieved through a cell-to-cell communication mechanism known as Quorum Sensing (QS) (Bassler & Miller, 2001). Bacteria produce these diffusible signaling molecules called autoinducer as they grow and replicate allowing them to count and asses their cell density (Yi-Hu et al., 2002). Detection of their minimal threshold concentration leads to the alternation of gene expression which triggers their communal behavior (Bassler & Miller, 2001). This behavior depends on the particular gene that is being transcribed. In essence, if a single cell were to carry out tasks such as bioluminescence or production of antibiotics; it would not be very efficient to do so single-handedly. Therefore, The QS mechanism is exploited to allow bacteria to work collectively to mirror a multi-cellular organism (Yi-Hu et al., 2002).

This year, the University of Calgary's iGEM teams is using this natural mechanism, found in *Vibrio harveyi,* and tailoring it to target biofilms. The international Genetically Engineered Machines (iGEM) competition is a synthetic biology competition that is annually held at the

Massachusetts Institute of Technology (MIT). iGEM requires each team to use the Biobrick construction techniques which contains the standard restriction sites after each biological sequence that encodes a particular function (Knight, 2003). Using this technique Biobricked parts can be readily assembled with other Biobrick parts (Knight, 2003). The goal of this year's project is to mirror the AI-2 signalling cascade, which is naturally found in the bioluminescent marine bacteria *Vibrio harveyi*, and adapt it to *Escherichia coli*.

The AI-2 induced signaling cascade is a series of proteins found in the periplasm and cytoplasmic space of the cell that respond to the threshold concentration of AI-2 in its surroundings (Park et al., 2008). In the natural system, the *Lux P* protein, found in the perplasm, binds to AI-2 and interacts with another membrane bound protein *LuxQ (*Waters & Bassler, 2005). At low cell density, when the concentration of AI-2 is proportionally low, *LuxQ* acts as a kinase and autophosphorylates (Freeman & Bassler, 1999) and leads to the phosphorylated form of the regulator protein, *LuxO* (Figure 1) (Waters & Bassler). Phosphorylated *LuxO* forms a complex with σ54 (transcription factor) and activates transcription of the five regulatory RNAs, or sRNA, called qrr1 to qrr4 (Tu & Bassler, 2007). These RNAs act to destabilize the mRNA encoding the *LuxR* protein which is needed to activate the light emitting luciferase operon *LuxCDABE* (Tu &Bassler, 2007). Thus, light is not emitted at lower cell density. However, in the presence of high concentration of AI-2, the process is reversed leading to dephosphoylated form of *LuxO* and consequently bacteria glows as an outcome.

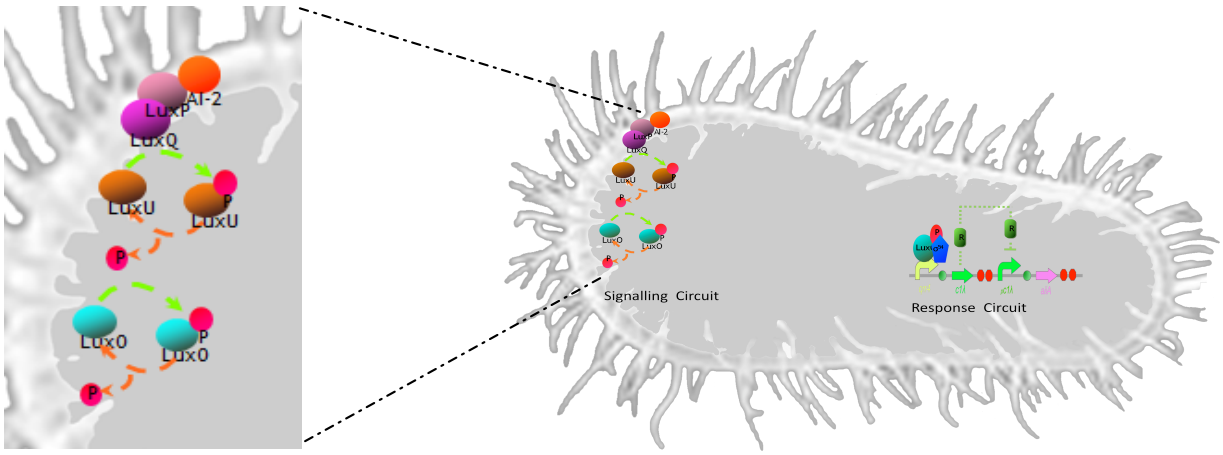

Figure 1. A typical *E.coli* cell with the signaling cascade embedded in the periplasm and cytoplasmic space. The light green arrows indicate phosphorylation of the receptor and regulator proteins, *LuxPQ* and *LuxOU* respectively, in the absence of AI-2 in the surroundings. The orange arrows indicate a dephosphorylation cascade which occurs in the presence of AI-2, where *LuxP* does not bind to a phosphate group and lead to an unphosphorylated *LuxO*, which does not bind to σ54 or qrr4 promoter. Diagram is designed by Georgijevic. S, (2008).

This QS signaling system is characterized in the Biobrick format in *E.coli* bacteria with a constitutive σ70 (BBa_R0040) promoter and double terminators (BBa_B0015). However, to account for the small sRNA which act as inverters in the natural system, a Biobricked inverter (BBa_Q04510) has been added downstream of the qrr4 promoter (Pqrr4) in the *E.coli*. Naturally, in *Vibrio harveyi*, qrr4 appears to be the strongest quorum regulating RNA (sRNA) (Tu & Bassler, 2007). Although this strength is not reflected in *E.coli*, this promoter was still adapted since this experiment is using the natural AI-2 signalling system from *Vibrio harveyi* (Tu & Bassler, 2007). Adding this downstream of Pqrr4 will enable it to repress or allow transcription of the gene of interest based on the concentration of AI-2 and phosphorylation or dephosphorylation of the signaling cascade. Transcription of this target gene allows the cell to express a particular trait as the outcome (Fuqua & Greengberg, 1998).

Since the Pqrr4 initiates a series of biological processes to produce or inhibit an outcome, it has been integrated a part of what is known as the response circuit. As termed, the response circuit *responds* to the phosphorylation and dephosphorylation of the signaling cascade by repressing or expressing the gene of interest: autoinducer inactivation enzyme, aiiA. The aiiA gene is originally isolated from *Bacillus species*, 240B1 strain (iGEM registry, 2009). This gene encodes an enzyme which catalyzes the degradation of AHL, thereby disrupting QS (Bassler & Miller, 2001). Thus, these series of promoters, ribosome binding sites (rbs), c1λ inverter and gene of interest forms the response circuit in the cell (Figure 2).

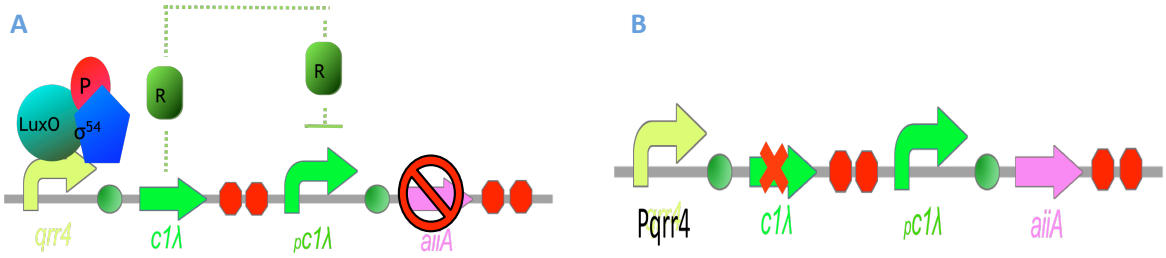

Figure 2. The response circuit is housed within the cytoplasm of the cell. In the presence of AI-2 allows phosphorylated LuxO and σ54 (transcriptional factor) to bind to qrr4 promoter and initiate transcription of the inverter (c1λ). Again, c1λ is the Biobricked inverter chosen to mirror the sRNA natural inverters of *Vibrio harveyi* in our laboratory strain of *E.coli*. Expression of c1λ protein represses the c1λ promoter inhibiting any further transcription. As a result aiiA is not expressed (Figure 2.A). However, in the absence of AI-2, unphosphorylated LuxO does not bind to σ54, therefore, inhibits transcription of Pqrr4. Thus, c1λ is not transcribed and aiiA is expressed (Figure 2.B).

In this year's iGEM project, Team Calgary is providing a novel approach to target and prevent the formation of biofilm. Biofilms are the aggregation of microorganisms on the surface of a solid material (Sawhney & Berry, 2009). In order to prevent biofilm aggregation, it is essential to

cease communication among these colonies that use AHL for the purpose of Quorum Sensing. Since these microbial accumulations remain quite firm and difficult to penetrate (Saldarriaga et al., 2009), it is imperative to cease the force that is allowing them to detect and remain bound to neighboring bacteria. This paper focuses on the construction, function and expression of aiiA in the response circuit and the subsequent enzymatic degradation of AHL (Bassler & Miller, 2001) also known as Quorum Quenching (Czajkowski & Jafra 2009). This will disable bacteria from "counting" their population density, which in turn will prevent synchronized activities, such as formation of biofilm. The goal of this year's iGEM project is to facilitate quorum quenching by coupling it with the synthetic AI-2 quorum-sensing mechanism.

In order to carry out the project, each biological part of the response circuit is taken from the registry. These parts have been Biobricked, or in other words, flanked with the standard restriction sites to allow small fragments of DNA to readily assemble to a larger system (Shetty, Endy & Knight, 2008). Figure 3 summaries the construction of the biological parts that make up the response circuit and Figure 4 illustrates the goal and possible outcome of piecing together individual Biobrick parts.

The Response Circuit

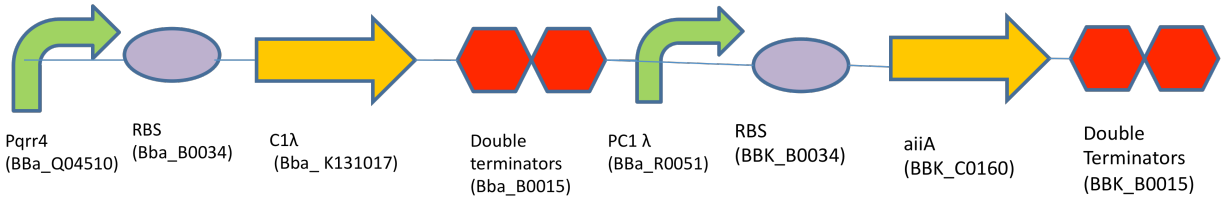

Figure 3. The response circuit will be constructed in psB1AK3 which is a high copy plasmid with Ampicillin and Kanamycin resistance. It is lead by a promoter that controls transcription of the downstream DNA sequence. The rbs is situated before the protein domain or gene of interest to allow binding of ribosome to initiate translation of the subsequent parts. The following c1λ protein, found in the registry, acts as the inverter in the system. The qrr4 promoter is chosen since it shows a dramatic increase in bioluminescence in natural system of *Vibrio harveyi*, thus appears to be the strongest promoter (Tu & Bassler, 2007). Green is the standard color for promoters in the iGEM Registry (2009) and red represents double terminators. The double terminators are meant to reinforce termination of transcription.

Goal

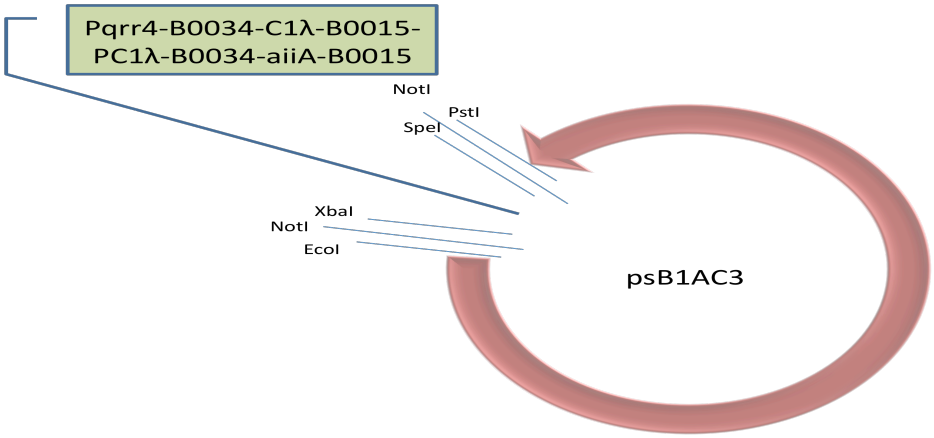

Figure 4. Each Biobrick part exists in different plasmids. For example, Pqrr4 is in psB1AC3 while aiiA is in psB1A2 "AK" simply implies that the plasmid contains Ampicillin and Kanamycin resistance. "AC" indicates Ampicillin and Chloramphenicol resistance. However, cloning each fragment after another, by digesting the linear fragments in the indicated restriction sites (*EcoRI*, *NotI*, *XbaI*, and *SpeI*, *NotI*, *PstI*) the entire construct must be transformed into one plasmid, the psB1AC3. This plasmid was chosen due to the sequential order of cloning followed during construction of these parts. psB1AC3 is a high copy plasmid. The process of assembling any two or more BioBrick part is very similar as it conforms to the same Biobrick techniques and results in a new composite Biobrick part with the same standard restriction sites.

Materials & Method

The iGEM registry provides a variety of standardized Biobricked parts to help each team assemble their biological systems. Each BioBricked part is flanked with *EcoR I, NotI, XbaI* and *SpeI, NotI, PstI* on the left and right sides, respectively, of each BioBricked gene sequence (Figure4) (Shetty, Endy & Knight, 2008). This standard construction technique enables two or more BioBrick parts to readily assemble into larger, multi-component genetic system (Shetty, Endy & Knight, 2008).

Primers	Sequence	T_m
BBK CP -F	CACCTGACGTCTAAGAAACC	60
BBK CP -R	AGGAAGCCTGCATAACGCG	60
aiiA -F	GAAGCTTTATTTCGTCCCAG	58
aiiA -R	GGGAACACTTTACATCCCC	58

Table 1. BBK CP-F and BBK CP-R are general custom made construction primers (Kubik, 2009) that anneal to the vector approximately 250bp upsteam and downstream of the multiple cloning sites. AiiA-F and aiiA-R are gene-specific primers designed specifically to anneal to the aiiA sequence to ensure the gene is successfully constructed. They are also custom primers used for gene-specific PCR (Moinul & Kubik, 2009). These primers were synthesized from base pairs 14 bp downstream of the start codon of the aiiA sequence (Figure 6).

Plasmids	Type of Plasmid	Source
psB1AK3	Construction plasmid containing double terminators (BBa_B0015) Amp^R and Kan^R	iGEM Registry
psB1A2	Construction plasmid containing aiiA (BBa_C0160) insert Amp^R	iGEM Registry
psB1A2	Construction plasmid containing RBS (BBa_B0034) Amp^R	iGEM Registry

Table 2. All of these plasmids have a high copy number.

For the purpose of our experiments, chemically competent laboratory strain of Top 10 *E.coli* bacteria was used. All liquid cultures and agar plates were made from *Luria-Bertani* (LB) broth. Often one or more antibiotics were used on the plates and broth: Chloramphenicol (35ng/µL), Kanamycin (35ng/µL) and Ampicillin (100ng/µL).

Construction of aiiA and B0015

All of these Biobricked biological parts are taken from the iGEM registry and amplified using polymerase chain reaction (pcr). Using the restriction digest construction protocol, from Qiagen, aiiA is digested with *XbaI* and *SpeI* as the insert and cloned in front of the double terminators, in their respective plasmid, psB1AK3. Serving as the vector, psB1AK3 is digested with *EcoRI* and *XbaI* (Invitrogen, CA). This forms a scar between *XbaI* and *SpeI*, which allows the restriction sites to become unrecognizable and prevent it from breaking (Figure 6).

Following the old iGEM construction techniques and based on the concentrations of the psB1A2 and psB1AK3 plasmids, 21 µL of insert is digested with 6.25 µL of vector. The insert and vector were placed in the water bath overnight at 37 ^{o}C, although the protocol requires a minimum of one hour. Then both insert and vector were placed into the 65 ^{o}C heating block for 10 minutes to ensure that the enzymes were deactivated.

Next, the vector is treated with Antarctic phosphatase (New England Biolabs, ON) to prevent the plasmid from ligating back onto itself without the insert in place. Following the construction procedure, the vector was heat shocked at 65 ^{o}C for 10 minutes and ligated with 10µL 2x Quick Ligase Buffer (New England Biolabs, ON) and 1 µL Quick Ligase. This ligation allows a scar to from where *XbaI* and *SpeI* was liagted (Figure 5) and forms a construct with the same BBK restriction sites on each side of the construct.

21µL of the ligation reaction is then transformed into chemically competent laboratory strain of Top 10 *E.coli* cells. The Top 10 cells are used for this transformation instead of XL Gold strains because the XL Gold cells are chemically capable of up-taking much larger DNA fragments than Top 10. Since this is a small construct (885bp), Top 10 will suffice. 25µL and 50µL of ligation reaction were plated on two LB plates containing 50ng/µL Kanamycin antibiotic. Cultures were left to grow overnight at 37 ^{o}C. The cells were grown on Kan plates because the vector, containing the construct is AK, meaning it has Ampicillin and Kanamycin resistance.

Colony PCR

To verify the size and sequence of the above construction, three chronological processes were followed: Colony PCR, restriction digest and sequencing. In colony PCR, a Mastermix containing dNTPs, BBK CP (BBK Construction Primers-F and CP-R), and platinum *Taq* polymerase (Invitrogen, CA), is prepared. With CP primers, the T*aq* DNA Polymerase catalyzes the incorporation of dNTPs into DNA. 50µL of Mastermix is used for each colony PCR. The initial denaturation process was set to 6 minutes. Annealing and extention times, for 36 cycles, were set to 55 ^{o}C and 72 ^{o}C respectively with 1 minute for extension as followed by the general rule of 1min/kb. The size of the construct was expected to be 885bp.

Mini-prep: Isolating plasmids

To isolate the psB1AK3 plasmid from overnight cultures of *E.coli* in 5 ml of LB medium, GenElute Plasmid Miniprep kit (Sigma-Aldrich, MO) is used. Mini-prep will allow us to retrieve the plasmids from the cells and determine their concentrations by specking them.

Restriction Digest

This procedure was the diagnostics for verifying the size of the construct and comparing it to the uncut plasmid. 12µL of isolated BBK plasmid is digested using React buffer 3 (Invitrogen, CA) and *Not I* (Invitrogen, CA). *NotI* cuts the gene on the Biobricked *NotI* restriction sites on each side of the construct. The digest is placed in 37 ^{o}C water bath for 2 hours, as mentioned in the manufacturer's guide. The AK plasmid is 3189bp but including the 885 bp construct, the total size of the uncut plasmid is expected to be ~4.1kb.

Gene-specific (aiiA) PCR

To verify the existence of aiiA in the BBK_C0160 DNA fragment, a PCR was run with platinum *Taq* polymerase (Invitrogen, CA) and aiiA-F and aiiA-R (Moinul & Kubik, 2009) primers (Figure 7). Again, the enzyme is screens the colonies with the aiiA-F and aiiA-R primers. These primers are designed to anneal to only aiiA, thus verifying the existence of the gene in the construct. The initial denaturation is set to 30 seconds at 94 ^{o}C and extension to 50 seconds in 72 ^{o}C. The

aiiA gene was taken from colony 4 of aiiA colony PCR After performing a gel electrophoresis in 1% agarose gel, bands were shown, meaning primers annealed and gene exists.

Following a successful construction of aiiA-B0015, RBS (B0034) was constructed from its psB1A2 plasmid into the AK plasmid, in front of aiiA using the same Biobrick construction techniques described above. In this case, B0034 is the insert that is being ligated to the psB1AK3 vector containing the aiiA-B0015 construct. The RBS was cloned in front of the aiiA because RBS is a RNA sequence which allows the binding of ribosomes to initiate translation. The subsequent growth of Top 10 competent cells will ensure that the vector with Kanamycin resistance, containing the B0034-aiiA-B0015, is successfully transformed in the cell. Then successive verification procedures: colony PCR, plasmid isolation and verification digest will follow in that respective order.

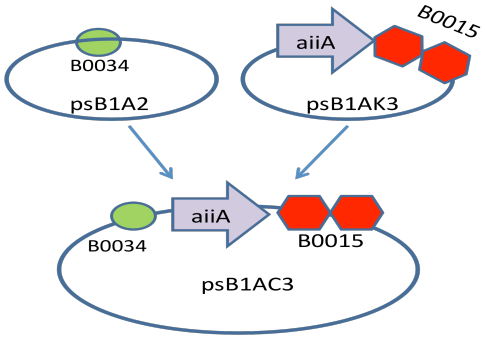

Figure 5. The B0034 insert was digested with *SpeI* and *EcoRI* and cloned in front of aiiA-B0015 vector, in psB1AK3, which was digested with *EcoRI* and *XbaI*. The ligation of *XbaI* and *SpeI* formed a scar which makes the site unrecognizable and prevents it from breaking. The construct is expected to be 897bp after it was run on a 1% agarose gel at 90V.

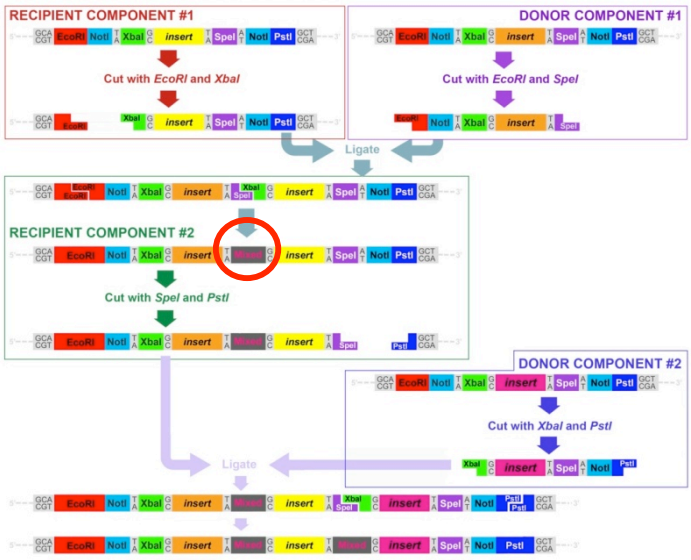

Figure 6. The Biobrick construction techniques illustrate the locations and order of the restriction sites before and after a gene of interest or construct The red circle indicates the formation of a scar after ligating two fragments of DNA that have been digested with *XbaI* and *SpeI*. This scar makes the site unrecognizable, thus preventing it from breaking the construct. Desgined by Georgijevic, S. (2007).

Results

Synthesis of aiiA-specific Forward and Reverse primers

aiiA-F

5′atgacagtaaagaagctttatttcgtcccagcaggtcgttgtatgttggatcattcgtctgttaatagtacatta
acaccaggagaattattagacttaccggtttggtgttatcttttggagactgaagaaggacctattttagtagatac
aggtatgccagaaagtgcagttaataatgaaggtctttttaacggtacatttgtcgaagggcaggttttaccgaaaa
tgactgaagaagatagaatcgtgaatattttaaaacgggttggttatgagccggaagaccttctttatattattagt
tctcacttgcattttgatcatgcaggaggaaatggcgcttttataaatacaccaatcattgtacagcgtgctgaata
tgaggcggcgcagcatagcgaagaatatttgaaagaatgtatattgccgaatttaaactacaaaatcattgaaggtg
attatgaagtcgtaccaggagttcaattattgcatacaccaggccatactccagggcatcaatcgctattaattgag
acagaaaaatccggtcctgtattattaacgattgatgcatcgtatacgaaagagaattttgaaaatgaagtgccatt
tgcgggatttgattcagaattagctttatcttcaattaaacgtttaaaagaagtggtgatgaaagagaagccgattg
ttttctttggacatgatatagagcaggaaaaggggatgtaaagtgttcccctgaatatatataataa3′

aiiA-R

Figure 7. The custom made aiiA-F and aiiA-R primers were synthesized from the autoinducer inactivation (aiiA) gene sequence shown above. The optimal T_m (55 - 60 ^oC) was first determined from the formula 4(G/C) + 2(A/T) = T_m. Based on this, the sequences for the primers were determined near the beginning and end of the aiiA sequence. These primers ensure that aiiA exists in the constructed plasmid by annealing to this sequence in these specific sites. The yellow represents the sequence for each primer. The formation of a band in PCR gel electrophoresis confirms annealing of primers and existence of the gene.

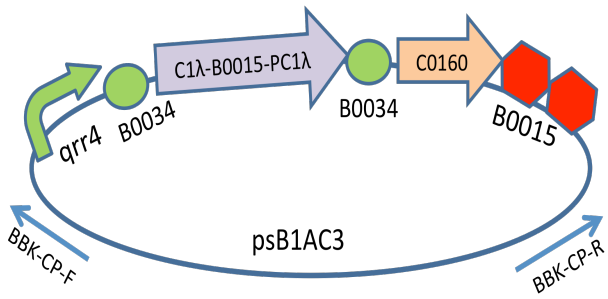

Figure 8. Sequencing is the final method of verification to ensure the success of the construction. The constructs of the response circuit is sequenced using custom BBK-CP-F and BBK-CP-R primers (Kubik, 2009). These primers anneal 250 bp upstream and downstream of the Biobricked multiple cloning sites.

5' GGCGTATCACGAGGCAGAATTTCAGATAAAAAAAATCCTTAGCTTTCGCTAAGGAT

EcoRI NotI XbaI

GATTTCTGGAATTCGCGGCCGCTTCTAGAGAAAGAGGAGAAATACTAGATGACAGT

AAAGAAGCTTTATTTCGTCCCAGCAGGTCGTTGTATGTTGGATCATTCGTCTGTTAATAGTACATTAACACCAGGAG
AATTATTAGACTTACCGGTTTGGTGTTATCTTTTGGAGACTGAAGAAGGACCTATTTTAGTAGATACAGGTATGCC
AGAAAGTGCAGTTAATAATGAAGGTCTTTTTAACGGTACATTTGTCGAAGGGCAGGTTTTACCGAAAATGACTGA
AGAAGATAGAATCGTGAATATTTTAAAACGGGTTGGTTATGAGCCGGAAGACCTTCTTTATATTATTAGTTCTCACT
TGCATTTTGATCATGCAGGAGGAAATGGCGCTTTTATAAATACACCAATCATTGTACAGCGTGCTGAATATGAGGC
GGCGCAGCATAGCGAAGAATATTTGAAAGAATGTATATTGCCGAATTTAAACTACAAAATCATTGAAGGTGATTAT
GAAGTCGTACCAGGAGTTCAATTATTGCATACACCAGGCCATACTCCAGGGCATCAATCGCTATTAATTGAGACAG
AAAAATCCGGTCCTGTATTATTAACGATTGATGCATCGTATACGAAAGAGAATTTTGAAAATGAAGTGCCATTTGC
GGGATTTGATTCAGAATTAGCTTTATCTTCAATTAAACGTTTAAAAGAAGTGGTGATGAAAGAGAAGCCGATTGTT
TTCTTTGGACATGATATAGAGCAGGAAAGGGGATGTAAAGTGTTCCCTGAATATATATAATAATACTAGAGCCAG
GCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTT SpeI G NotI EcoRI CTCTC

TACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGTAGCGGCCGCTGCAGTCCGGC
AAAAAGGGCAAGGTGTCACCACCCTGCCCTTTTTCTTTAAACCGAAAAGATTACTT 3'

Figure 9. The sequencing results for the construction of B0034-C0160-B0015 in psB1AK3 flanked with Biobrick restriction sites. This construct, taken from colony 3 of Top 10 competent cells, was sequenced using BBK CP-F primers. The size of the construct is 897bp. The grey color represents the autoinducer inactivation enzyme (aiiA). The double terminators are coded in red. The biobrick restriction sites: *XbaI*, *SpeI*, *EcoRI*, *NotI* and *PstI* are coded in blue, dark green, maroon, teal and purple respectively.

Figure 10. The sequencing results for the construction of C0160-B0015 in psB1AK3 flanked with Biobrick restriction sites. This construct, taken from colony 2 of Top 10 competent cells, was sequenced using BBK CP-F primers which anneals approximately 250bp before the Biobricked construct. The size of the construct is 885bp. The grey color represents the autoinducer inactivation enzyme (aiiA). The double terminators are coded in red. The biobrick restriction sites: *XbaI*, *SpeI*, *EcoRI*, *NotI* and *PstI* are coded in blue, dark green, maroon, teal and purple respectively.

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1    ATGACAGTAAAGAAGCTTTATTTCGTCCCAGCAGGTCGTTGTATGTTGGATCATTCGTCT  60
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1    ATGACAGTAAAGAAGCTTTATTTCGTCCCAGCAGGTCGTTGTATGTTGGATCATTCGTCT  60

61   GTTAATAGTACATTAACACCAGGAGAATTATTAGACTTACCGGTTTGGTGTTATCTTTTG  120
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
61   GTTAATAGTACATTAACACCAGGAGAATTATTAGACTTACCGGTTTGGTGTTATCTTTTG  120

121  GAGACTGAAGAAGGACCTATTTTAGTAGATACAGGTATGCCAGAAAGTGCAGTTAATAAT  180
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
121  GAGACTGAAGAAGGACCTATTTTAGTAGATACAGGTATGCCAGAAAGTGCAGTTAATAAT  180

181  GAAGGTCTTTTTAACGGTACATTTGTCGAAGGGCAGGTTTTACCGAAAATGACTGAAGAA  240
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
181  GAAGGTCTTTTTAACGGTACATTTGTCGAAGGGCAGGTTTTACCGAAAATGACTGAAGAA  240

241  GATAGAATCGTGAATATTTTAAAACGGGTTGGTTATGAGCCGGAAGACCTTCTTTATATT  300
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
241  GATAGAATCGTGAATATTTTAAAACGGGTTGGTTATGAGCCGGAAGACCTTCTTTATATT  300

301  ATTAGTTCTCACTTGCATTTTGATCATGCAGGAGGAAATGGCGCTTTTATAAATACACCA  360
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
301  ATTAGTTCTCACTTGCATTTTGATCATGCAGGAGGAAATGGCGCTTTTATAAATACACCA  360

361  ATCATTGTACAGCGTGCTGAATATGAGGCGGCGCAGCATAGCGAAGAATATTTGAAAGAA  420
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
361  ATCATTGTACAGCGTGCTGAATATGAGGCGGCGCAGCATAGCGAAGAATATTTGAAAGAA  420

421  TGTATATTGCCGAATTTAAACTACAAAATCATTGAAGGTGATTATGAAGTCGTACCAGGA  480
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
421  TGTATATTGCCGAATTTAAACTACAAAATCATTGAAGGTGATTATGAAGTCGTACCAGGA  480

481  GTTCAATTATTGCATACACCAGGCCATACTCCAGGGCATCAATCGCTATTAATTGAGACA  540
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
481  GTTCAATTATTGCATACACCAGGCCATACTCCAGGGCATCAATCGCTATTAATTGAGACA  540

541  GAAAAATCCGGTCCTGTATTATTAACGATTGATGCATCGTATACGAAAGAGAATTTTGAA  600
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
541  GAAAAATCCGGTCCTGTATTATTAACGATTGATGCATCGTATACGAAAGAGAATTTTGAA  600

601  AATGAAGTGCCATTTGCGGGATTTGATTCAGAATTAGCTTTATCTTCAATTAAACGTTTA  660
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
601  AATGAAGTGCCATTTGCGGGATTTGATTCAGAATTAGCTTTATCTTCAATTAAACGTTTA  660

661  AAAGAAGTGGTGATGAAAGAGAAGCCGATTGTTTTCTTTGGACATGATATAGAGCAGGAA  720
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
661  AAAGAAGTGGTGATGAAAGAGAAGCCGATTGTTTTCTTTGGACATGATATAGAGCAGGAA  720

721  AGGGGATGTAAAGTGTTCCCTGAATATATATAATAA  756
     ||||||||||||||||||||||||||||||||||||
721  AGGGGATGTAAAGTGTTCCCTGAATATATATAATAA  756
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Figure 11. The distribution of genes is homologous to the published *Bacillus* sp. strain 204B1 *aiiA* sequence among the sequenced bacterial genomes available on the National Center for Biotechnology Information (NCBI) database. The aiiA protein is putative metallo hydrolase (Dong, 2000).

Discussion

The overall goal of this year's iGEM wetlab project is to engineer the cell-to-cell communication system in *E. coli* to quorum quench using a quorum sensing mechanism. The ultimate goal is to break down biofilm. This can be achieved by construction the synthetic AI-2 signalling cascade followed by a response circuit which contains the autoinducer inactivation enzyme (aiiA). aiiA facilitates enzymatic degradation of the signaling molecule used by the microorganisms within the biofilm for communication (Yi-Hu et al., 2002). Breaking down this molecule prevents the bacteria from monitoring their cell density and coordinating such communal activities (Yi-Hu et al., 2002).

Thus far, only a segment of the response circuit has been constructed using the rbs (BBa_B0034), autoinducer inactivator enzyme (BBa_C0160) and double terminators (BBa_B0015) are biobricked parts. They were originally taken from the iGEM registry and have already been cloned into psB1AK3 plasmid. The next step is to piece together the remaining segments of the response circuit including the c1λ (inverter) and the Pqrr4 promoter to the aiiA construct.

Again, the Pqrr4 allows for the expression of aiiA in the presence of AI-2 when the promoter is not bound to the LuxO- σ54 complex (Tu & Bassler, 2007). This prevents the transcription of the promoter and the inverter, allowing for the expression of aiiA (Tu & Bassler, 2007). The c1λ inverter consists of the following parts: c1λ gene, terminators and Pc1λ (promoter). The c1λ, which currently exists in psB2K3 plasmid, will be cloned behind the Pqrr4 (psB1AC3) promoter in psB1AC3 plasmid. Despite its large size (987kb) in comparison to qrr4 promoter (275bp), c1λ is being cloned into the promoter plasmid because it is in a plasmid with Kanamycin resistance. The latter construct, B0034-aiiA-B0015, is also in a Kan resistant plasmid (psB1AK3). As such, following construction and transformation, the cells cannot be plated on to a Kan plate or any other antibiotic resistant plates because by plating it on Kan, may produce multiple colonies but there is no way to determining if the constructed plasmid was transformed in the colonies that grew. Obviously, the transformed bacteria, then, cannot be plated on other antibiotic LB agar it is only resistant to Kan. Therefore, by constructing on an AC plasmid, it allows us to grow the cells on a Chloramphenicol plate. The growth of those colonies will ensure that they have the AC plasmid.

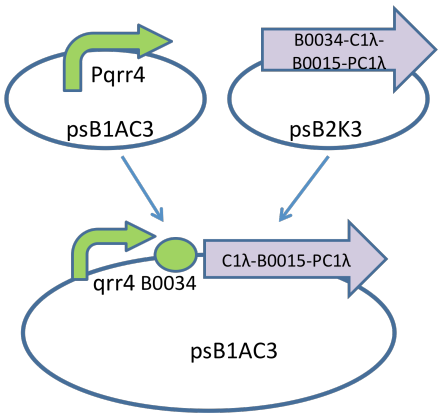

Figure 12. The cloning of Pqrr4-c1λ into psB1AC3 plasmid. c1λ is quad-part inverter that is composed of four sub-parts: a ribosome binding site, repressor protein (c1λ), double terminators and a promoter (Pc1λ), all regulated by the encoded repressor protein (iGEM Registry, 2009). This quad-part inverter insert is digested with *EcoRI* and *SpeI* and cloned behind the vector Pqrr4, which is digested with *EcoRI* and *XbaI* (Invitrogen, CA). The size of this final construct is expected to be 1262bp.

Now that most of the response circuit has been cloned into psB1AC3 plasmid, the final fragment, B0034-aiiA-B0015 construct will be cloned from psB1AK3 plasmid into psB1AC3 (Figure 13). The B0034-aiiA-B0015 construct will be digested behind the c1λ in the psB1AC3 plasmid simply because it is a much smaller fragment than the Pqrr4-inverter fragment. Obviously, it is much easier to construct a smaller construct, therefore, a higher rate of success in just one trial.

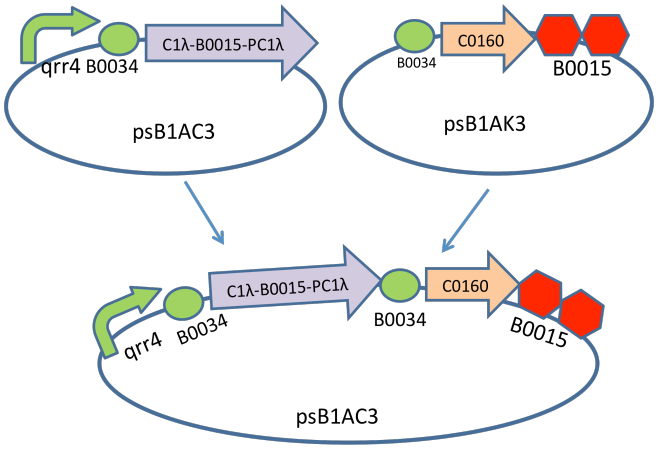

Figure 13. The aiiA construct will be digested with *EcoRI* and *PstI* (Invitrogen, CA) as the insert and Pqrr4-B0034-c1λ-B0015-Pc1λ- B0034-C0160-B0015 (vector) in psB1AC3 will be digested with *EcoRI* and *XbaI* (Invitrogen, CA). The B0034-C0160-B0015 construct will be digested behind the c1λ in the psB1AC3 plasmid to allow the inverter to control the expression of aiiA in varying levels of AI-2. The expected size of the construct is 2159bp.

To test the functionality of the response circuit to ensure that it, in can fact, degrades biofilm, a simple laboratory test on LB agar can be performed. By integrating last year's iGEM project with this year's AI-2 quorum sensing model, the Champion cell may be used to compare the level of GFP (green fluorescent protein) in the cell.

Originally, the University of Calgary's iGEM 2008 project built the "Champion Cell" in *E.coli* which acts as a biosensor and responds to the quorum sensing pheromones, AI-1 and AI-2 produced from "Bad Guy 1" and "Bad Guy 2" bacteria respectively (Kubik et al., 2008). In response to the detection of these pheromones, the engineered *E.coli* initiates the transcription of bacteriocins (ie. Colicin). In addition to constructing the system, the Champion cell reported the presence of Bad guy 1 and Bad guy 2 by glowing green or red (Kubik et al., 2008) in tandem with the specific Colicin as determined by the presence of either AI-1 or AI-2.

To test the response circuit, Bad Guy 1 can be reprogrammed to produce AI-2 along with Bad Guy 2. Then bioluminescence of last year's Champion cell can be compared to the bioluminescence of the cell after the degradation of AHL by this year's engineered *E.coli*, which houses the aiiA gene in the response circuit.

Bad Guy 1 and 2 should be allowed to grow overnight at 37 ^{o}C. In doing so, the bacteria can begin to accumulate and quorum sense using AI-2. The engineered *E.coli*, containing the aiiA response circuit, can then be released in the Bad Guy-infested environment to disrupt their communication. In the presence of AI-2, Pqrr4 will not be transcribed, which in turn, prevents transcription of the subsequent c1λ protein. As a result, Pc1λ should not be repressed; thus, generating the expression of aiiA. When aiiA is expressed, enzymatic degradation of AHL will attenuate the signal. Introducing the Champion cell to this environment, now, would activate or deactivate the *TetR* promoter (Kubik et al., 2008), which drives the expression of GFP in the presence or absence of AHL, respectively. Compared to the initial glow of the Champion cell in the presence of Bad Guy bacteria in the beginning of the test, a diminishing glow now would indicate that the AHL concentration has decreased substantially. This observation would allow us to infer that the response circuit is functional. After successfully determining the efficacy of the system, it can be introduced to target biofilm in industrial pipelines where they may be problematic.

In summary, the Biobrick format of construction has been very useful in the construction of the response circuit. It provides an innovative and advantageous approach to the construction of biological systems. This standard technique facilitates the assembly of BioBricked parts to formulate another composite Biobrick component which has similar restrictions sites to allow it to propagate. One special feature of Biobrick methods is that since cells transformed with Biobrick vectors produce the *ccdB* toxic protein, adding another biobrick part to the cloning site of this vector can remove the toxin (Shetty, Endy, Knight, 2008). This simply adds to the one of

several advantages of using Biobrick standards parts: inclusion of transcriptional terminators and high copy origin in the cloning site, to name a few (Shetty, Endy, Knight, 2008).

Conclusions

Bacteria sense their density and coordinate activities using pheromones, such as autoinducer-2 pheromones (Su-Jin et al, 2008). This natural system is cloned from *Vibrio harveyi* into *E.coli* to construct a response circuit in the *E.coli* to target and break down biofilm. The autoinducer enzyme, responsible for the degradation of the universal autoinducer, AHL, which will be transcribed in the presence of the minimal threshold level of AHL and launch a communal attack to disrupt communication with the biofilm. The University of Calgary iGEM team is in the process of constructing a quorum-coupled-quorum-quenching system.

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