

Construction of two Mutant Protein Genetic Circuits Using Biobrick Construction Techniques

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Abstract:

Through a process called quorum sensing, bacteria use pheromone-like molecules termed autoinducers to communicate with each other, monitoring their own cell density. When a critical density level is reached, they are able to alter gene expression on a population level. This system exists in nature where it is used by microorganisms in a variety of activities, such as the induction of virulence and the formation of biofilms. The 2009 University of Calgary iGEM team set out to construct an AI-2 quorum sensing system in *Escherichia coli*. For this project, two mutant protein circuits were created in order to produce the LuxOD47E and LuxOD47A proteins which mimic the phosphorylated and dephosphorylated forms of the *Vibrio harveyi* LuxO protein. Both forms of the LuxO protein are involved in the natural system. These circuits shall be used to test the functionality of the *qrr4* promoter used in turn to test our signaling system.

Keywords: Quorum Sensing, Biobrick, Synthetic Biology, Autoinducer-2,

1. Introduction

Quorum Sensing (QS) is a process through which microorganisms communicate with each other to coordinate their behavior¹. Through the use of pheromone-like molecules termed autoinducers, organisms are able to monitor their population density and, when at a critical point, change their gene expression. This population-wide change results in the coordination of group activities such as the induction of virulence or the formation of biofilms². There are two general types of quorum-sensing systems: LuxI/LuxR systems in gram negative bacteria and oligopeptide/two-component circuits found in gram-positive bacteria³. Both types of systems exist in nature in a variety of microorganisms. One example is *Vibrio. Harveyi*, a marine bacterial species that takes part in a symbiotic relationship with the Hawaiian Bobtailed Squid⁴. *V. harveyi* uses three different autoinducers in quorum sensing processes: autoinducer-1 which is an acylated homoserine lactone (AHL), CA-1 and autoinducer-2 (AI-2)⁵. These autoinducers are used to coordinate the expression of Luciferase, which is used by the squid as a mechanism to escape predation⁶.

Both AHL and AI-2 are autoinducers; however there are several key differences between them. AHL, produced by LuxI-like proteins, has only been found to be used by gram-negative bacteria while AI-2 is used by both gram-negative and gram-positive bacteria⁷. Many species of gram-negative bacteria make use of AHL, however due to slight modifications in the AHL molecules they produce as well as modifications in their AHL receptors; it is a species-specific signaling system⁸. AI-2 on the other hand, has been hypothesized to be a universal signaling molecule as it is known to be produced by over 50% of bacterial species via the synthetase *luxS*⁹. In this sense, AHL is used for intraspecies communication while AI-2 is hypothesized to be used for interspecies communication.

The AHL signaling system was successfully constructed by Canton et al. In 2008¹⁰. Although this system has been characterized and added to the Registry of Standard Biological Parts, this is currently the only signaling system present in the registry (parts.mit.edu). The addition of a second system, particular one making use of AI-2, would be useful for a number of reasons. For one, AI-2, unlike AHL, makes use of a phosphorylation cascade, thus allowing for the amplification of the signal.¹¹ Because of its proposed universality, an AI-2 signaling system also has the potential to receive and respond to signals from other species of bacteria; something that cannot be achieved with the AHL system. Finally, a second system also offers the possibility to disrupt other signaling systems, such as the AHL system. Through a process known as quorum quenching, this could allow the disruption of a variety of bacterial activities such as biofilm formation or the induction of virulence¹².

To this end, we, the University of Calgary iGEM team are exploring the AI-2 signaling pathway as a second quorum sensing system to contribute to the Registry of standard biological parts. We will be taking this system from *Vibrio harveyi* and making it functional in *Escherichia coli*. In nature, this system involves the periplasmic, AI-2 binding protein LuxP and adjacently bound protein kinase LuxQ as well as cytoplasmic proteins LuxO and LuxU¹³. In the absence of AI-2, LuxQ autophosphorylates and acts as a kinase, phosphorylating LuxU which in turn phosphorylates LuxO¹⁴. Phosphorylated LuxO binds to transcription factor σ 54 and activates the transcription of genes encoding five regulatory small RNAs (sRNAs) termed Qrr1-5. The sRNAs bind to and destabilize the mRNA encoding LuxR, a transcriptional activator, required for activation of the transcription of luxCDABE, the Luciferase operon¹⁵. Therefore when the population density of the bacteria is low, no bioluminescence is expressed.

When present in the environment, AI-2 binds to LuxP which undergoes a conformational change. The adjacently bound protein, LuxQ, contains both an N-terminal periplasmic sensory domain in the membrane and a C-terminal response regulator domain in the intercellular space. The binding of AI-2 to LuxP causes LuxQ to act as a phosphatase, removing a phosphate from LuxU¹⁶. Non-specific

phosphatases eventually cause the loss of a phosphate group from LuxO. The dephosphorylated form of LuxO is then unable to bind to transcription factor σ 54, inhibiting transcription of the sRNAs¹⁷.

We obtained cloned genes encoding for the proteins involved in the signaling system (LuxPQ and LuxOU) as well as the downstream *qrr4* promoter from Dr. Bonnie Bassler (Princeton, NJ). Our system makes use of the *qrr4* promoter in place of sRNAs. Phosphorylated LuxO binds to transcription factor σ 54 and activates the *qrr4* promoter, initiating transcription of any genes downstream (figure 1). In order to visualize and measure the performance of this signaling system, a reporter circuit was constructed using the *qrr4* promoter and a gene that codes for green fluorescent protein (GFP) as well as the B0015 terminator. The functionality of this circuit will in turn be tested through the use of two mutant circuits containing sequences coding for the LuxOD47E and LuxOD47A proteins (figures 2, 3). LuxOD47E mimics the phosphorylated, active form of the LuxO protein while LuxOD47A mimics the dephosphorylated, inactive form. Finally, a response circuit will also be constructed using the following Biobricked components: *qrr4* promoter (BBa_K131017), RBS (BBa_B0034), *cl* lambda repressor (BBa_CO051), double terminator (BBa_B0015), *cl* regulated promoter (BBa_R0051), RBS (BBa_B0034), autoinducer inactivation enzyme (*aiiA*) (BBa_CO160) and double terminator (BBa_B0015). *Cl* lambda, when expressed and coupled with the *cl* lambda repressible promoter (BBa_R0051) acts as an inverter, binding to and repressing the *cl* lambda repressible promoter, allowing for the expression of our gene of interest, *aiiA*, in the presence of AI-2¹⁸. *aiiA* encodes an enzyme which catalyzes the degradation of AHL, thereby disrupting QS. AHL is an important autoinducer in gram-negative bacteria involved in biofilm formation¹⁹. As such this degradation of AHL will be used to prevent the formation of biofilms, the targeted output of our system.

The construction of our system will be executed using BioBrick cloning techniques (figure 4). A standardized method of DNA assembly, Biobrick cloning techniques involve the addition of specific restriction sites in front and behind the gene of interest²⁰. *EcoRI*, *NotI* and *XbaI* restriction sites are added to the prefix of genes, while *SpeI*, *NotI* and *PstI* restriction sites are added to the suffix of genes²¹. With the use of these restriction sites, DNA assembly can not only be made more reliable and predictable, but parts can be interchanged and outsourced²². This standardization initiative is an important part of Synthetic Biology: an emerging field of science where novel biological devices are constructed through the application of engineering principle²³. By using natural and synthetic parts, synthetic biology is able to, through the design of living systems; allow organisms to perform tasks different from those that they would in nature²⁴. Another initiative through which standardization is being achieved is through the ever-growing Standard Registry of Biological Parts. Founded in 2003 by the Massachusetts Institute of Technology (MIT), this online registry provides information on over 3200 genetic parts that can be used in conjunction with each other to build a variety of synthetic devices and systems²⁵.

In this paper, we describe our procedure to develop two independent circuits containing genes encoding the LuxOD47A and LuxOD47E mutant proteins using the Biobrick standard. Circuits also include the BioBrick part J13002, containing the R0040 promoter and the B0034 ribosomal binding site (RBS) as well as the BioBrick B0015 terminator. With these circuits, the functionality of the *qrr4* promoter will be able to take place, which will be used to test our signalling system. The addition of this second signalling system to the registry of standard biological parts will pave the way for a variety of different applications where the fine-tuned coordination of bacteria is required. Our system specifically will be used to prevent the formation of biofilms by degrading AHL molecules, however this system could also be used in

many diverse applications such as in the delivery of drugs or in bioremediation such as the cleaning up of oil spills.

2. Methods/ Materials

2.2 Bacterial Strains, Media, Antibiotics, Primers and Vectors

All necessary plasmids and primers are listed in table 1. The *luxOD47E* and *luxOD47A* DNA coding sequences were originally cloned from *V. Harveyi* into a cosmid, and then mutated by Dr. Bonnie Bassler (Princeton, NJ). The genes of interest, *luxOD47E* and *luxOD47A* were then cloned into a pCR 2.1-TOPO vector (Invitrogen, CA). The J13002 promoter and Ribosomal binding site (RBS) were received from the Registry of Standard Biological Parts (Cambridge, MA). All primers were supplied by the University of Calgary DNA Synthesis Lab (University of Calgary, Alberta). All constructs were cloned into competent TOP 10 *E. coli* cells (F^- *mcrA* $\Delta(mrr-hsdRMS-mcrBC)$ $\phi80lacZ\Delta M15$ $\Delta lacX74$ *recA1* *araD139* $\Delta(ara-leu)$ 7697 *galU* *galK* *rpsL* (Str^R) *endA1* *nupG* λ^-). Testing of the circuits was performed in KT1144 cosmids containing the *qrr4* promoter and a gene encoding GFP. Liquid cultures and agar plates were made with Luria-Bertani(LB) broth. Ampicillin, chloramphenicol and kanamycin were used in final concentrations of 100ng/ μ L, 35ng/ μ L and 50ng/ μ L respectively.

2.2 Amplification, Cloning and Verification of Genes Coding for Mutant Proteins

LuxOD47E and *luxOD47A* in 2.1 pCR-TOPO vectors were amplified through gradient Polymerase Chain Reaction (PCR) amplification with *luxO*-RS-F* and *luxO*-RS-R primers and pPFX (Invitrogen, CA). Cycling conditions were as follows: 94°C for five minutes, 36 x (94 °C for 15 seconds, 55-68 °C for 30 seconds and 68 °C for 90 seconds), 68°C for 15 minutes and then held at 4 °C . The PCR products were run on a

1% agarose gel at 120 volts with GeneRuler DNA ladder. This resulted in linear strands of *luxOD47E*/*luxOD47A* DNA with the Biobrick prefix and suffix. This DNA was then purified using the Qiaquick PCR product purification kit (Qiagen, MD) according to the directions of the manufacturer. The concentration of the DNA was then measured with a 1000 Nanodrop Spectrophotometer. Biobrick construction technique was used to get *luxOD47E* and *luxOD47A* into the PSB1AC3 vector. *luxOD47E*/*luxOD47A* and the psB1AC3 vector with the *ccdB* gene were cut with *EcoRI* and *PstI* restriction enzymes (Invitrogen, CA) and with *XbaI* and *PstI* restriction enzymes (Invitrogen, CA). Approximately 200 ng of vector and 600ng of linear insert were digested separately for 2 hours in 37 °C water bath. The vector was treated with Antarctic phosphatase (New England Biolabs, ON) according to the directions of the manufacturer. Vector and insert were ligated with QuikLigase (Invitrogen, CA) in accordance with the manufacturer's directions. Ligated product was then transformed into chemically competent TOP10 cells through standard heat shock and plated on suitable antibiotic-plates.

Verification was done in three ways. First a colony PCR was run with Platinum Thermus aquaticus polymerase (p taq) (Invitrogen, CA) and gene-specific *luxO* forward and reverse primers. Cycling conditions were followed as per gradient PCR with the initial denaturing step extended to 6 minutes. PCR product was visualized on a 1% agarose gel with Generuler DNA ladder. Overnight cultures of successful colonies were grown and plasmids of cultures were isolated using the Qiaprep Spin Miniprep kit (Qiagen, MD) in accordance with the manufacturer's directions. A restriction digest with *NotI* restriction enzymes (Invitrogen, CA) was performed in accordance with the manufacturer's instructions. Digested DNA was visualized on a 1% agarose gel run at 120 volts with GeneRuler DNA ladder and uncut DNA for each colony as a positive control. Finally 10 µL of plasmid (100ng/1 kb) of one successful colony was then sent down for sequencing with BBK-CP-F and BBK-CP-R primers at the University of Calgary DNA sequencing Facility (University Core DNA Services, Calgary, Alberta, Canada).

2.3 Circuit Construction

All construction was performed using standard Biobrick techniques (figure 4). J13002, containing both the R0040 Tetracycline repressible promoter (tetR) as well as the B0034 ribosomal binding site (RBS) was added to *luxOD47E*. Both insert and vector were digested with *EcoRI*/*PstI* restriction enzymes (Invitrogen, CA) and with *XbaI*/*PstI* restriction enzymes (Invitrogen, CA) according to the directions of the manufacturer. Vector was then treated with Antarctic Phosphatase (New England BioLabs, ON). Ligation of insert and vector was performed with QuikLigase (Invitrogen, CA). Ligated J13002-*luxOD47A* in pSB1AC3 vector was then transformed into TOP 10 *E. coli* cells and plated.

B0015 was added to *luxOD47A* using standard Biobrick construction techniques. Both insert and vector were digested with *EcoRI*/*PstI* restriction enzymes (Invitrogen, CA) and with *XbaI*/*PstI* restriction enzymes (Invitrogen, CA) according to the directions of the manufacturer. Vector was then treated with Antarctic Phosphatase (New England BioLabs, ON). Ligation of insert and vector was performed with QuikLigase (Invitrogen, CA). Ligated J13002-*luxOD47A* in pSB1AC3 vector was then transformed into chemically competent TOP 10 *E. coli* cells following standard heat shock procedure and plated.

B0015 and J13002-*luxOD47E* were digested with *XbaI*/*PstI* restriction enzymes (Invitrogen, CA) according to the directions of the manufacturer. Antarctic Phosphatase treatment (New England BioLabs, ON) was performed on the vector according to the supplier's instructions and ligation was performed with QuikLigase (Invitrogen, CA) following the procedure set out by the manufacturer. Ligated product was then transformed into TOP 10 *E. coli* cells following standard heat-shock procedure. J13002 was added to the *luxOD47E*-B0015 through a construction digest using *XbaI*/*PstI* restriction enzymes (CA) and *XbaI*/*PstI* restriction enzymes (Invitrogen, CA) according to the directions of the manufacturer. After ligation, products were transformed into TOP 10 *E. coli* cells and plated. Finally *luxOD47A* was then cloned into the pSB1AC3 vector for future testing following similar Biobrick construction techniques.

Construction was verified using the same three step procedure used to verify the *luxOD47E/luxOD47A* genes.

2.4 Testing of Completed Circuits

Completed circuits were transformed into chemically competent TOP10 *E. coli* cells containing plasmids with the *qrr4* promoter and a gene encoding GFP, as well as into KT1144 cosmids also containing the *qrr4* promoter and a gene encoding for GFP. Overnight cultures were prepared in LB broth with appropriate antibiotic resistance. GFP expression of these cultures as well as of 1:10 and 1:100 dilutions were measured using a Synergy HT plate reader. Cell cultures containing plasmid with a positive control circuit (a constitutive promoter and a gene encoding GFP) were also made and tested to establish high levels of GFP expression as a point of comparison.

3 Results

3.1 Gene Verification and Circuit Construction

LuxOD47E and *luxOD47A* were successfully Biobricked using gradient PCR with gene specific primers containing BioBrick restriction sites. Biobricked genes were then successfully moved into the psB1AC3 vector (*luxOD47E*) and the psB1AK3 vector (*luxOD47A*). Sequencing results were compared to a known sequence of the *Vibrio harveyi* LuxO protein (figure 5,6). J13002 was then successfully added to *luxOD47E* while B0015 was added to *luxOD47A*. B0015 was then successfully cloned into the J130002-*luxOD47E* construct while J13002 was cloned into the *luxOD47A*-B0015 construct. *luxOD47A* was then successfully transformed into the PSB1AC3 vector for future testing.

All constructs were verified using a colony PCR and restriction digest using *NotI* restriction enzymes (Invitrogen, CA). Products were visualized on a 1% agarose gel run with (data not shown). All constructs

were then sequenced using the BBK-CP-F and BBK-CP-R sequencing primers (figures 7-10) at the University of Calgary DNA sequencing Facility (University Core DNA Services, Calgary, Alberta, Canada).

3.2 Testing of Completed Circuits

GFP expression levels were measured using a Synergy HT plate reader (figure 11).

4 Discussion

The initial goal was to create two independent circuits producing mutated forms of the *Vibrio harveyi* LuxO protein: LuxOD47E and LuxOD7E. When constructed with a constitutive promoter and RBS site (BBa_J13002) and a terminator (BBa_B0015), these can be used to test the functionality of the qrr4 promoter which will be used in further testing of our system. As the phosphorylated form of LuxO binds to the qrr4 promoter, expressed LuxOD47E should also bind to the promoter whereas LuxOD47A should not. To this end, when our mutant circuits are transformed into a plasmid containing the qrr4 promoter and a gene encoding GFP, fluorescence levels should be higher for the *luxOD47E* circuit than for the *luxOD47A* circuit.

Although the construction of the J13002-*luxOD47A*-B0015 circuit was relatively quick, there were many setbacks during the construction of the J13002-LuxOD47E-B0015 circuit. Adding the Biobrick prefix and suffix to LuxOD47E proved to be a very difficult step, requiring five separate attempts before a positive result was reached. This emphasizes the point that Biobrick cloning techniques are not very effective. They are time consuming, taking a minimum of 3 days in order to isolate plasmid, and they are not always reliable. As discussed previously though, we needed to use this cloning technique as the Biobrick standard is what is used in the Registry of Standard Biological Parts.

Looking back at this experiment, more attention should have been placed on which vector each circuit was constructed in. As the circuit to test these mutant circuits was constructed in a vector with ampicillin and kanamycin resistance, a last minute plasmid switch had to be done on the J13002-*LuxOD47A*-B0015 circuit in order to move it into a vector with ampicillin and Chloramphenicol resistance to maintain antibiotic selection pressure when the two plasmids were transformed together. If we had just constructed both circuits in the pSB1AC3 vector in the beginning however, this step would not have been necessary and the testing could have started earlier.

On top of issues with the Biobrick cloning techniques used, progress was also slowed down due to contamination issues. There was a great deal of contamination in negative control lanes during the initial amplification PCR of *luxOD47E* in the 2.1pCR-TOPO vector. Some of this may have been due to sharing the same primer stocks between multiple people. Because *luxOD47E* and *luxOD47A* are the same size (1362 base pairs) and only differ by one base pair mutation, in a restriction digest or a PCR using gene-specific *luxO* primers, bands representing these two genes would look the exact same and only DNA sequencing could differentiate the two. For this reason, separate primer stocks, buffers, etc should have been used for the construction of these two circuits.

It also would have been helpful if we had constructed *luxOD47E*-B0015 first, instead of J13002-*luxOD47E*. This would have facilitated our construction of the B0034-*luxOD47E*-B0015-Pqrr4 circuit that was later made. If we had had sequenced *luxOD47E*-B0015, it would have cut down on one construction step.

With these circuits fully constructed and sequenced, the next step is to finish the testing of the two mutant circuits in chemically competent KT1144 cells containing cosmids with *qrr4* and a gene encoding for GFP. The mutant circuits will also simultaneously be tested in chemically competent TOP 10 E. coli cells containing our reporter circuit: Pqrr4 (K131017) and a gene encoding GFP (I13500) in order to test

the functionality of the *qrr4* promoter and we complete the testing of our system with the testing of our signaling circuit.

Initial results have yielded expected results. Cultures of cells containing the reporter circuit (*qrr4* promoter driving the expression of a gene coding for GFP) and the mutant circuit (J13002-*luxOD47E*-B0015) show higher GFP expression levels than cultures of cells containing the reporter alone. As well, cultures of KT1144 cells, containing J13002-*luxOD47E*-B0015 also show higher GFP expression levels than cultures with KT1144 cells alone. These are expected results as the mutant circuit (J13002-*luxOD47E*-B0015) should produce LuxOD47E which can bind to the *qrr4* promoter in the reporter and KT1144 cells, activating it and initiating transcription of GFP. This suggests that both our mutant circuits and the *qrr4* promoter are functioning. Although there is some expression in cultures without this circuit, this is likely due to the leaky nature of promoters. The next step is to follow the same testing procedure with the J13002-*luxOD47A*-B0015 circuit and see how this result compares to the cultures without J13002-*luxOD47E*-B0015. They should show similar levels of GFP expression as *luxOD47A* cannot bind to the *qrr4* promoter, and as a result, cannot initiate transcription of GFP in either the reporter cells or the KT1144 cells.

Another future direction is to finish construction of the B0034-*luxOD47E*-B0015-P*qrr4* circuit. We have submitted B0034-*luxOD47E* to the registry however, and this part, with the addition of a terminator followed by the *qrr4* promoter could be used in a variety of applications. LuxOD47E is one of only a small group of proteins that acts directly on a promoter, in this case the *qrr4* promoter. Because of this, any promoter could be placed in front of this circuit and any gene could be placed downstream and transcribed. This essentially acts as a control point in a circuit and as such, could be used in a variety of other projects.

Both completed mutant circuits have some future applications. J13002-luxOD47A-B0015 acts as a control circuit, that can be used, along with LuxO D47E, to test the functionality of the qrr4 promoter (as was demonstrated in this experiment). As the LuxOD47A protein mimics the unphosphorylated form of the LuxO protein, this protein should not bind to the qrr4 promoter and as a result, there should not be any transcription of genes placed downstream. As a result, if anyone else wanted to make use of this promoter in any way, these circuits could be used as a testing mechanism, should they be required. On top of that, if someone wanted to set about biobricking another of the qrr promoters (1-5) at some point, these circuits could be used as a test mechanism as well. Because the strength of the qrr promoters varies across bacterial species, if someone were to try to set up a quorum sensing system in another species of bacteria, this could be important in that case.

In conclusion these circuits are very important in the testing process of our system. With the functionality of the qrr4 promoter verified, we can move on to the testing of our signaling circuit, putting us one step closer to the addition of a second signaling system, responsive to AI-2, to the Registry of Standard Biological Parts.

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Tables/ Figures

Primers	Sequence	Annealing Temperature
<i>LuxO-F primer</i>	ATGGTAGAAGACACCGCATC	60 °C
<i>LuxO-R primer</i>	TCATACGTTTTGTTTTTCGTCC	60 °C
<i>BBK-CP-F primer</i>	CACCTGACGTCTAAGAAACC	60 °C
<i>BBK-CP-R primer</i>	AGGAAGCCTGCATAACGCG	60 °C
<i>LuxO-RS-F* primer</i>	GAATTCGCGGCCGCTTCTAGATGGTAGAAGACACCGCATC	60 °C
<i>LuxO-RS-R primer</i>	CTGCAGCGGCCGCTACTAGTTCATACGTTTTGTTTTTCGTCC	60 °C
Vector/ Parts	Comments	Reference
<i>PCR-2.1 TOPO TA vector</i>	Amp ^R and Kan ^R ,	Invitrogen
<i>PsBIAC3 vector</i>	Amp ^R and Chlor ^R	iGEM registry
<i>BBa_J13002</i>	In pSB1A2	iGEM Registry
<i>BBa_B0015</i>	In pSB1AK3	iGEM Registry

Table 1. Table 1. Primers plasmid backbones and BioBrick parts used during cloning of genes and construction of circuits. *LuxO-F* and *LuxO-R* primers are gene-specific primers used for verification PCR of genes in 2.1 TOPO TA vector. *LuxO-RS-F** and *LuxO-RS-R* are gene-specific primers with biobrick prefix and suffix and were used during amplification PCR to add the BioBrick prefix (*EcoRI*, *NotI* and *XbaI*) and suffix (*SpeI*, *NotI* and *PstI*) to the genes of interest. *BBK-CP-F* and *BBK-CP-R* are custom primers used for sequencing and construction verification PCR. They anneal approximately 250 base pairs upstream and downstream of the multiple cloning site. Both pCR-

2.1TOPO TA and psBIAC3 have the *ccdB* gene incorporated to act as a positive selection factor. All of the plasmids are high-copy number. Both BBa_J13002 and BBa_B0015 are BioBrick parts obtained from the Registry of Standard Biological Parts.

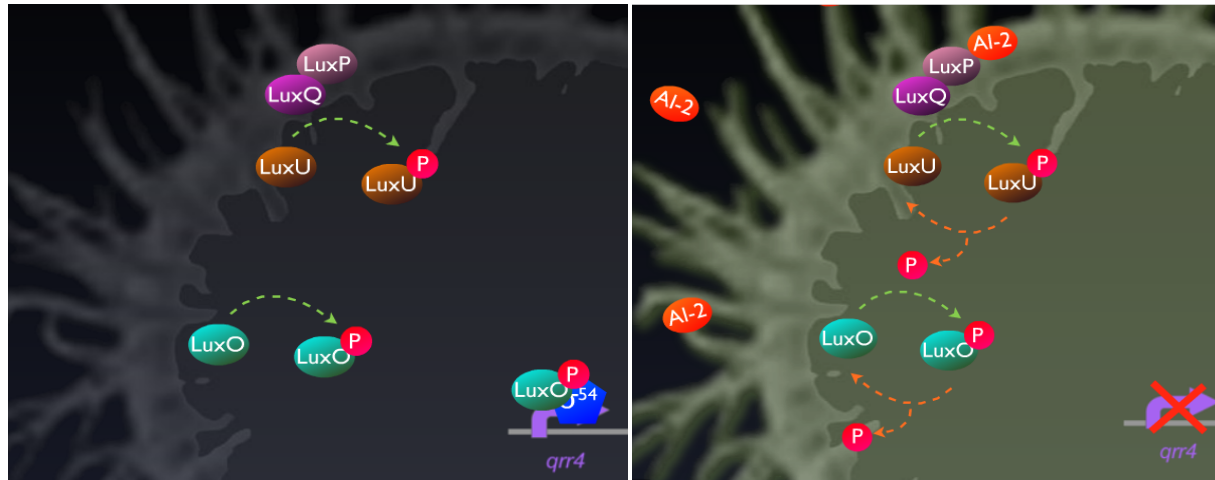


Figure 1. Schematic diagram of the signaling pathway for the AI-2 QS system showing the phosphorylation cascade in the absence of AI-2 and the dephosphorylation cascade in the presence of AI-2. In the absence of AI-2, LuxQ autophosphorylates and acts as a kinase, phosphorylating LuxU which in turn phosphorylates LuxO. The phosphorylated form of LuxO is able to bind to Sigma 54 and the *qrr4* promoter resulting in transcription of any gene located downstream of *qrr4*. When present in the environment however, AI-2 binds to LuxP which undergoes a conformational change. The adjacently bound protein, LuxQ, contains both an N-terminal periplasmic sensory domain in the membrane and a C-terminal response regulator domain in the intercellular space. The binding of AI-2 to LuxP causes LuxQ to act as a phosphatase, removing a phosphate from LuxU²⁶. Non-specific phosphatases eventually cause the loss of a phosphate group from LuxO. The dephosphorylated form of LuxO is then unable to bind to sigma factor 54 and the *qrr4* promoter, inhibiting transcription of any gene placed downstream of the *qrr4* promoter.

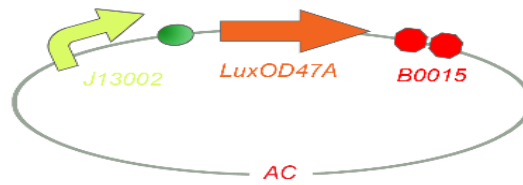


Figure 2. Mutant protein circuit containing the J13002 promoter and RBS site, luxOD47A with Biobrick prefix (*EcoRI*, *NotI*, *XbaI*) and suffix (*SpeI*, *NotI*, *PstI*) and B0015 terminator in the psB1AC3 vector.

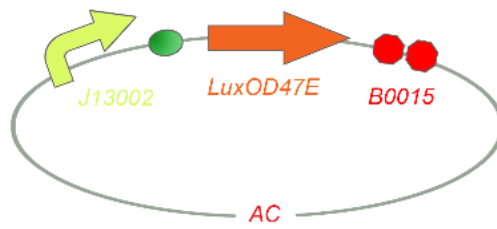


Figure 3. Mutant protein circuit containing the J13002 promoter and RBS site, luxOD47e with Biobrick prefix (EcoRI, NotI, XbaI) and suffix (SpeI, NotI, PstI) and B0015 terminator in the psB1AC3 vector.

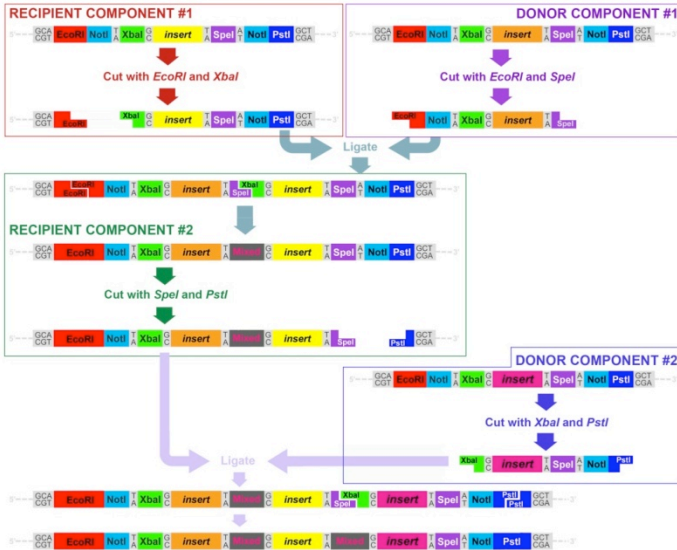


Figure 4. Diagram Illustrating Biobrick cloning techniques, created by Sonja Georgijevic.

lcl|17681 unnamed protein product
Length=453

Score = 943 bits (2437), Expect = 0.0, Method: Compositional matrix adjust.
Identities = 452/453 (99%), Positives = 453/453 (100%), Gaps = 0/453 (0%)

LuxO	1	MVEDTASVAALYRSYLTPLGIDINIVGTGRDAIESLNHRIPDLILL	LRLPDMTGMDVLH	60
LuxOD47E	1	MVEDTASVAALYRSYLTPLGIDINIVGTGRDAIESLNHRIPDLILL	LRLPDMTGMDVLH	60
LuxO	61	AVKKSHPDVPIIFMTAHGSIDTAVEAMRHGSQDFLIKPC	EADRLRVTVNNAIRKATKLN	120
LuxOD47E	61	AVKKSHPDVPIIFMTAHGSIDTAVEAMRHGSQDFLIKPC	EADRLRVTVNNAIRKATKLN	120
LuxO	121	EADNPGNQNYQGFIGSSQTMQQVYRTIDSAASSKASIFITGESGTGKEVCAEAIHAASKR		180
LuxOD47E	121	EADNPGNQNYQGFIGSSQTMQQVYRTIDSAASSKASIFITGESGTGKEVCAEAIHAASKR		180
LuxO	181	GDKPFIAINCAAIPKDLIESELF GHVKG AFTGAANDRQGA	ELADGGTFLFDELCEMDLD	240
LuxOD47E	181	GDKPFIAINCAAIPKDLIESELF GHVKG AFTGAANDRQGA	ELADGGTFLFDELCEMDLD	240
LuxO	241	LQTKLLRFIQGTGTFQKVGSSKMKSVDVRFVCATNRDPWKEVQEGRFREDLYYRLYVIPLH		300
LuxOD47E	241	LQTKLLRFIQGTGTFQKVGSSKMKSVDVRFVCATNRDPWKEVQEGRFREDLYYRLYVIPLH		300
LuxO	301	LPPLRERGKDVIEIAYSLLGYMSHEEGKSFVRFAQDVIERFNSYEWPGNVRQLQNVLRNI		360
LuxOD47E	301	LPPLRERGKDVIEIAYSLLGYMSHEEGKSFVRFAQDVIERFNSYEWPGNVRQLQNVLRNI		360
LuxO	361	VVLNNGKEITLDMPLPPPLNQPVVRQSVAKFIEPDIMTVSDIMPLWMTEKMAIEQAIQACE		420
LuxOD47E	361	VVLNNGKEITLDMPLPPPLNQPVVRQSVAKFIEPDIMTVSDIMPLWMTEKMAIEQAIQACE		420
LuxO	421	GNIPRAAGYLDVSPSTIYRKLQAWNSKDEKQNV		453
LuxOD47E	421	GNIPRAAGYLDVSPSTIYRKLQAWNSKDEKQNV		453

Figure 5. Sequence alignment of LuxOD47E with LuxO²⁷. The highlighted area shows the location of the single base mutation at base pair 47, resulting in a change of a single produced amino acid.

lcl|55613 unnamed protein product
Length=453

Score = 942 bits (2434), Expect = 0.0, Method: Compositional matrix adjust.
Identities = 452/453 (99%), Positives = 452/453 (99%), Gaps = 0/453 (0%)

LuxO	1	MVEDTASVAALYRSYLTPLGIDINIVGTGRDAIESLNHRIPDLILL	DLRLPDMTGMDVLH	60
LuxOD47A	1	MVEDTASVAALYRSYLTPLGIDINIVGTGRDAIESLNHRIPDLILL	LRLPDMTGMDVLH	60
LuxO	61	AVKKSHPDVPIIFMTAHGSIDTAVEAMRHGSQDFLIKPCADRLRVTVNNAIRKATKLN		120
LuxOD47A	61	AVKKSHPDVPIIFMTAHGSIDTAVEAMRHGSQDFLIKPCADRLRVTVNNAIRKATKLN		120
LuxO	121	EADNPGNQNYQGFIGSSQTMQQVYRTIDSAASSKASIFITGESGTGKEVCAEAIHAASKR		180
LuxOD47A	121	EADNPGNQNYQGFIGSSQTMQQVYRTIDSAASSKASIFITGESGTGKEVCAEAIHAASKR		180
LuxO	181	GDKPFIAINCAAIPKDLIESELF GHVKG AFTGAANDRQGAAELADGGTFLFDELCEMDLD		240
LuxOD47A	181	GDKPFIAINCAAIPKDLIESELF GHVKG AFTGAANDRQGAAELADGGTFLFDELCEMDLD		240
LuxO	241	L QTKLLRFIQGTGTFQKVGSSKMKSV D VRFVCATNRDPWKEVQEGRFREDLYYRLYVIPLH		300
LuxOD47A	241	L QTKLLRFIQGTGTFQKVGSSKMKSV D VRFVCATNRDPWKEVQEGRFREDLYYRLYVIPLH		300
LuxO	301	LPPLRERGKDVIEIAYSLLGYMSHEEGKSFVRFAQDVIERFNSYEWPGNVRQLQNVLRN		360
LuxOD47A	301	LPPLRERGKDVIEIAYSLLGYMSHEEGKSFVRFAQDVIERFNSYEWPGNVRQLQNVLRN		360
LuxO	361	VVLNNGKEITLDMLPPPLNQPVVRQSVAKFIEPDIMTVSDIMPLWMTEKMAIEQAIQACE		420
LuxOD47A	361	VVLNNGKEITLDMLPPPLNQPVVRQSVAKFIEPDIMTVSDIMPLWMTEKMAIEQAIQACE		420
LuxO	421	GNIPRAAGYLDVSPSTIYRKLQAWNSKDEKQNV		453
LuxOD47A	421	GNIPRAAGYLDVSPSTIYRKLQAWNSKDEKQNV		453

Figure 6. Sequence alignment of LuxOD47A with LuxO²⁸. The highlighted area shows the location of the single base mutation at base pair 47, resulting in a change of a single produced amino acid.

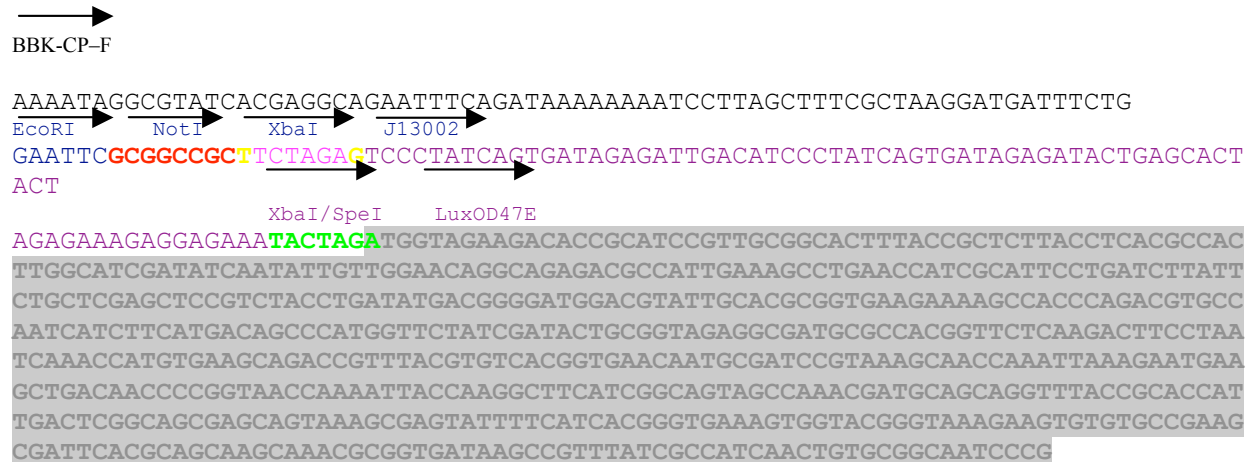


Figure7. Forward DNA sequence of the J13002-LuxOD47E construct, sequenced with the BBK-CP-F primer which anneals 250 base pairs upstream of the gene of interest. The blue region represents the EcoRI restriction site, the red region represents the NotI restriction site, the pink region the XbaI restriction site, the purple region the biobrick part: Bba_J13002, the green region the XbaI/SpeI mixed restriction site, and the grey region the gene of interest: LuxOD47E.

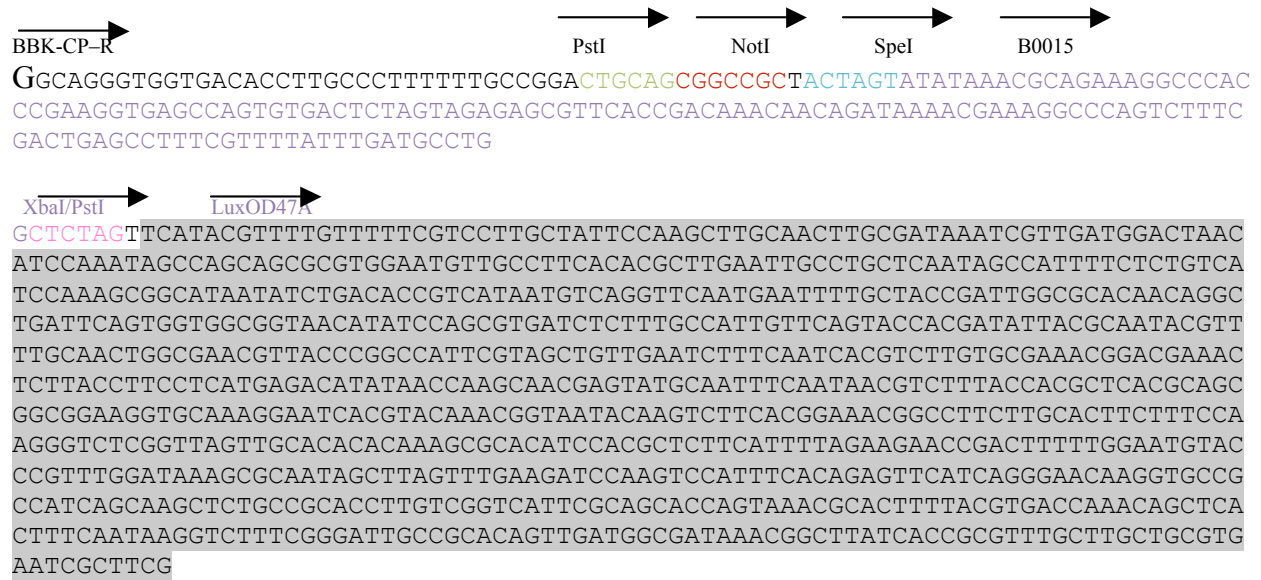


Figure 8. Reverse DNA sequence of the LuxOD47A-B0015 construct, sequenced with the BBK-CP-R primer which anneals 250 base pairs downstream of the gene of interest. The green region represents the PstI restriction site, the red region represents the NotI restriction site, the blue region represents the SpeI restriction site the purple region the biobrick part: Bba_B0015, the pink region the XbaI/PstI mixed restriction site and the grey region the gene of interest: LuxOD47A.

BBK-CP-R
 GGCAGGGTGGTGACACCTTGCCCTTTTTTGCCGGA

PstI NotI SpeI B0015
 GTCAGCGGCTACTAGTATATAAACGCAGAAAGGCCAC
 CCGAAGGTGAGCCAGTGTGACTCTAGTAGAGAGCGTTCACCGACAAACAACAGATAAAACGAAAGGCCAGTCTTTC
 GACTGAGCCTTTCGTTTTATTTGATGCCTG

XbaI/PstI LuxOD47E
 GCTCTAGTTTCATACGTTTTGTTTTTCGTCCTTGCTATTCCAAGCTTGCAACTTGCATAAATCGTTGATGGACTAAC
 ATCCAAATAGCCAGCAGCGCGTGGAATGTTGCCTTCACACGCTTGAATTGCCTGCTCAATAGCCATTTTCTCTGTCA
 TCCAAAGCGGCATAATATCTGACACCGTCATAATGTCAGGTTCAATGAATTTTGCTACCGATTGGCGCACAACAGGC
 TGATTCAGTGGTGGCGGTAACATATCCAGCGTGATCTCTTTGCCATTGTTTCAGTACCACGATATTACGCAATACGTT
 TTGCAACTGGCGAACGTTACCCGGCCATTTCGTAGCTGTTGAATCTTTCAATCACGTCCTTGTGCGAAACGGACGAAAC
 TCTTACCTTCCTCATGAGACATATAACCAAGCAACGAGTATGCAATTTCAATAACGTCCTTACCACGCTCACGCAGC
 GGCGGAAGGTGCAAAGGAATCACGTACAAACGGTAATACAAGTCTTCACGAAACGGCCTTCTTGCACTTCTTTCCA
 AGGGTCTCGGTTAGTTGCACACACAAAGCGCACATCCACGCTCTTCATTTTAGAAGAACCGACTTTTTGGAATGTAC
 CCGTTTGGATAAAGCGCAATAGCTTAGTTTGAAGATCCAAGTCCATTTACAGAGTTCATCAGGGAACAAGGTGCCG
 CCATCAGCAAGCTCTGCCGCACCTTGTCGGTCATTTCGCAGCACCAGTAAACGCACCTTTACGTGACCAACAGCTCA
 CTTTCAATAAGGTCTTTCGGGATTGCCGCACAGTTGATGGCGATAAACGGCTTATCACC GCGTTTGCTTGCTGCGTG
 AATCGCTTCG

Figure 9. Reverse DNA sequence of the J13002-LuxOD47E-B0015 construct, sequenced with the BBK-CP-R primer which anneals 250 base pairs downstream of the gene of interest. The green region represents the PstI restriction site, the red region represents the NotI restriction site, the blue region the SpeI restriction site, the purple region the biobrick part: Bba_J13002, the pink region the mixed XbaI/SpeI restriction site, and the grey region the gene of interest: LuxOD47E.



Figure 10. Forward DNA sequence of the J13002-LuxOD47A-B0015 construct, sequenced with the BBK-CP-F primer which anneals 250 base pairs upstream of the gene of interest. The blue region represents the EcoRI restriction site, the red region represents the NotI restriction site, the pink region the XbaI restriction site, the purple region the biobrick part: Bba_J13002, the green region the XbaI/SpeI mixed restriction site, and the grey region the gene of interest: LuxOD47A.

Fluorescent levels of different cultures (5 hours after dilution in Plate reader)

Blank			KT1144 w/o mutant	Reporter w/o mutant	KT1144 w/ mutant	Reporter w/ mutant	R0040 + GFP	R0040 +GFP 1/10	R0040 +GFP 1/50	R0040 +GFP 1/100	Blank:	5411.0
	1	2	3	4	5	6	7	8	9	10	11	12
A		0	810	8492	4219	17022	70495	26431	6818	2727		

Figure 11. Levels of GFP expression of cultures of cells containing plasmids of J13002-luxOD47E-B0015 and the reporter circuit (*qrr4* promoter (BBa_K131017) with a gene encoding GFP (Bba_I13500)) as well as cultures of cells containing plasmid of J13002-luxOD47E-B0015 in KT1144 cosmids containing the *qrr4* promoter and a gene encoding for GFP. Measurements were taken with a HT Synergy plate reader. R0040+GFP was a positive control circuit containing a constitutive promoter and a gene encoding for GFP. It was used to generate high expression levels of GFP as a means of comparison.