Abstract

Title of Document: Bacterial Cross Talk in Mixed Culture

Christina Kenny Giblin

Directed by: Dr William Bentley, Professor
Department of Bioengineering

Quorum sensing is the term used to describe signaling pathways used by bacteria for intra and interspecies communication. Autoinducer 2 (AI2) is one signal molecule known to be involved in quorum sensing that stands out because it appears to be expressed across a wide variety of bacterial species. A genetic switch has been created exploits the AI2 quorum sensing pathway by turning on a target marker gene, green fluorescent protein, in the presence of AI2. The purpose of this investigation was to characterize this genetic switch and to investigate its behavior in mixed culture. Specifically to see if *E. coli* can respond to AI2 from other *E. coli* cells. It was found that the switch could be turned on by synthetic AI2 and AI2 being made by other cells growing in mixed culture as well by autoexpression.

Bacterial Cross Talk in Mixed Culture

by

Christina K. Giblin

Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park in partial fulfillment
of the requirements for the degree of
Master of Science
2007

Advisory Committee:

Professor William E. Bentley, Chairman/Advisor
Professor Richard V. Calabrese
Professor Kyu Yong Choi

Acknowlegements

I would like to thank Dr William Bentley, my advisor, for his guidance and support. I would also like to thank the members of the Bentley lab, especially Chen Yu Tsao for his guidance, help running experiments and for use of his cell lines. Finally, I would like to thank my committee: Dr Richard Calabrese and Dr Kyu Yong Choi.

List of Tables

List of Figures

Chapter 1: Introduction

1.1 Quorum Sensing

Bacterial cells communicate via small signaling molecules in a process known as quorum sensing. Winzer et al[1] proposed specific criteria that distinguished signal molecules from other bacterial metabolites. First, production of the molecule must occur during a specific growth stage, under specific physiological conditions or environmental stimuli. Second, that the signal molecule accumulates in the supernatant and the cell membrane has a receptor for it. Third, that the signal molecule, above a threshold concentration leads to a coordinated response in the cell population and fourth that the response produced involves something other than breaking down the molecule.[1]

Quorum sensing regulates many different cellular processes that depend on the cells acting in concert. Some processes that have been studied include biofilm formation, bioluminescence and motility. Gram positive bacteria use secreted peptides while Gram negative bacteria, like *Escherichia coli*, use small chemical molecules, usually an acetylated homoserine lactone (AHL), as their autoinducers.[2, 3] Quorum sensing acts as a function of cell density giving it potential to be exploited for applications as diverse as recombinant protein synthesis and the discovery of novel antibiotics. Producing recombinant protein is stressful to cells and can slow cell growth. If production can be delayed until a higher cell density is reached, the ultimate yield may be higher. A tunable system, in which initiation of recombinant protein production is controlled by conditions set by the designer of the system, is one goal of metabolic engineers trying to optimize protein production. In the development of new drugs to fight drug resistant strains,

quorum sensing pathways can be manipulated to disrupt cell to cell communication in harmful bacteria. There are currently no such drugs available.

AHL autoinducers are used by gram negative bacteria to communicate. They are derived from S-adenosyl methionine (SAM) and act using a basic "LuxI/LuxR" system. LuxI-like proteins synthesize specific AHLs, which diffuse out of the cell and accumulate extracellularly. At high AHL concentration, LuxR-like proteins bind to the AHL and the LuxR-AHL complex then binds to a target gene promoter, activating transcription. While Gram negative bacteria use this basic "LuxI/LuxR" system, they have added additional regulatory mechanisms that tailor the quorum sensing to their specific needs allowing AHL quorum sensing to control a wide variety of cell behaviors including expression of virulence factors, biofilm formation and expression of genes required for mating between bacterial cells.[4]

Gram positive bacteria communicate via oligopeptides that are transported out of the cell with an ATP binding cassette transporter complex (ABC). The oligopeptides are detected extracellularly by a sensor kinase when they accumulate to a certain level, causing a phosphorylation/dephosphorylation process in which a phosphorylated regulatory protein ultimately stimulates expression of target genes.[4]

1.2 AI2: The Signal Molecule and Pathway

While quite a few signal molecules have been discovered, Autoinducer 2 (AI2) stands out as being particularly useful as it has been found in a broad range of both Gram positive and Gram negative bacteria. It has been proposed that AI2 is involved in interspecies communication and that it can operate on a multicellular level.[3] As a

universal signal molecule shared by a wide variety of bacteria types, research on AI2 and its pathway can be used to tackle a diverse range of problems. AI2 can be used to manipulate bacterial behavior while knowledge of the signal pathway can be used to disrupt harmful biofilm formation.[5, 6] Delisa et al[7, 8] investigated regulation of gene expression in *Escherichia coli* cells in response to AI2. DNA microarrays showed that 5.6% of the genes in *E. coli* were either upregulated or down regulated including genes involved in biofilm formation, cell motility, signal transduction, small molecule metabolism, DNA replication and cell division.[7, 8]

AI2 production is dependent on the LuxS enzyme. LuxS cleaves the AI2 precursor, S-ribosylhomoserine into homocystein and a molecule that shows AI2 activity.[1] Cells with a *luxS* deletion (MDAI2 in this study) do not exhibit AI2 activity confirming the necessity of LuxS in the AI2 biosynthesis pathway. LuxS "knockout" cells can be used to investigate cell response under controlled AI2 stimulation.

The LuxS responsive operon (*lsr* operon) controls the expression of the following seven genes: *lsrA, lsrB, lsrC, lsrD, tam, lsrR* and *lsrK*. The *lsr* operon is upregulated when phosphorylated AI2 binds to the operon repressor, LsrR. The general pathway of AI2 is as follows. Once AI2 is synthesized by LuxS, it may be secreted and accumulate extracellularly. Basal expression of the *lsr* operon yields low levels of the Lsr transporter (LsrACDB) complex which uptakes AI2. AI2 that is either already intracellular or has been imported by the Lsr transporter is phosphorylated by LsrK. Phospho-AI2 is able to block the Lsr repressor. Once the *lsr* operon is derepressed, more Lsr transporter can be produced and more extracellular AI2 is taken up by the cell. LsrF and LsrG break down phospho-AI2.[9]

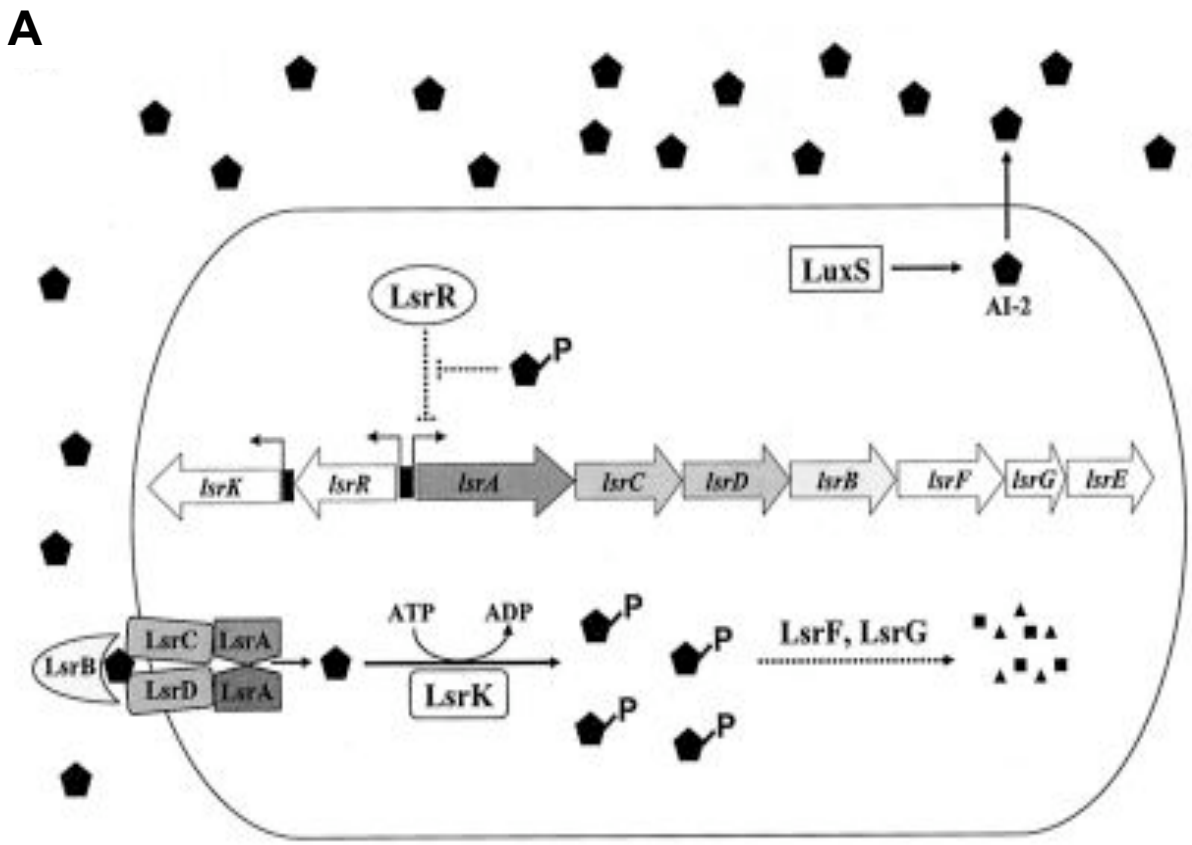

Figure 1: This diagram shows the proposed pathway of AI2 (pentagons). AI2 is synthesized by LuxS and diffuses out of the cell. At high extracellular concentrations, AI2 is transported back into the cell by the Lsr Transporter complex where it is phosphorylated by LsrK. Phospho-AI2 inhibits LsrR, derepressing the *lsr* operon. Excess phospho-AI2 may be degraded by LsrF and LsrG. Diagram from Xavier *et al*.[9]

1.3 *Vibrio harveyi*

Vibrio harveyi is a Gram negative, bioluminescent bacteria that has been extensively studied by the Bassler group.[2, 4-6, 9-15] *V. harveyi* has been found to have parallel quorum sensing circuits that regulate its bioluminescence. Mok, et al[11] clarified the mechanism by which AI-1 and AI2 work "synergistically" in *V. harveyi*. They proposed that AI-1 is for intra-species signaling while AI2 is responsible for interspecies signaling, allowing *V. harveyi* to distinguish between different environmental situations. This observation is supported by the fact that AI-1, an AHL signal molecule, is highly specific and has not been found outside the *Vibrio* species. AI2, however, has been found across a wide variety of Gram positive and negative bacteria and is increasingly being considered a universal signal molecule.[11]

AI2 in *V. harveyi* fits the signal molecule criteria from Winzer et al.[1] First, it is produced during a specific growth stage: logarithmic growth. Second, it accumulates extracellularly. Third, it triggers a coordinated response, bioluminescence, through a signal cascade, once it reaches a threshold concentration. Finally, the response, bioluminescence, is something that goes beyond metabolizing the signal molecule.[1]

Surrette et al[12] have developed an assay through their research to detect AI2 activity. Filtered conditioned media is added to the *V. harveyi* strain BB170. If AI2 is present, bioluminescence is detected. If no AI2, or low levels of AI2 are present the bioluminescence does not increase relative to the negative control, fresh media.[12] The drawback of their assay is that it is not quantifiable, so only relative levels of AI2 and trends in AI2 activity over time can be determined.

1.4 Escherichia coli

The presence of AI2 expression in *Escherichia coli* was discovered by Surette and Bassler using *V. harveyi*.[12] They found that the extracellular activity of AI2 varied with the growth phase of *E. coli*.[5, 12] Previous research in our lab studied the effect of environmental stimuli on AI2 levels. *E. coli* is commonly used in bioengineering research because it has been thoroughly studied, is cheap and fast to grow and easy to manipulate genetically. These factors make it a prime candidate for developing a novel system that exploits the AI2 quorum sensing pathway. Possibilities for such a system could include one that uses the AI2 signal to turn on a recombinant target protein or interrupting quorum sensing and subsequently blocking biofilm formation. Once such applications have been developed in a well studied system such as *E. coli* they can be expanded to other types of bacteria. Since such a wide variety of bacteria possess AI2 signal capabilities, potential applications could have high impact. In this research project, AI2 was used as a signal molecule to turn on a genetic switch, producing a target protein.

1.5 The Genetic Switch System

Based on our laboratory's latest research about quorum sensing in *E. coli*, we have constructed a genetic switch system using the native AI2 quorum sensing circuit. The objective of this design was to have a target protein produced in response to AI2, that is, switched on or off by the presence or absence of the AI2 quorum sensing molecule. The T7 RNA polymerase gene (T7RP) was inserted downstream of the *lsr* operon. The target protein, green fluorescent protein (GFP), was inserted into a second plasmid

downstream from the T7 promoter. These two vectors were cloned into two strains of *E. coli*: a wild type (W3110) and a LuxS "knockout" (MDAI2) that is not capable of producing AI2 and is therefore missing the key piece of the quorum sensing circuit. The layout of the two plasmids is shown in Figure 2. The bacterial strains and plasmids used to create this system are listed in Table 1.

In the off state, the Lsr repressor (LsrR) is bound to the *lsr* promoter blocking transcription of T7RP and subsequently GFP (Figure 3). When phospho-AI2 inactivates LsrR, derepressing the *lsr* operon, T7RP is produced. T7RP induces the T7 promoter leading to the expression of the target gene (Figure 4). Therefore, the expression of GFP is indirectly regulated by the AI2 quorum sensing pathway.

Table 1: Bacterial strains and plasmids used in this study.[16]

Strain/plasmid	Relevant genotype and property	Source or reference
Strains		
E. coli		
W3110	K12 strain, wild type, λ^-, F^-, IN(*rrnD-rrnE*)1, *rph-1*s	Genetic Stock Center Yale University, New Haven, CT
MDAI2	W3110 *luxS*::Tc^r W3110-derived *luxS* mutant strain	(DeLisa et al. 2001a)
BL21	B strain, F^-*ompT [dcm][lon]hsdS*($r_B^-M_B^-$)*gal*	Novagen
V. harveyi		
BB170	BB120 *luxN*::Tn5 (sensor 1^- ,sensor 2^+), Km^r	(Bassler et al. 1993)
Plasmids		
pCT5	pFZY1 derivative, containing *lsr* promoter fused with *T7RPol*, Ap^r	This study
pCT6	pFZY1 derivative, containing *lsrR* and *lsrR* promoter region fused with *T7RPol*, Ap^r	This study
pET200/GFP	pET200 derivative, containing *gfpuv*, Km^r	This study
pTrcHis-LuxS	pTrcHisC derivative, containing *luxS* from W3110, Ap^r	(Barrios et al. 2006)
pTrcHis-Pfs	pTrcHisC derivative, containing *pfs* from W3110, Ap^r	(Barrios et al. 2006)

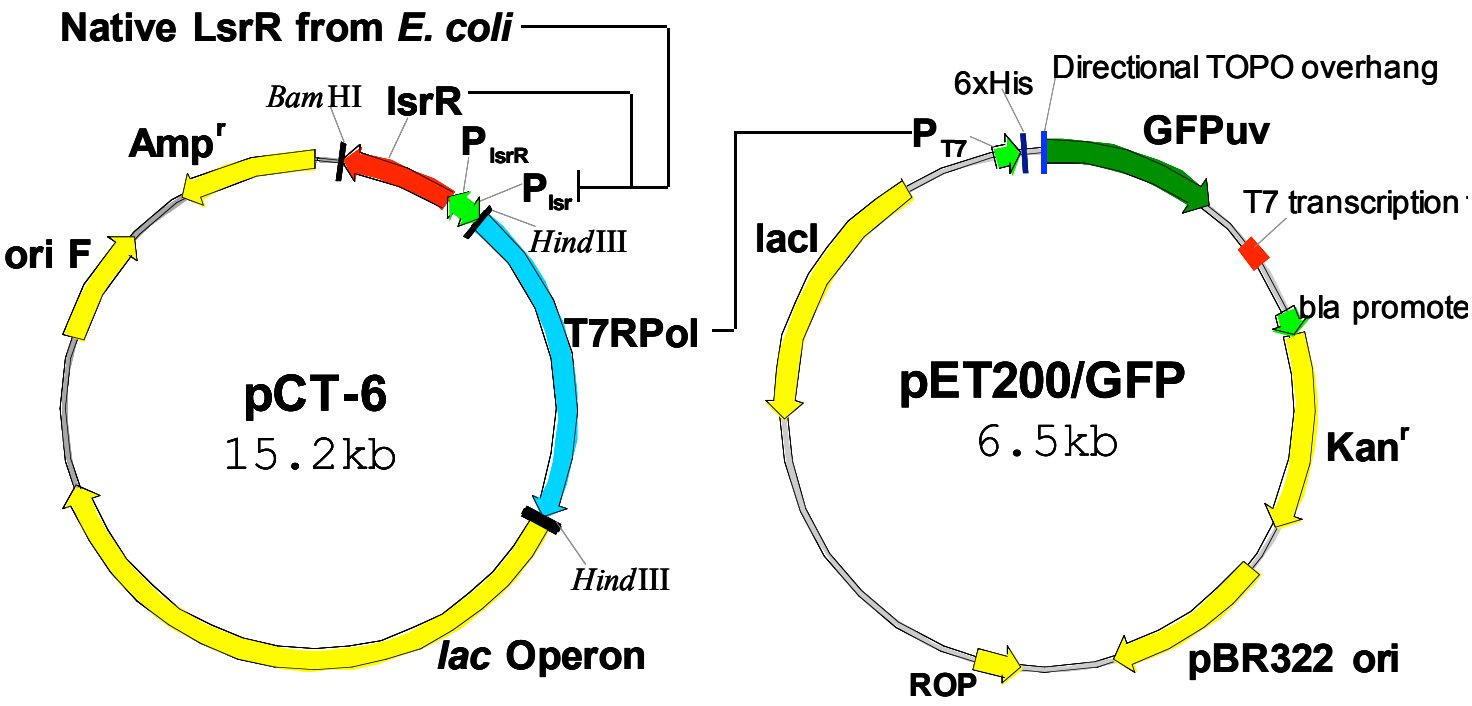

Figure 2: The lay out of the two plasmids in the genetic switch system. Key features: plasmid one has the T7RP gene downstream of the *lsr* operon and includes ampicillin resistance ensuring incorporation of the plasmid into transfected cells. Plasmid two has the GFP gene downstream of the T7 promoter and includes Kanamycin resistance.[16]

Figure 3: The OFF State- In the off state, LsrR blocks the transcription of the lsr operon, preventing transcription of T7RP. If no T7RP is produced the second plasmid, remains silent and the target protein, GFP is not produced. (Adapted from Tsao).[16]

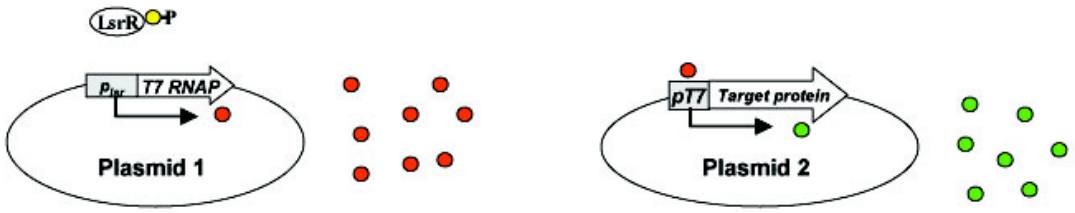

Figure 4: The ON State- In the on state, Phospho-AI2 binds to the LsrR, freeing the lsr operon. T7RP is transcribed and activates the T7 promoter. The target protein, GFP is produced. (Adapted from Tsao). [16]

1.6 Project Motivation

The motivation for this research is threefold. First, to characterize the genetic switch system that has been cloned into the two strains of *E. coli*. This unique inducible system is controlled by a naturally occurring molecule, AI2. Other inducible systems may use molecules like Isopropyl β-D-1-thiogalactopyranoside (IPTG), which has to be added to the culture at a specific growth phase to induce recombinant protein production. AI2 is known to act in a density dependent way, eliminating the need to monitor cell density and manually induce recombinant protein production.

Second is to investigate cross talk in mixed culture. It has already been determined that some *V. harveyi* strains can respond to signal molecules of other bacterial species. In this investigation, the genetic switch system is used to determine if *E. coli* can respond to an AI2 signal from other *E. coli* cells. If it can, then the investigation can be expanded to see if it can respond to AI2 from other bacterial species. To date, no reports exist on the signaling between different *E. coli* strains

Third is to develop a novel method to detect and quantify AI2. The current way of detecting AI2 is through an AI2 activity assay with *V. harveyi* BB170 using filtered conditioned media. If the genetic switch system works it could be used to test for AI2 directly, without having to filter or otherwise process samples. Flow cytometry could be used to quantify the degree of fluorescence from the target protein GFP and relate that back to the presence and amount of AI2. The ultimate goal of this project is to learn more about this genetic switch circuit that has been developed by our laboratory and to investigate potential applications for it.

It is hypothesized that in the presence of AI2, the *lsr* promoter can be induced and turn on the genetic switch. W3110 (pCT6 + pET200 GFP) will auto-express GFP while the MDAI2 (pCT6 + pET200 GFP) strain will not express GFP unless AI2 has been added to the growth media. It is also predicted that W3110 cells grown in mixed culture with MDAI2 (pCT6 + pET200 GFP) will produce enough AI2 to turn the genetic quorum sensing switch on and that adding MDAI2 to MDAI2 (pCT6 + pET200 GFP) will have no effect. These would confirm previous preliminary research conducted by Tsao[16] and show that *E. coli* is capable of communicating with other *E. coli* strains.

Chapter 2: Materials and Methods

2.1 Overview

The general setup of these experiments was a follows. A flask was inoculated with either wild type or LuxS "knockout" cells with the genetic switch cloned in. In some cases, synthetic AI2, conditioned media or a second strain of *E. coli* was also added. Hourly samples were taken and used for finding the optical density (to measure growth rate) and for taking images which were later counted. These samples were also spun down. Total mRNA was extracted from the cell pellet so that levels of T7RP and GFP mRNA could be measured and an AI2 activity assay was performed using the conditioned media.

2.2 Cells and Cell Maintenance

The bacterial strains and plasmids used in this study are listed in table 1. All *E. coli* derived cells were grown in Luria Broth (25g LB powder, 1L water). All *V. harveyi* derived cells were grown in AB media as described by Greenberg et al.[17]

Overnight cultures of MDAI2 (LuxS knockout) cells had 0.4μL/mL ampicilin and 5μL/mL kanamycin. One milliliter of overnight culture was seeded in 20mL of Luria Broth. The cells were placed on a shaker in a 30°C incubator. The optical density was read hourly, starting at zero hours using a spectrophotometer at 600nm wavelength. One milliliter samples were pipetted into semimicron polystyrene cuvettes. When the optical density rose above 0.4, cells were diluted 1:10 so that the OD measurements were within the linear range.

2.3 Gel Immobilization and Imaging

Cells were immobilized in a 1% agar gel. To do this, 5µL of well mixed, harvested cell sample was pipetted onto the center of a glass slide. Five microliters of melted agar was pipetted on top of the cells and they were briefly mixed, then a glass slide was immediately pressed on top while the agar was still fluid. If it was not done fast enough, the agar would split and the slide had to be redone.

The microscope used was an Olympus BX60 light microscope with an Olympus BXFLA reflected fluorescent light attachment. Images were taken with a Canon EOS-D60 digital camera under ultraviolet light with a 30 second exposure time and under white light using an automatic exposure.

2.4 Counting

The UV and white light images were counted manually using Image J (downloadable from http://rsb.info.nih.gov/ij). Each image was counted twice and the results were used to calculate the fraction of fluorescing cells.

2.5 *In vitro* AI2 Synthesis

AI2 was made *in vitro* and used in these experiments to eliminate cell effects in turning on the *lsr* promoter. Cultures of BL21 (pTrcHis-pfs) and BL21 (pTrcHis-LuxS) cells were grown at 37°C until they reached and OD_{600} of between 0.4 and 0.6 and were induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were harvested at 4.5 hours post induction by centrifugation at 14,000xg for 20 minutes at 4°C. Cells were lysed with BugBuster (Novagen) at room temperature for 40 minutes. The soluble cell

extracts were mixed with Co^{2+} affinity resin (BD Talon™, BD Biosciences) and the bound His6pfs and His6LuxS were washed three times with phosphate buffer (pH 7.4) to remove non-specifically bound proteins. The resin with immobilized Pfs and LuxS enzymes were mixed with 1mM S-adenosylhomocyteine (SAH) and incubated on a 37°C shaker for 6 hours. The enzymatic product was extracted using chloroform and recovered from the aqueous phase. The activity of the extracted product was confirmed by an AI2 assay where it was compared to a previously tested positive control.[2, 18]

2.6 AI2 Assay

An AI2 assay, using *Vibrio harveyi* BB170, was used to determine relative AI2 concentrations. As mentioned earlier, the main drawback is that this assay is not standardized and therefore is not quantitative. It is, however, useful to help elucidate trends in AI2 concentration over time and compare relative AI2 levels between individual samples

First, 20µL of filtered, conditioned media was pipetted into 5mL tubes. A negative control of LB and a positive control of a previous reaction with known AI2 were used to ensure the cells were active and to set a zero point.

A 1:5000 dilution was made of overnight BB170 culture and AB media. This was added to the 20µL sample for a final volume of 200µL. Samples were incubated at 30°C on a shaker for 3 hours. At the third hour, the amount of light being produced by the samples was read using a luminometer (EG&G Berthold). This reading was repeated every 30 minutes through the fifth hour. This method follows the one outlined by Surrette et al.[12]

2.7 mRNA Extraction

mRNA was extracted using the RNAqueous Kit (Ambion) according to the manufacturer's instructions. One milliliter of cell culture was harvested based on the cell density. The total RNA concentration was determined by measuring the absorbance of a 1:40 diluted sample at 260 nanometers using a spectrophotometer.

2.8 mRNA Quantification: Reverse Transcription PCR

cDNA was synthesized via reverse transcriptase PCR from 300ng of total RNA and random hexamers. The cDNA template was then amplified by PCR using gene specific primers for either GFP or T7RP (primers listed in Table 2). These PCR products were run on a 1% agarose gel and the band intensities were compared. The specific protocols for RT PCR and PCR that were followed are those recommended by Invitrogen for their "Superscript III First Strand Synthesis System for RT PCR" and "Accuprime Taq DNA Polymerase High Fidelity" respectively.

2.9 Mixed Culture Experiment: Investigating Cross Talk

For the mixed culture experiment 1 mL of MDAI2 (pCT6 + pET200 GFP) overnight culture was mixed with 1 mL of either MDAI2 or W3110 overnight cultures in a total of 20 mL LB. MDAI2 (pCT6 + pET200 GFP) was mixed with MDAI2 to eliminate the possibility that the circuit may be effected by the presence of other cells in their culture. The experiment was run for five hours at which point excessive leaky expression of GFP was observed in the negative control.

2.10 Supernatant Experiment

The mixed culture experiment was repeated using five hour, filtered, conditioned media obtained from MDAI2 and W3110 cells. Wang et al[3] showed that AI2 activity in W3110 cells reaches a peak around five hours. For this experiment the maximum possible amount of AI2 was desired. MDAI2 supernatant was used as a negative control to eliminate the possibility that the circuit may be effected by other extracellular metabolites that are found in spent media. For this experiment, MDAI2 (pCT6 + pET200 GFP) cells were grown in 5 mL of filtered conditioned media mixed with 15 mL of fresh LB.

Chapter 3: Results and Discussion

3.1 Characterizing the Quorum Sensing Circuit

In order to learn more about the genetic switch system, its behavior first needed to be characterized under the following conditions: self induction (native AI2 present), no induction (no AI2 present), and synthetic induction (AI2 made *in vitro*). The quorum sensing circuit was put into both wild type (W3110) and luxS knockout cells (MDAI2).

3.1.1 Auto-Expression: W3110 (pCT6+pEt200GFP)

To test that the circuit was working, W3110 (pCT6+pEt200GFP) cultures were examined under the conditions described in Chapter 2. Image analysis showed the self-inducing cells always fluoresced (Appendix, Figures 19-28). This is consistent with the design of the genetic switch system because the wild type cells make AI2, which turns on the T7 promoter that leads to GFP expression.

Preliminary counts showed relatively higher percentages of fluorescing cells Figure 5. Dividing cells might have lower levels of GFP and therefore may not show up on the image. The AI2 assay showed steadily increasing AI2 concentration over time, after an initial lag (Figure 6).

After three hours post inoculation, cells begin to make excess AI2, which is released into the supernatant. Figure 7 shows a initial lag in growth compared to other wild type cell strains. This result suggests that the GFP production slows growth at low cell densities by increasing the metabolic burden.

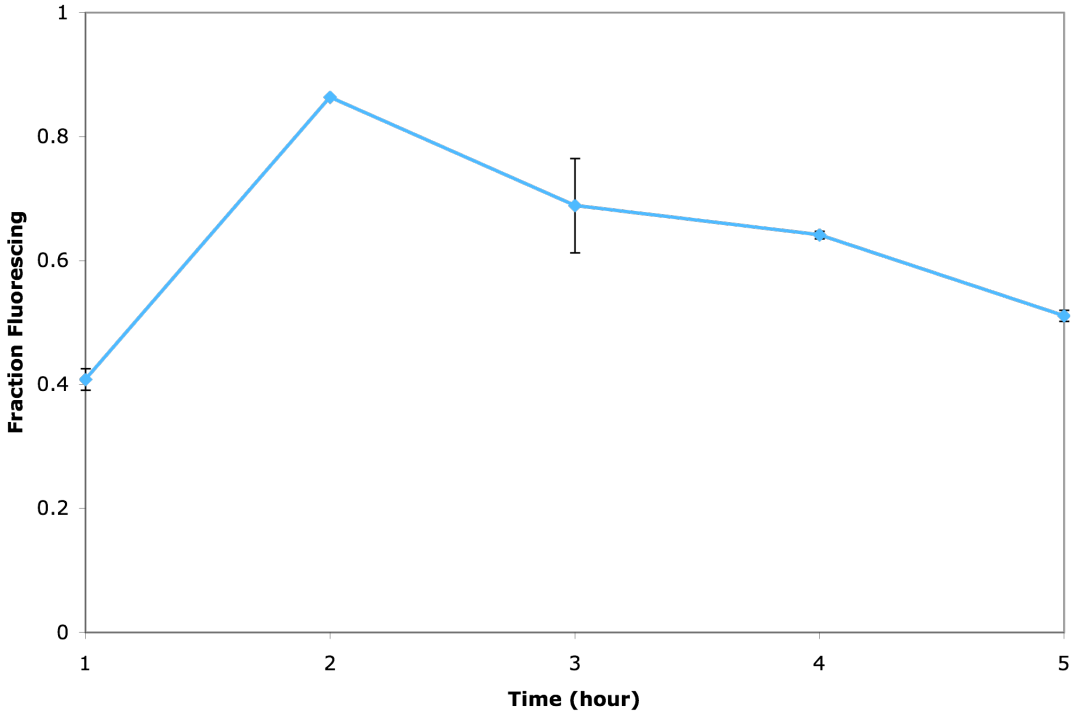

Figure 5: Fluorescence counts of W3110 (pCT6+pEt200GFP) show a fairly high degree of fluorescence

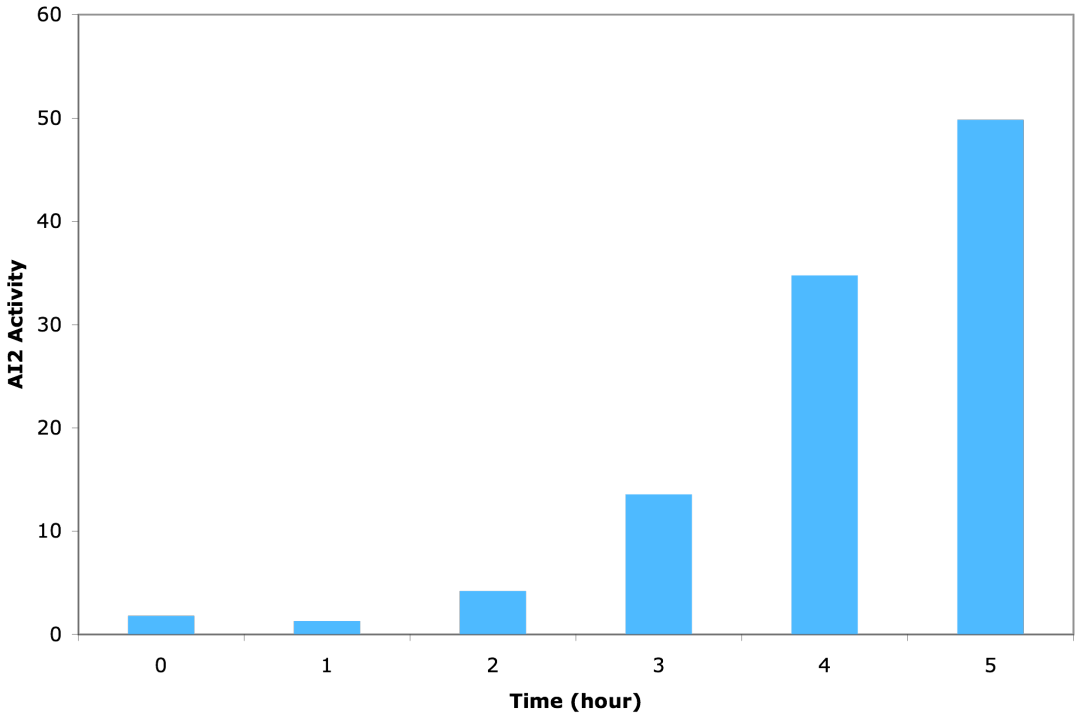

Figure 6: AI2 Assay of W3110 (pCT6+pEt200GFP) shows increasing AI2 activity with time.

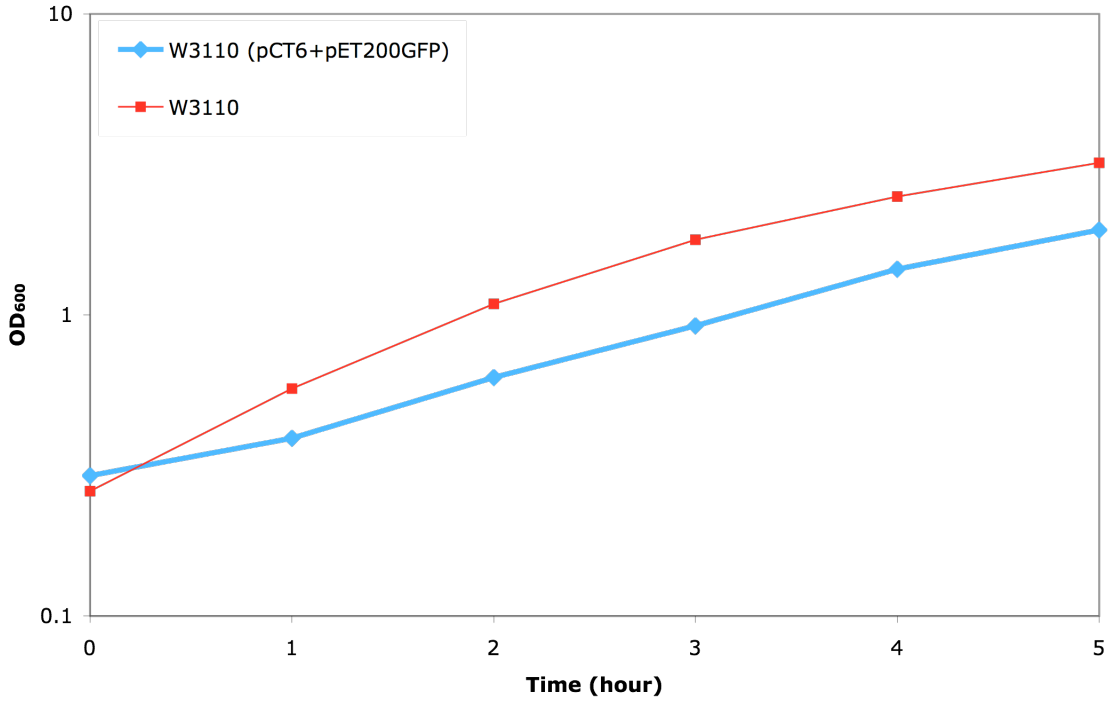

Figure 7: Growth curve of W3110(pCT6+pEt200GFP) compared to W3110 shows slower growth rate in the strain with the genetic switch.

3.1.2 LuxS Knockout: MDAI2 (pCT6+pEt200GFP)

LuxS knockout cells had the gene circuit but were not capable of producing AI2. Figure 8 shows that the growth rate of the cell line with the plasmid is close to that without the plasmid.

An AI2 assay confirmed that there was no AI2 present (Figure 9). Images showed that there was little to no GFP expression until late in the time course of the experiment. The GFP that was expressed could be explained by leaky expression in the circuit between T7RP and GFP. This was confirmed by RNA analysis. While T7RP was near zero compared to the wild type, GFP transcription for both was very high (Figure 10). This suggests that there is leaky expression of the genetic switch.

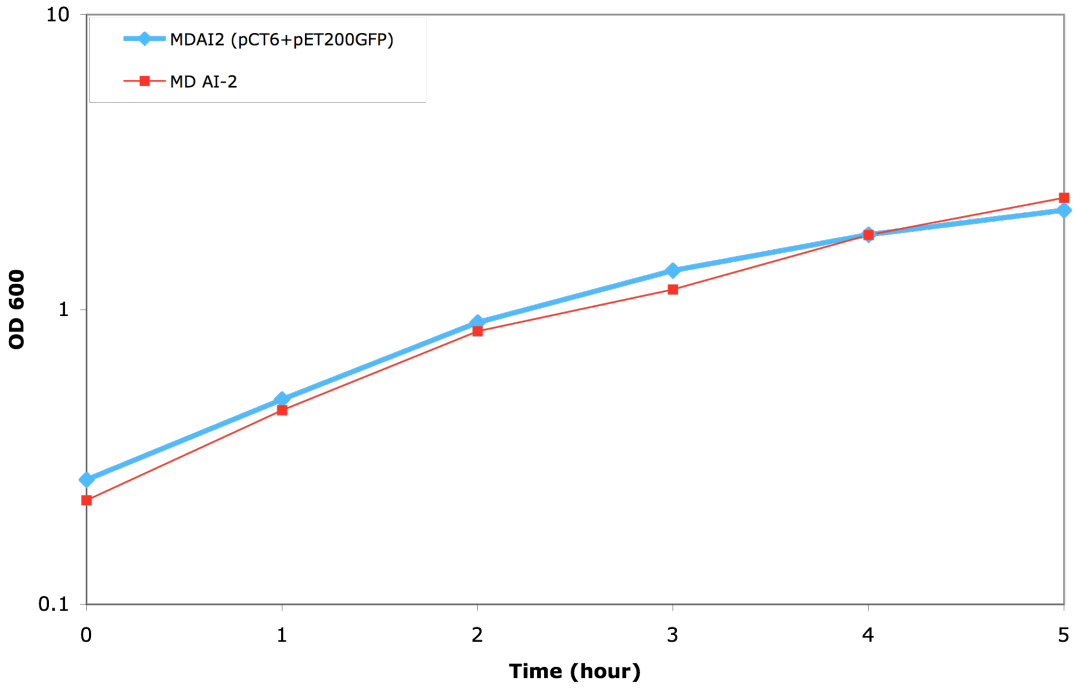

Figure 8: Growth Chart of MDAI2 (pCT6+pET200GFP) shows similar growth rate to MDAI2

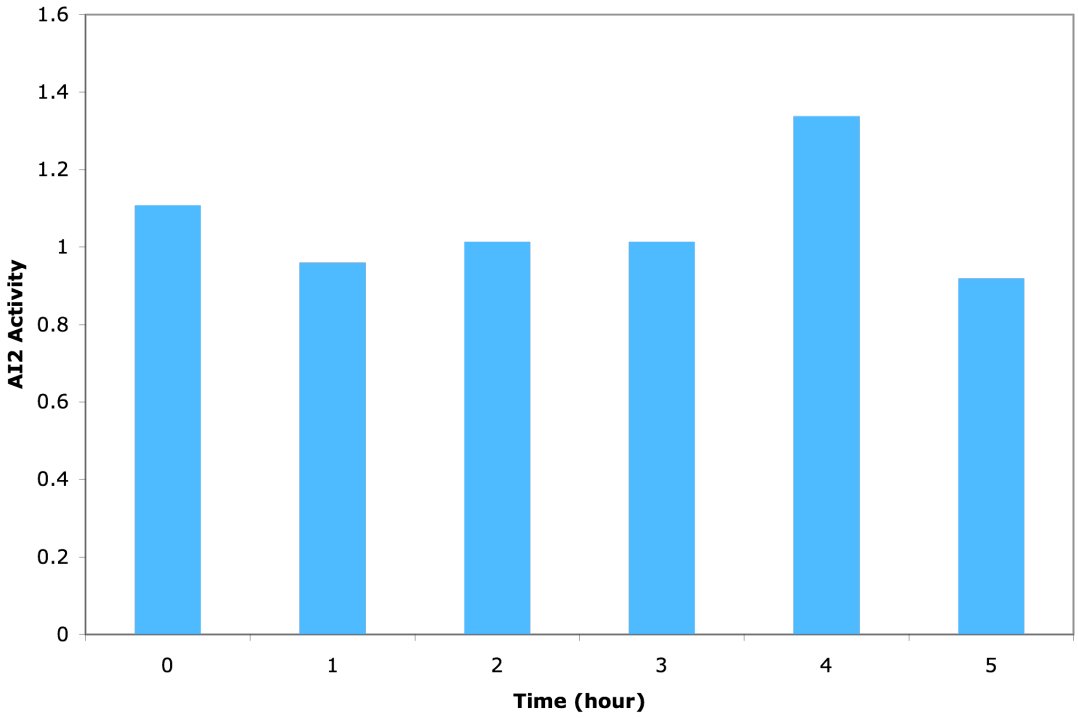

Figure 9: AI2 activity assay of MDAI2 (pCT6+pET200GFP) shows virtually no AI2 activity.

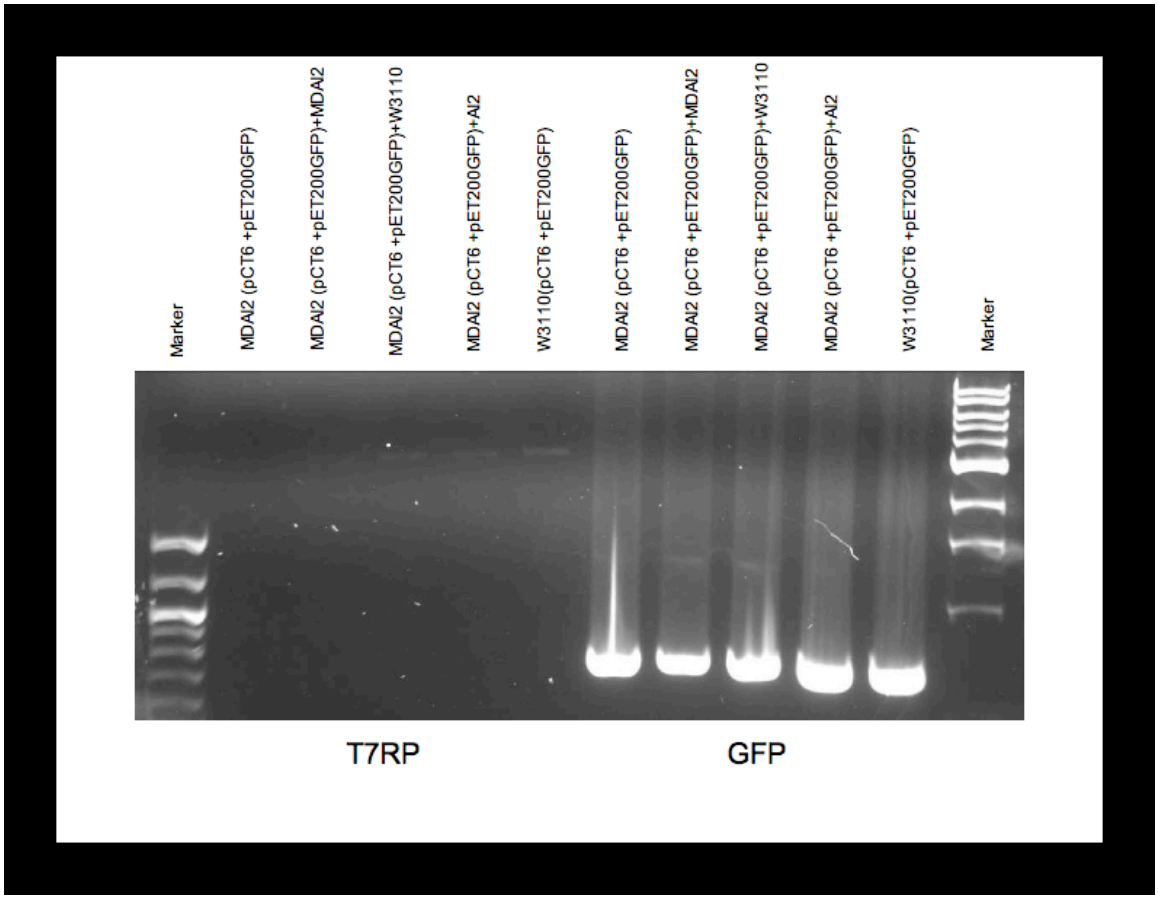

Figure 10: mRNA analysis

3.1.3 LuxS Knockout induced by Synthetic AI2: MD AI2 (pCT6+pEt200GFP)+AI2

To test the gene switch in the presence of AI2, synthetic AI2 that had been produced in vitro was introduced to the LuxS knockout culture. The growth curve was a slightly slower rate than other MD AI2 cultures, probably due to the stress on the cells to produce extra protein (Figure 11). Cells induced with AI2 fluoresced with intensity comparable to the wild type, W3110 (pCT6+pEt200GFP), after a two hour delay (Appendix, Figures 39-48). The lag is expected because once the gene is turned on, it takes time to transcribe and fold GFP.

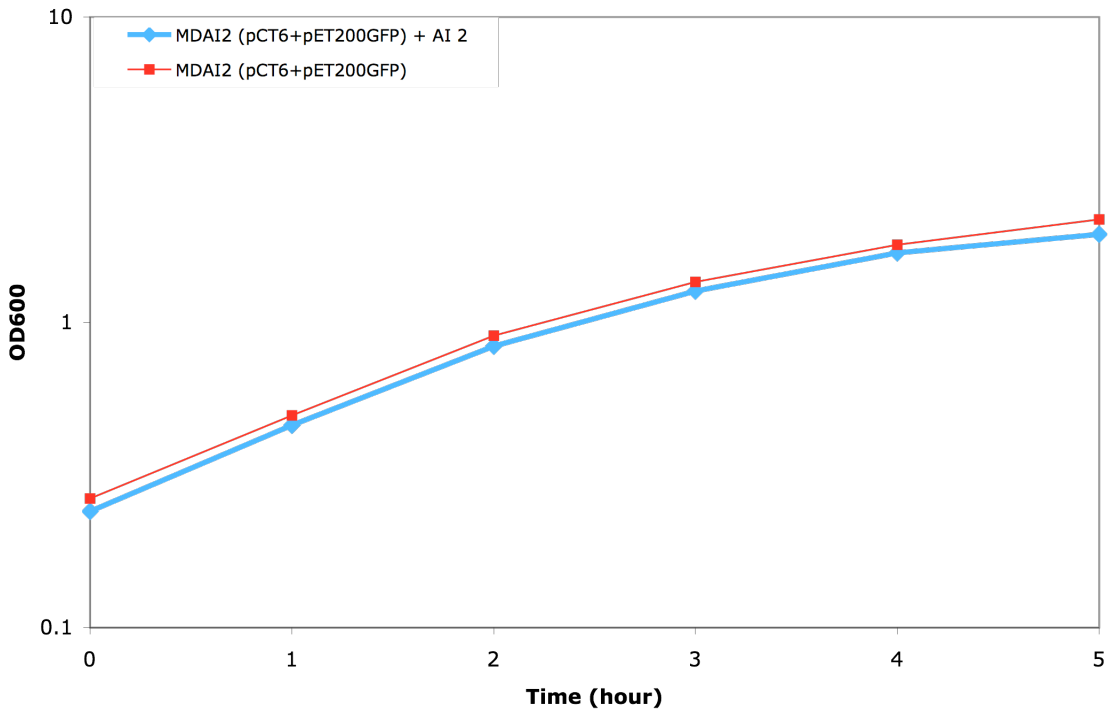

Figure 11: Growth curve comparing MDAI2 (pCT6+pET200GFP)+AI2 with MDAI2 (pCT6+pET200GFP)

The AI2 assay showed initially high levels of AI2 that decreased over the time course of the experiment (Figure 12). This supports the AI2 being taken up by the cells and turning on protein expression. The counts showed an increasing percent of fluorescing cells over time (Figure 13). The results confirm that the in vitro AI2 turns on the genetic switch. MDAI2 (pCT6+pEt200GFP) turns on sooner with AI2 present than without, that is, the genetic switch is turned on faster with synthetic AI2 than it is by leaky expression and the supernatant shows that the concentration of extracellular AI2 decreases as it is taken up by MDAI2 (pCT6+pEt200GFP).

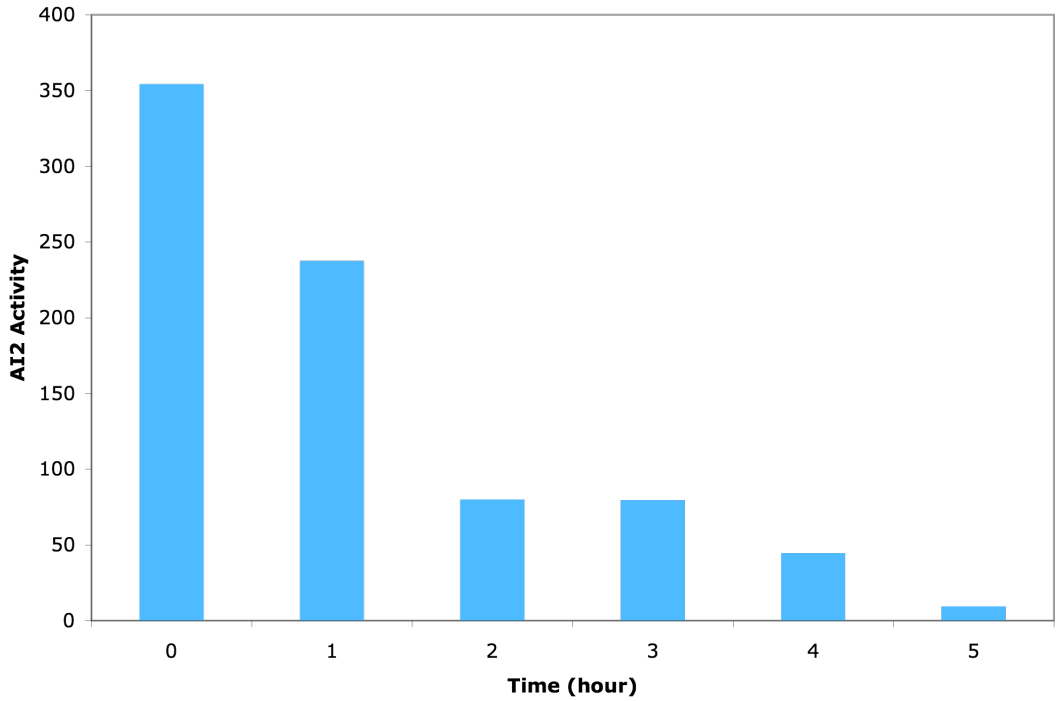

Figure 12: AI2 assay of MDAI2 (pCT6+pET200GFP) + AI2 show a decrease in AI2 activity over time, as AI2 gets taken up by MDAI2 (pCT6+pET200GFP) cells.

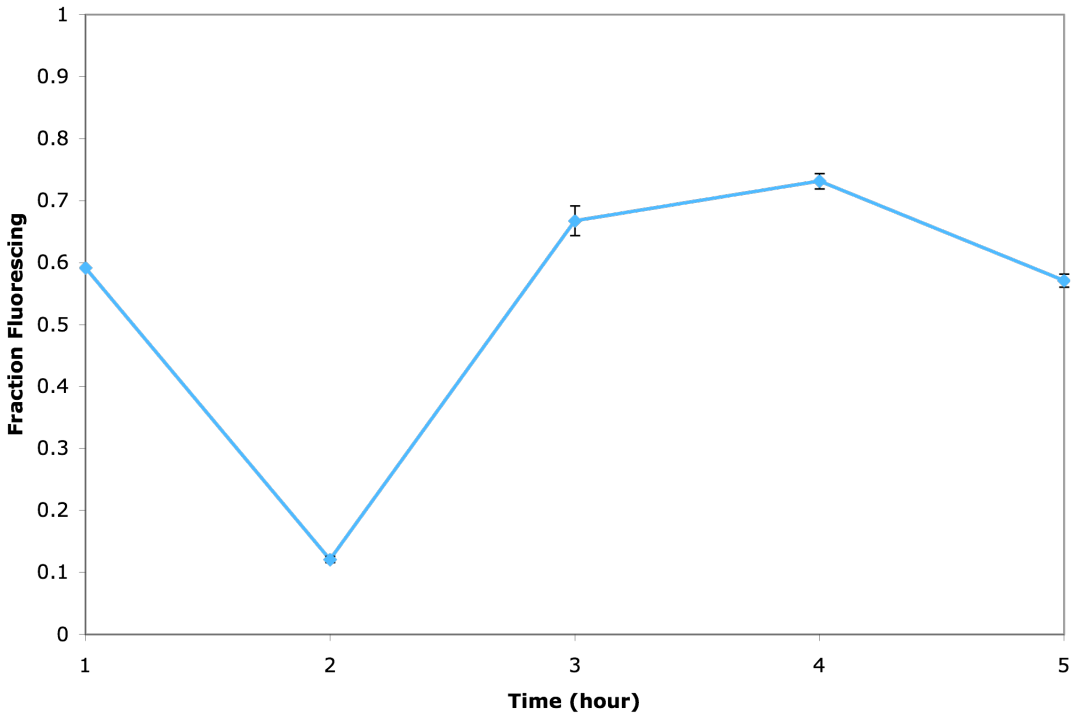

Figure 13: Fluorescence counts of MDAI2 (pCT6+pET200GFP) +AI2 show fairly high fluorescence.

3.2 Investigating Cross Talk in Mixed Culture

To determine if this is truly a communication system, MDAI2 (pCT6+pET200GFP) cells were grown in mixed culture with MDAI2 and W3110 cells respectively. It was observed that the system's response corresponds to communication via the AI2 quorum sensing circuit.

3.2.1 Mixed Culture LuxS knockout: MDAI2 (pCT6+pET200GFP) + MDAI2

The mixed culture of MDAI2 (pCT6+pET200GFP) with MDAI2 was considered as the negative control. It was used to confirm that the effect observed was not from the presence of another cell type and its metabolites but from the AI2 produced by the second cell type. This result resembled that of the knockout grown in monoculture (i.e. MDAI2 (pCT6+pET200GFP)). The AI2 assay showed similar results (figure not shown), AI2 activity was effectively zero (around that of the negative control). The images (Appendix, Figures 49-58) and counts showed that cells did not fluoresce until the fourth hour which, again, seems to be the result of leaky expression (Figure 14). This was confirmed by doing mRNA analysis (Figure 10).

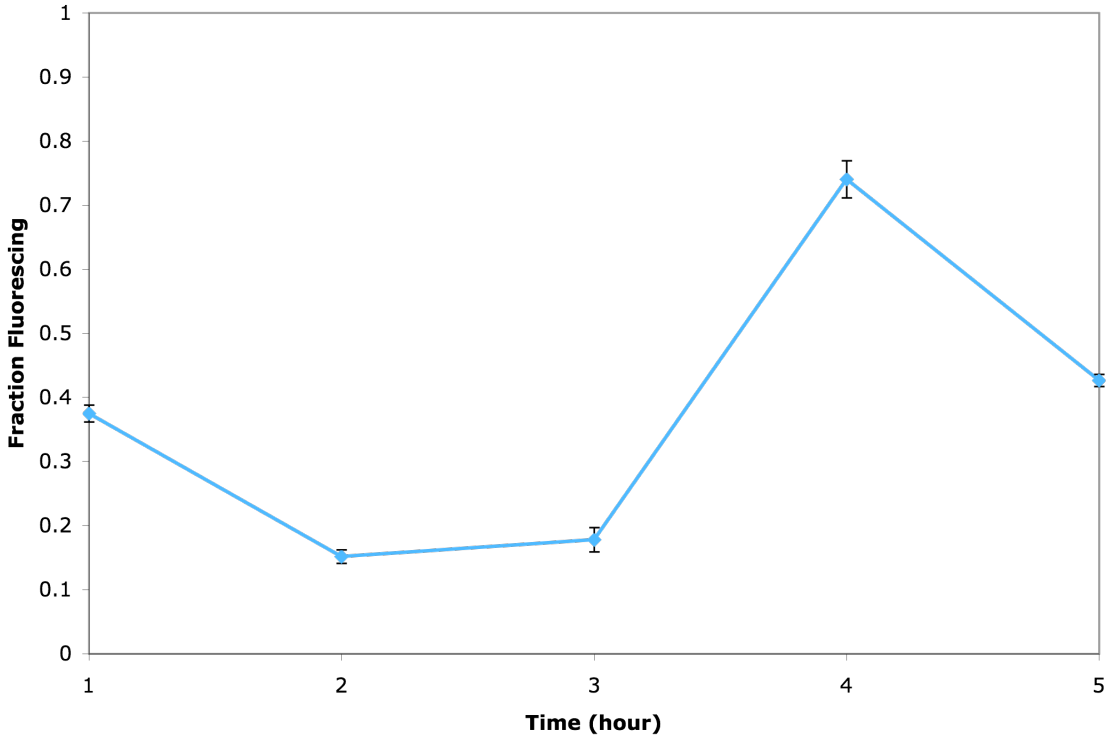

Figure 14: Fluorescent counts of MDAI2 (pCT6+pET200GFP) +MDAI2 show a jump in fluorescence at four hours, indicating the start of leaky expression.

3.2.2 Mixed Culture of LuxS knockout with Wildtype: MDAI2 (pCT6+pET200GFP) + W3110

A mixed culture of MDAI2 (pCT6+pET200GFP) and W3110 was grown to determine if AI2 communication exists between *E. coli* cell strains. The results of this experiment confirm that it does. The mix of the knockout and wild type cells led to some interesting results. The growth curve showed that while it started with around twice the optical density of W3110, by three hours, they were growing at similar densities (Figure 15). The AI2 assay was also interesting. Previous studies, by Wang et al[3] showed that in wild type cells (W3110) AI2 activity increased until about five hours when it reached a

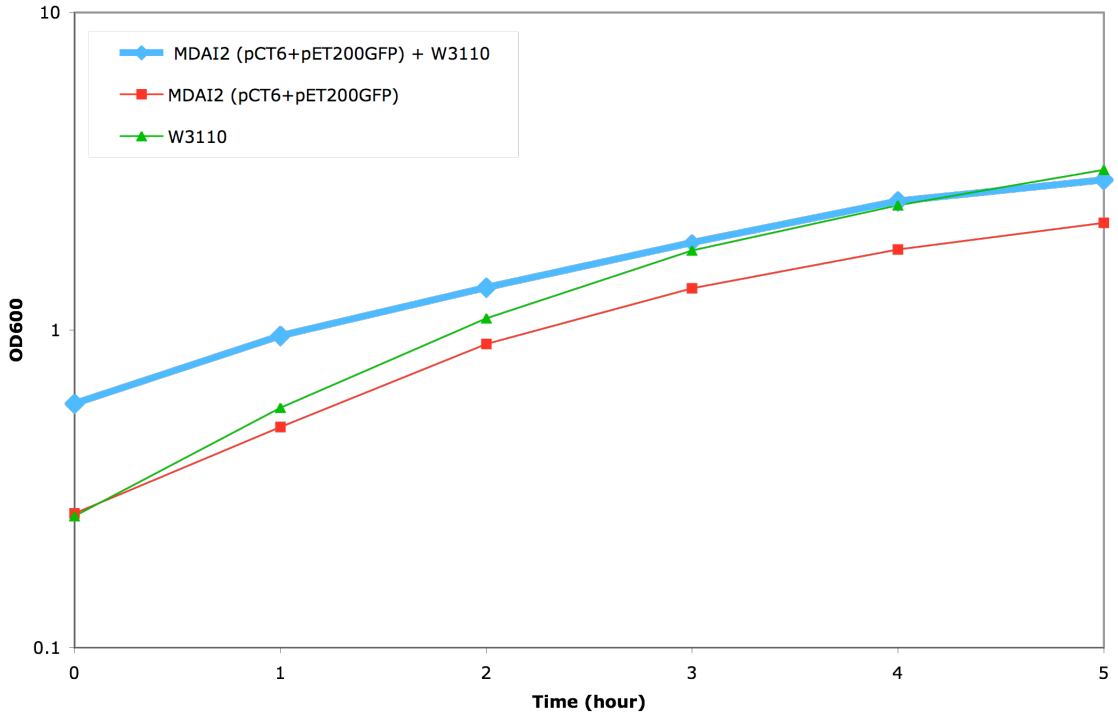

Figure 15: Growth Chart comparing MDAI2 (pCT6+pET200GFP) +W3110 to the growth rates of W3110 and MDAI2 (pCT6+pET200GFP)

peak then dropped to zero (Figure 16). He explained this phenomenon as a result of the cells uptaking the AI2 they had previously secreted as nutrients became scarce in the media.

In mixed culture, however, AI2 activity reached its peak around two hours then dropped (Figure 17). The extracellular AI2 was most likely taken up by the MDAI2 (pCT6+pET200GFP) cells and used to turn the gene circuit on using the same mechanism that took up the synthetic AI2 as described in 3.1.3. This is supported by the images (Appendix, Figure 59-68), the image counts (Figure 18) and the mRNA analysis (Figure 10). The images show cells fluorescing at 2 hours. The counts a show steadily increasing percent of fluorescing cells. The do not reach as high a percent as the in vitro

AI2 induced or self induced cells because it is a mixed culture and the wild type cells do not fluoresce. MDAI2 cells with the genetic switch can respond to AI2 from different strains of *E. coli*.

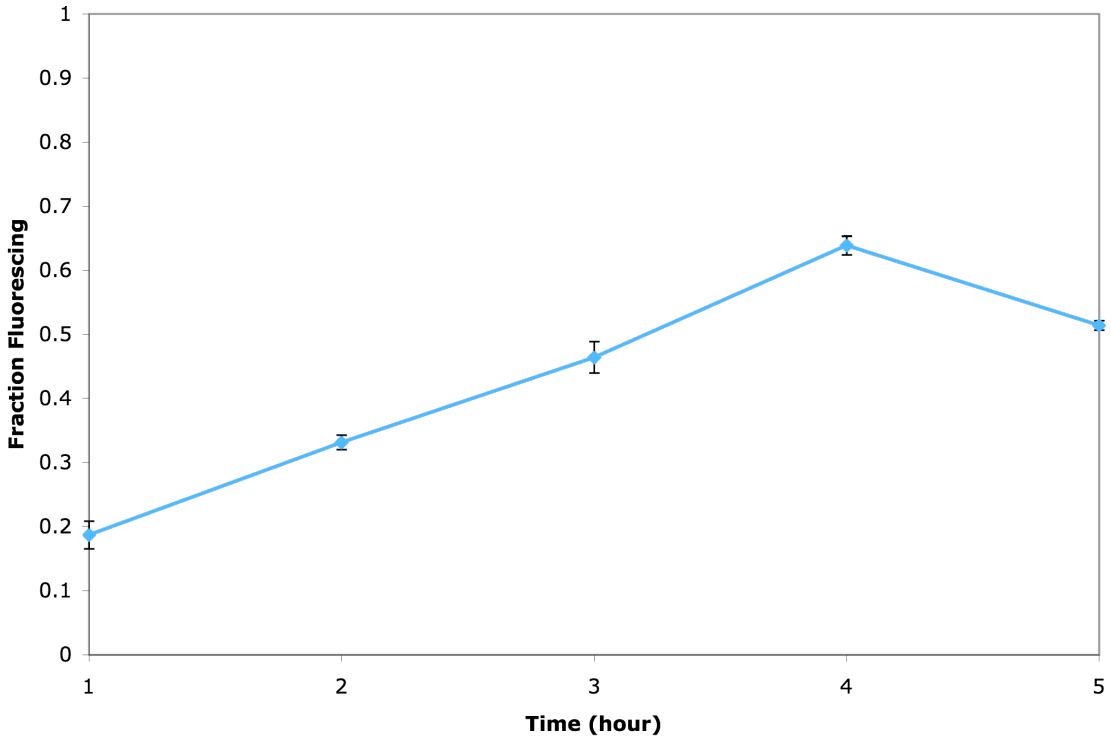

Figure 18:Fluorescence counts of MDAI2 (pCT6+pET200GFP) +W3110 show increasing fluorescence over time.

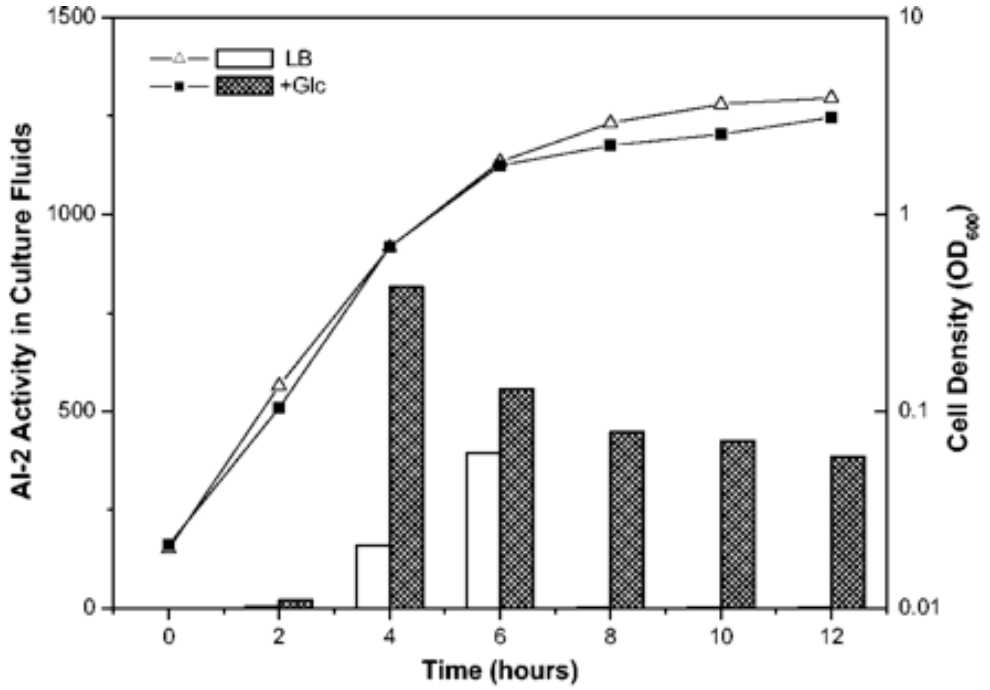

Figure 16: AI2 assay of W3110 from Wang et al shows AI2 activity in W3110 cells increases until about 6 hours then goes to zero.[3]

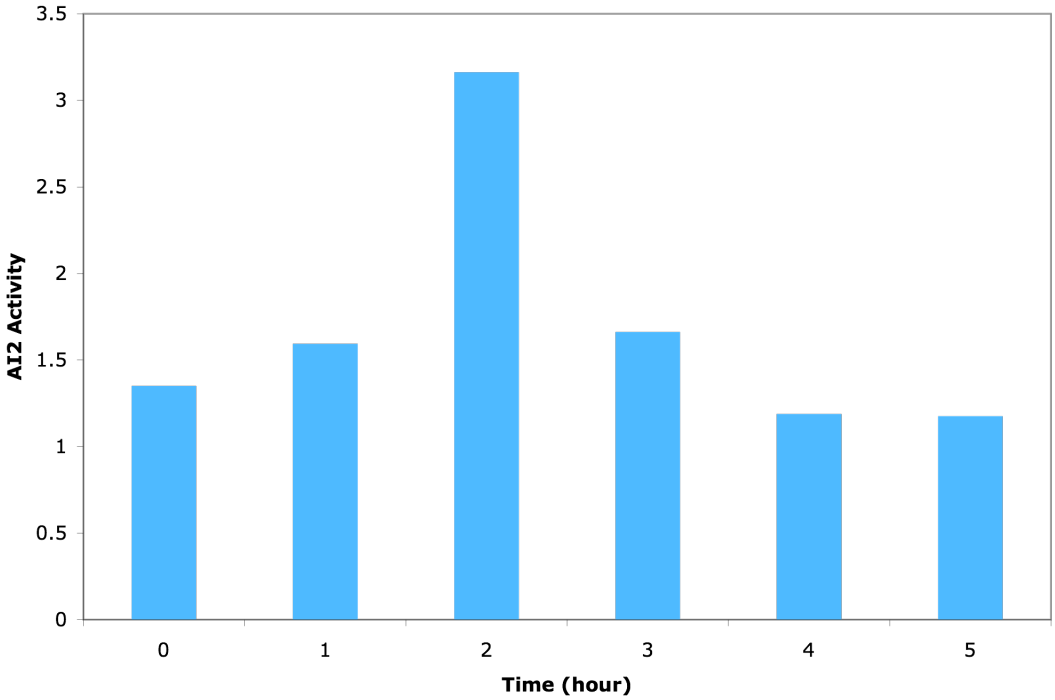

Figure 17: AI2 assay of MDAI2 (pCT6+pET200GFP) +W3110 show an increase of AI2 activity until 2 hours followed by a decrease to near zero.

3.3 Supernatant Comparison

The mixed culture experiments were repeated with five hour supernatants instead cells added to the MD AI2 (pCT6+pET200GFP). Five hour supernatants were chosen because they have the highest AI2 concentration according to Wang et al.[3] It was found that adding MDAI2 supernatant did not effect the extracellular AI2 concentration. The cells showed similar fluorescence results to those of the corresponding mixed culture with live cells. Therefore it can be concluded that the genetic circuit is not turned on by extracellular metabolites found in spent media. In the experiment where conditioned 5 hour W3110 media was added, it was found that the extracellular AI2 concentration decreased over time. This suggests that AI2 from the spent media was taken up by the MDAI2 (pCT6+pET200GFP) cells and used in the quorum sensing circuit. This result resembled that of adding in vitro AI2 to MDAI2 (pCT6+pET200GFP). It confirms that the extracellular AI2 from other E. coli strains, and not other metabolites, can turn on the quorum sensing switch.

Chapter 4: Conclusion and Future Work

The quorum sensing system investigated in this study can be used both to detect AI2 activity and to show communication by AI2 between Gram negative bacteria. In characterizing the genetic switch system it was found to respond to self made AI2 in the wild type strain, which means it is capable of autoexpression. The genetic switch also responded to synthetic AI2 added to cells that lacked the ability to synthesize AI2 (MDAI2). Most importantly, in investigating cross talk, it was found that *E. coli* is capable of responding to AI2 from other *E. coli* strains.

Future work should look into the following areas. The fraction of cells turned on by AI2 was inaccurate in mixed cultures because cells without the GFP plasmid were indistinguishable from cells with the plasmid. The supernatant experiments corrected that problem to a certain extent but it was limited in investigating cell communication that it had a fixed quantity of AI2. A membrane that is permeable to small molecules like AI2 could eliminate the cross culture mixing problem. If, say, the wild type cells were placed in dialysis tubing, AI2 could cross the membrane and turn on the circuit in MDAI2 (pCT6+pET200GFP) cells but the two *E. coli* strains would stay separate. In order to do this, a way of sterilizing the membrane such that there is no contamination between the cell types or from other microbes.

Flow cytometry could be used to improve counts of fluorescing cells and the total number of cells. This method would improve counts because it can look at a larger portion of the cell population, thereby giving a better picture of the fluorescence of the overall population. It also eliminates human error that comes from manually counting

images. Flow cytometry could also measure how brightly cells are fluorescing when they are expressing GFP.

This work focused on cross talk within one cell type, *E. coli*. Future studies should expand to investigate cross species communication and include Salmonella, *V. harveyi* and other bacteria strains that have been shown to express AI2.

References

1. Winzer, K.; Hardie, K. R.; Williams, P., Bacterial cell-to-cell communication: sorry, can't talk now - gone to lunch! *Curr Opin Microbiol* **2002,** 5, (2), 216-22.

2. Schauder, S.; Shokat, K.; Surette, M. G.; Bassler, B. L., The LuxS family of bacterial autoinducers: biosynthesis of a novel quorum-sensing signal molecule. *Mol Microbiol* **2001,** 41, (2), 463-76.

3. Wang, L.; Hashimoto, Y.; Tsao, C. Y.; Valdes, J. J.; Bentley, W. E., Cyclic AMP (cAMP) and cAMP receptor protein influence both synthesis and uptake of extracellular autoinducer 2 in Escherichia coli. *J Bacteriol* **2005,** 187, (6), 2066-76.

4. Schauder, S.; Bassler, B. L., The languages of bacteria. *Genes Dev* **2001,** 15, (12), 1468-80.

5. Surette, M. G.; Miller, M. B.; Bassler, B. L., Quorum sensing in Escherichia coli, Salmonella typhimurium, and Vibrio harveyi: a new family of genes responsible for autoinducer production. *Proc Natl Acad Sci U S A* **1999,** 96, (4), 1639-44.

6. Xavier, K. B.; Bassler, B. L., LuxS quorum sensing: more than just a numbers game. *Curr Opin Microbiol* **2003,** 6, (2), 191-7.

7. DeLisa, M. P.; Valdes, J. J.; Bentley, W. E., Mapping stress-induced changes in autoinducer AI-2 production in chemostat-cultivated Escherichia coli K-12. *J Bacteriol* **2001,** 183, (9), 2918-28.

8. DeLisa, M. P.; Wu, C. F.; Wang, L.; Valdes, J. J.; Bentley, W. E., DNA microarray-based identification of genes controlled by autoinducer 2-stimulated quorum sensing in Escherichia coli. *J Bacteriol* **2001,** 183, (18), 5239-47.

9. Xavier, K. B.; Bassler, B. L., Regulation of uptake and processing of the quorum-sensing autoinducer AI-2 in Escherichia coli. *J Bacteriol* **2005,** 187, (1), 238-48.

10. Bassler, B. L.; Wright, M.; Showalter, R. E.; Silverman, M. R., Intercellular signalling in Vibrio harveyi: sequence and function of genes regulating expression of luminescence. *Mol Microbiol* **1993,** 9, (4), 773-86.

11. Mok, K. C.; Wingreen, N. S.; Bassler, B. L., Vibrio harveyi quorum sensing: a coincidence detector for two autoinducers controls gene expression. *Embo J* **2003,** 22, (4), 870-81.

12. Surette, M. G.; Bassler, B. L., Quorum sensing in Escherichia coli and Salmonella typhimurium. *Proc Natl Acad Sci U S A* **1998,** 95, (12), 7046-50.

13. Surette, M. G.; Bassler, B. L., Regulation of autoinducer production in Salmonella typhimurium. *Mol Microbiol* **1999,** 31, (2), 585-95.

14. Taga, M. E.; Miller, S. T.; Bassler, B. L., Lsr-mediated transport and processing of AI-2 in Salmonella typhimurium. *Mol Microbiol* **2003,** 50, (4), 1411-27.

15. Taga, M. E.; Semmelhack, J. L.; Bassler, B. L., The LuxS-dependent autoinducer AI-2 controls the expression of an ABC transporter that functions in AI-2 uptake in Salmonella typhimurium. *Mol Microbiol* **2001,** 42, (3), 777-93.

16. Tsao, C. Y. Rewiring quorum sensing circuitry for recombinant protein production in *E. coli*. University of Maryland, College Park, MD, 2007.

17. Greenberg, E. P.; Hastings, J. W.; Ulitzur, S., Induction of Luciferase Synthesis in Beneckea-Harveyi by Other Marine-Bacteria. *Archives of Microbiology* **1979,** 120, (2), 87-91.

18. Barrios, A. F.; Zuo, R.; Ren, D.; Wood, T. K., Hha, YbaJ, and OmpA regulate Escherichia coli K12 biofilm formation and conjugation plasmids abolish motility. *Biotechnol Bioeng* **2006,** 93, (1), 188-200.

Appendix

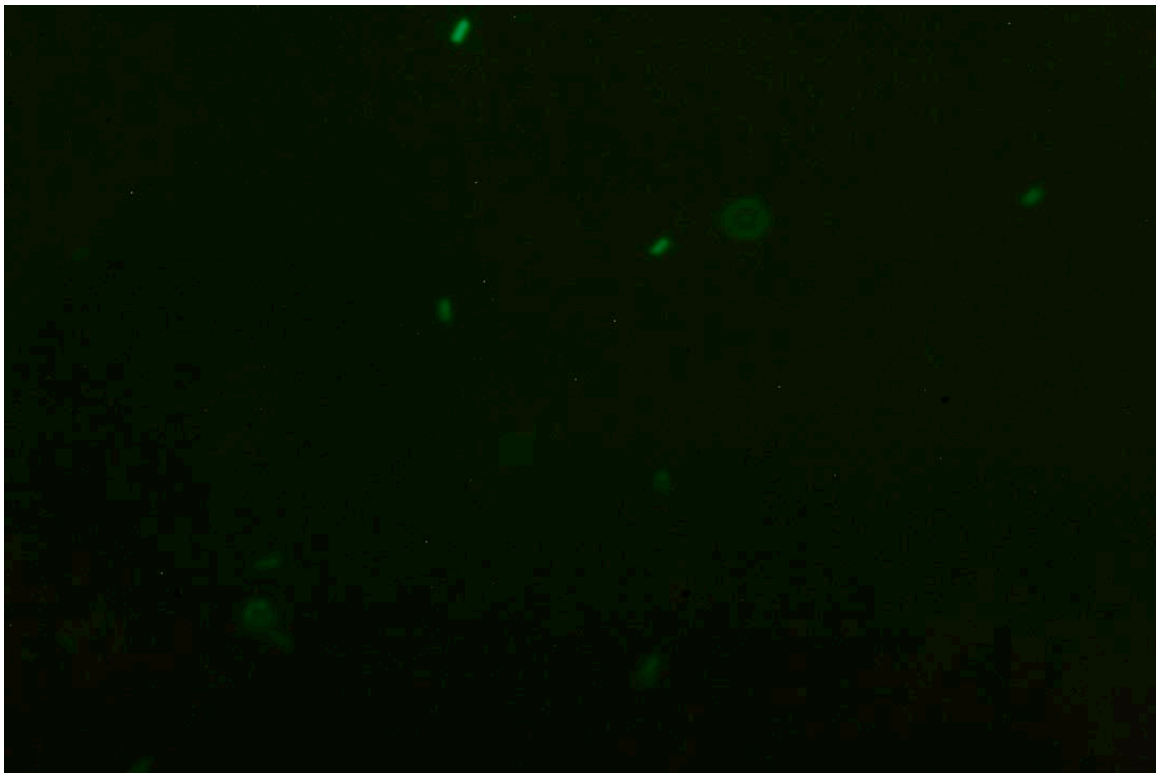

Figure 19: W3110(pCT6+pET200GFP) UV 1 hour

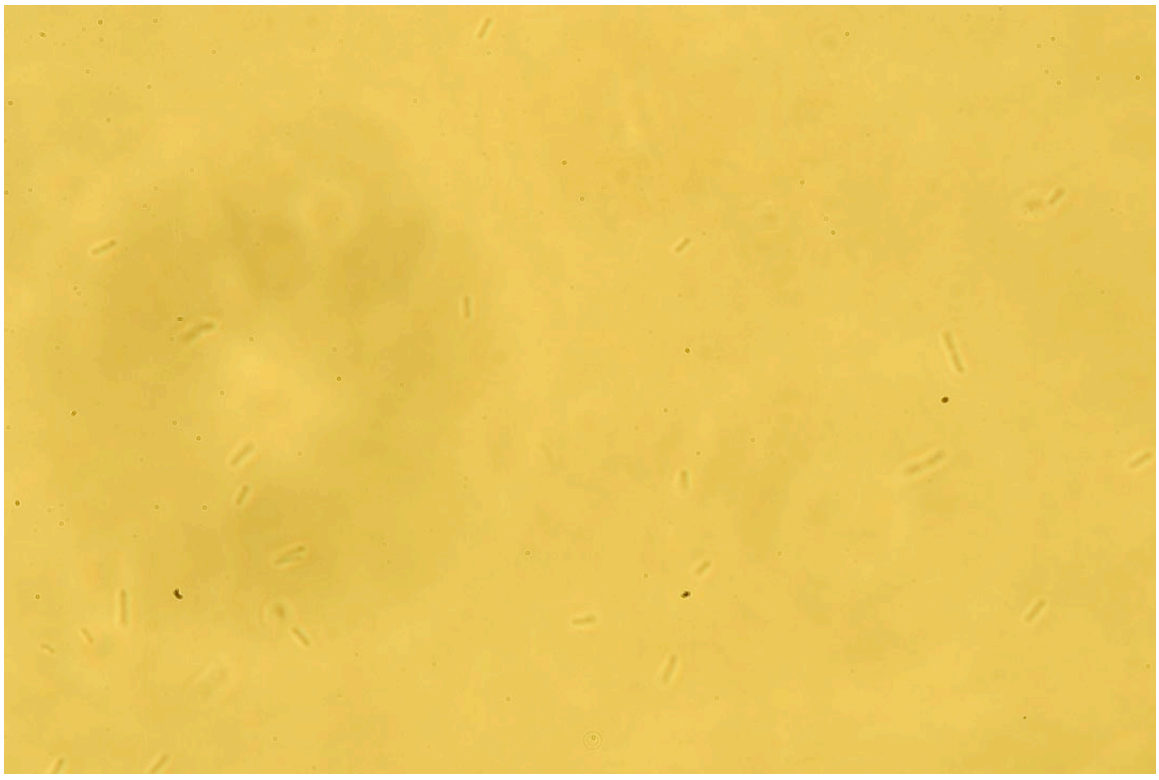

Figure 20: W3110(pCT6+pET200GFP) white 1 hour

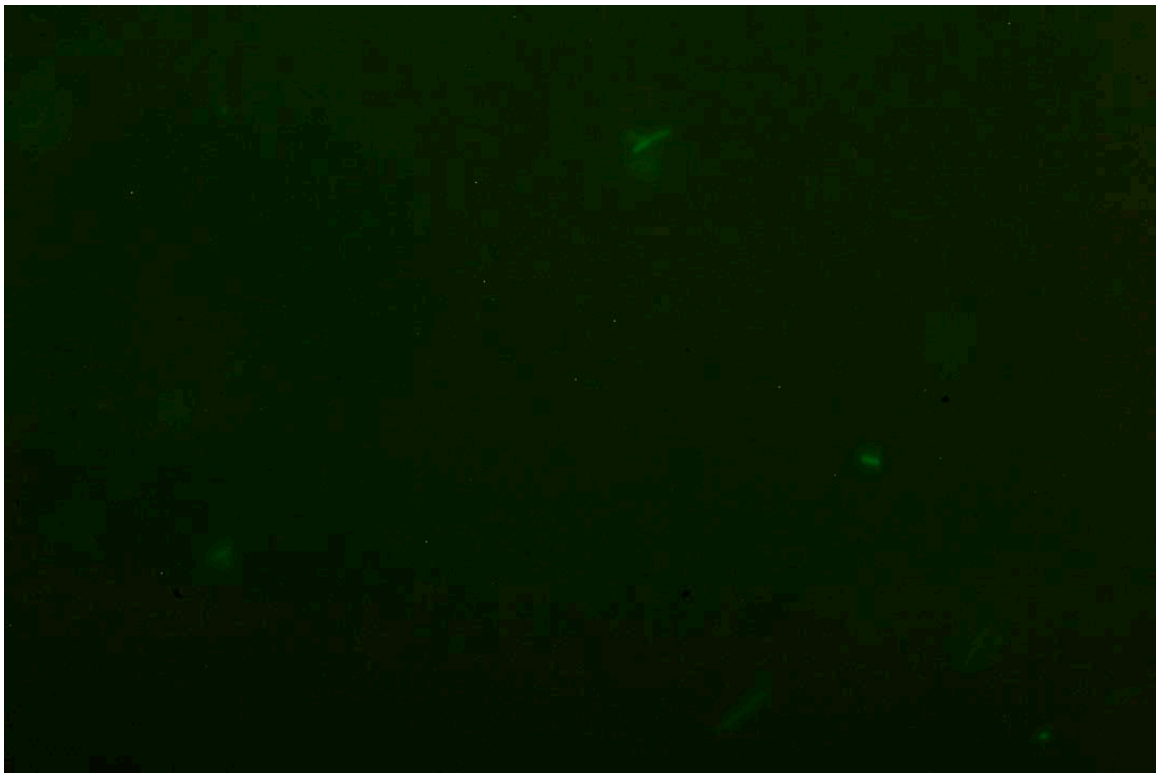

Figure 21: W3110(pCT6+pET200GFP) UV 2 hour

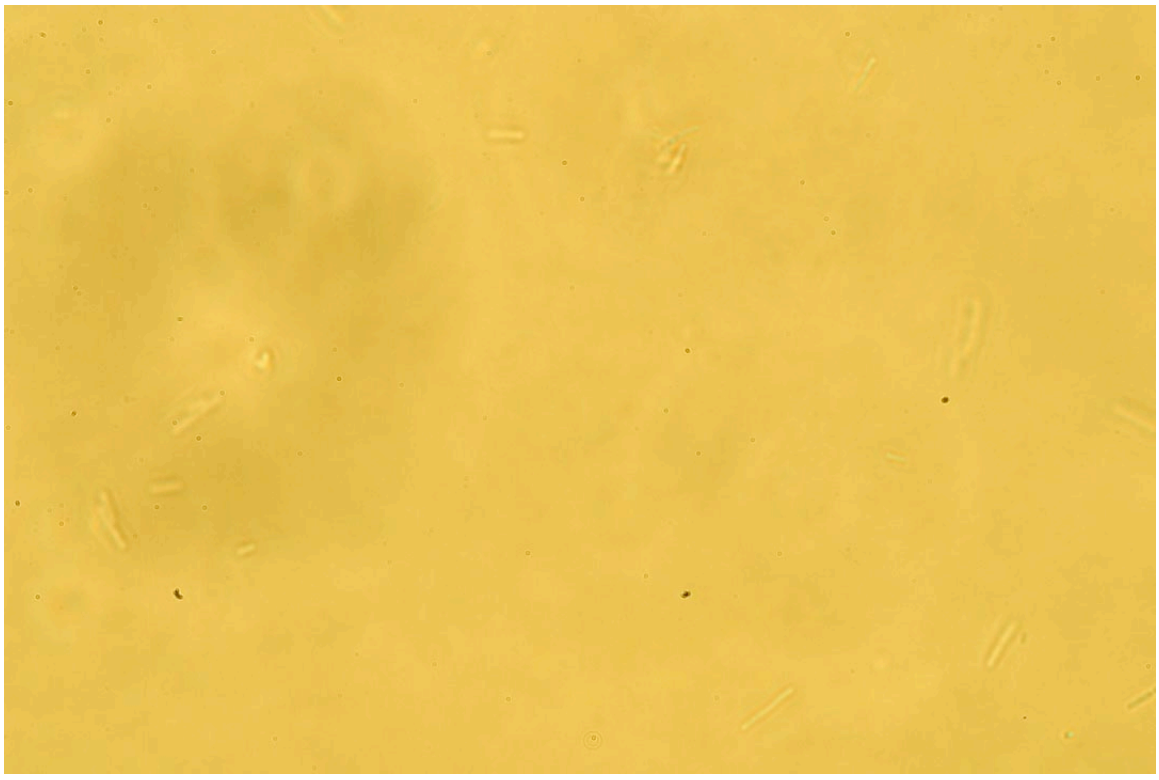

Figure 22: W3110(pCT6+pET200GFP) white 2 hour

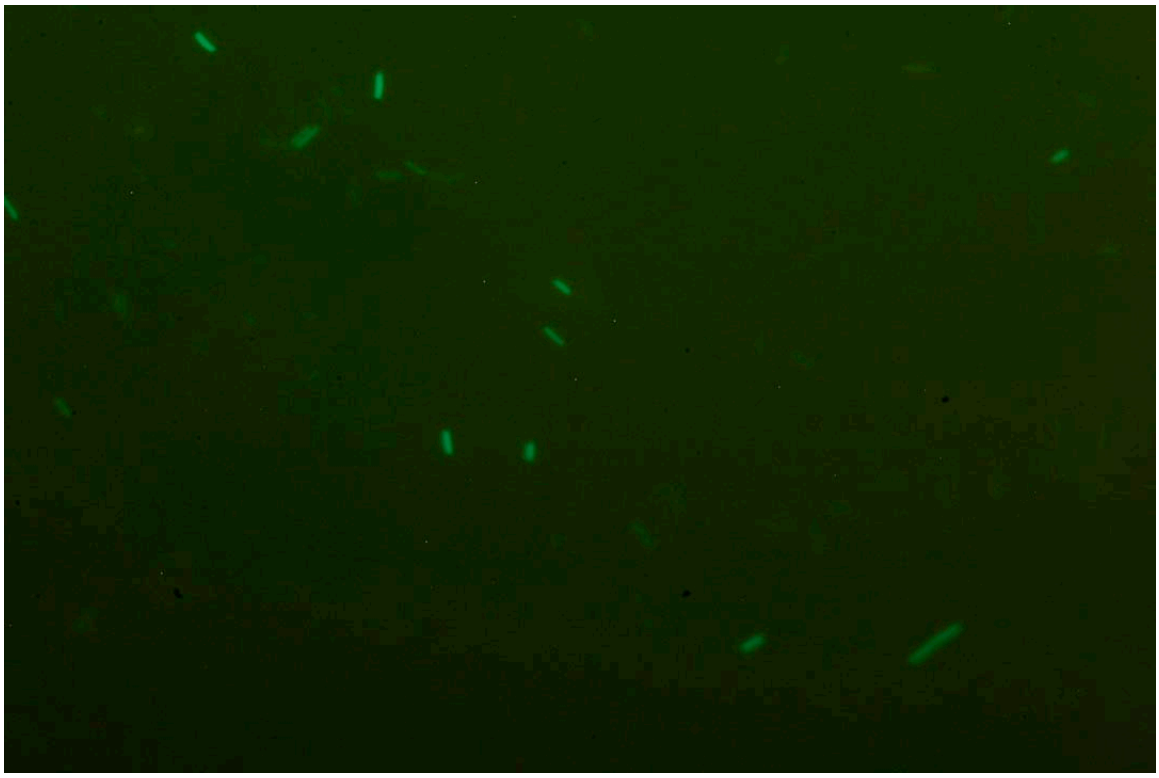

Figure 23: W3110(pCT6+pET200GFP) UV 3 hour

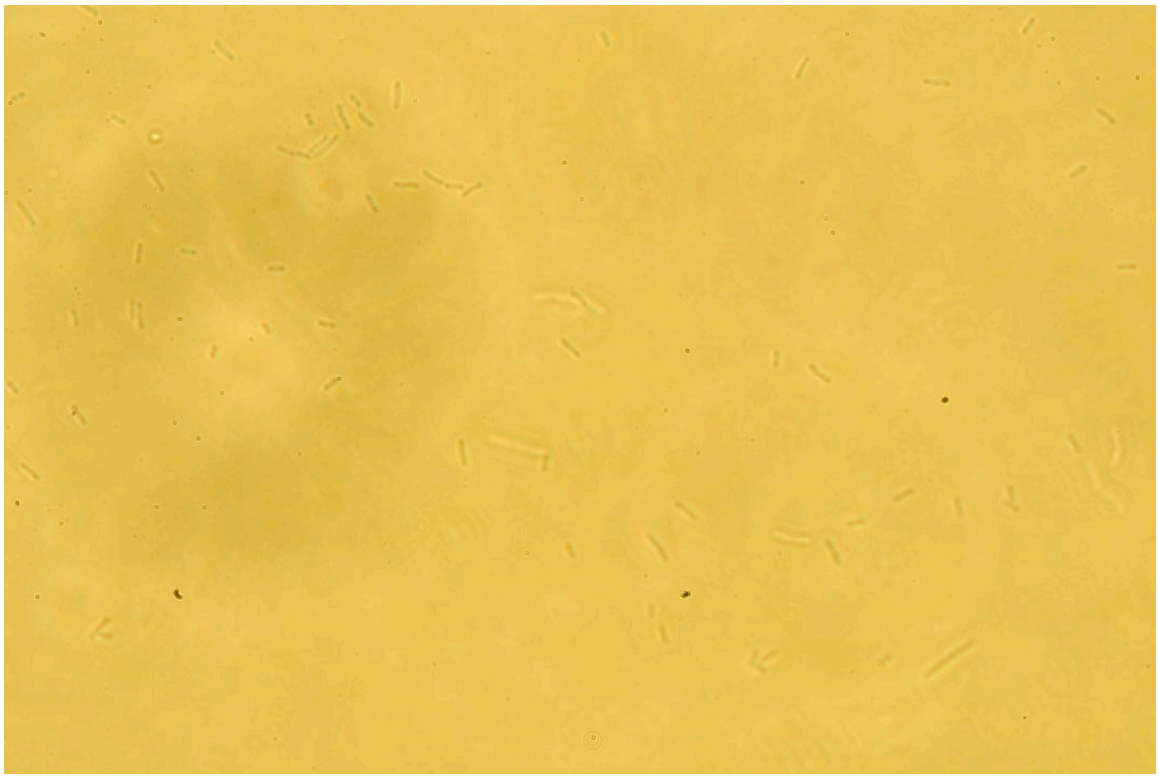

Figure 24: W3110(pCT6+pET200GFP) white 3 hour

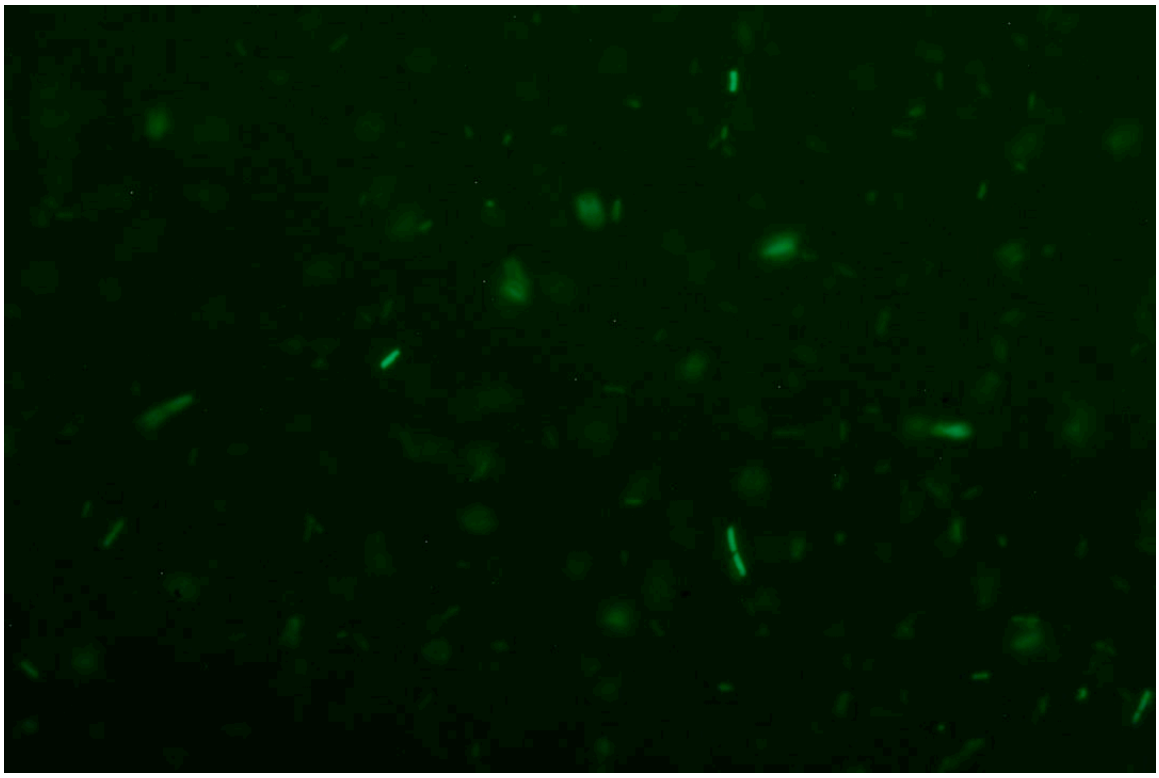

Figure 25: W3110(pCT6+pET200GFP) UV 4 hour

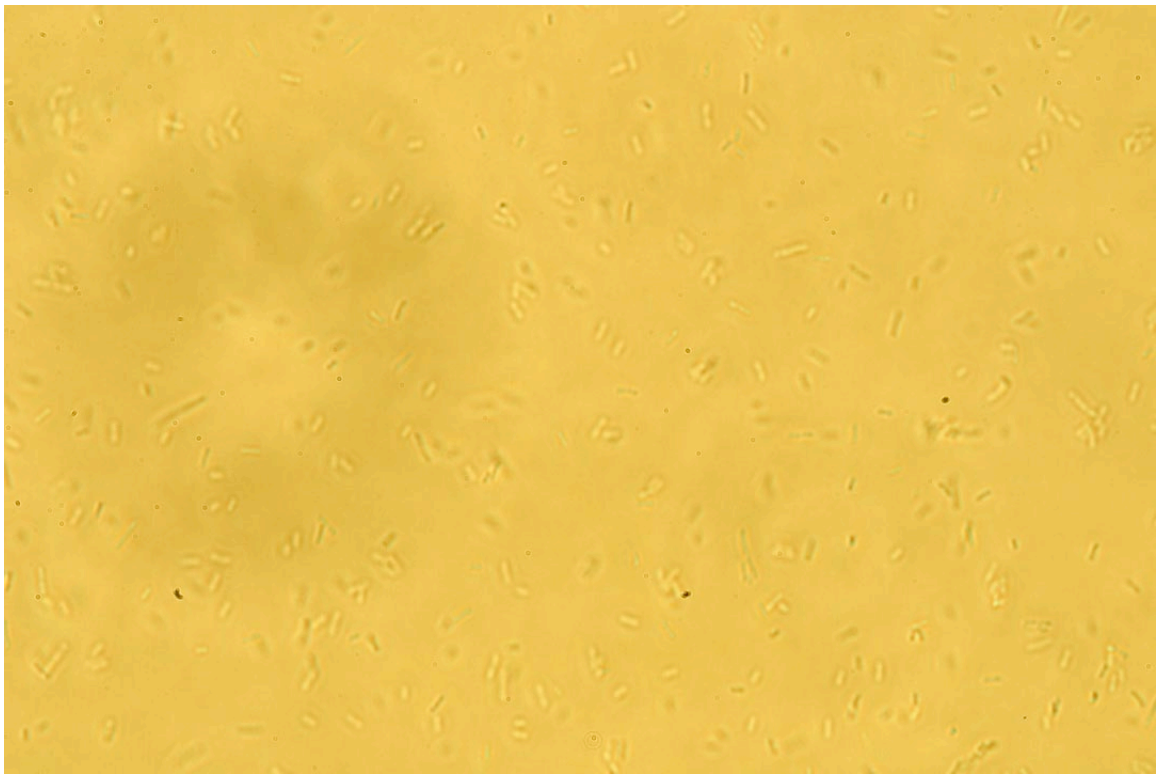

Figure 26: W3110(pCT6+pET200GFP) white 4 hour

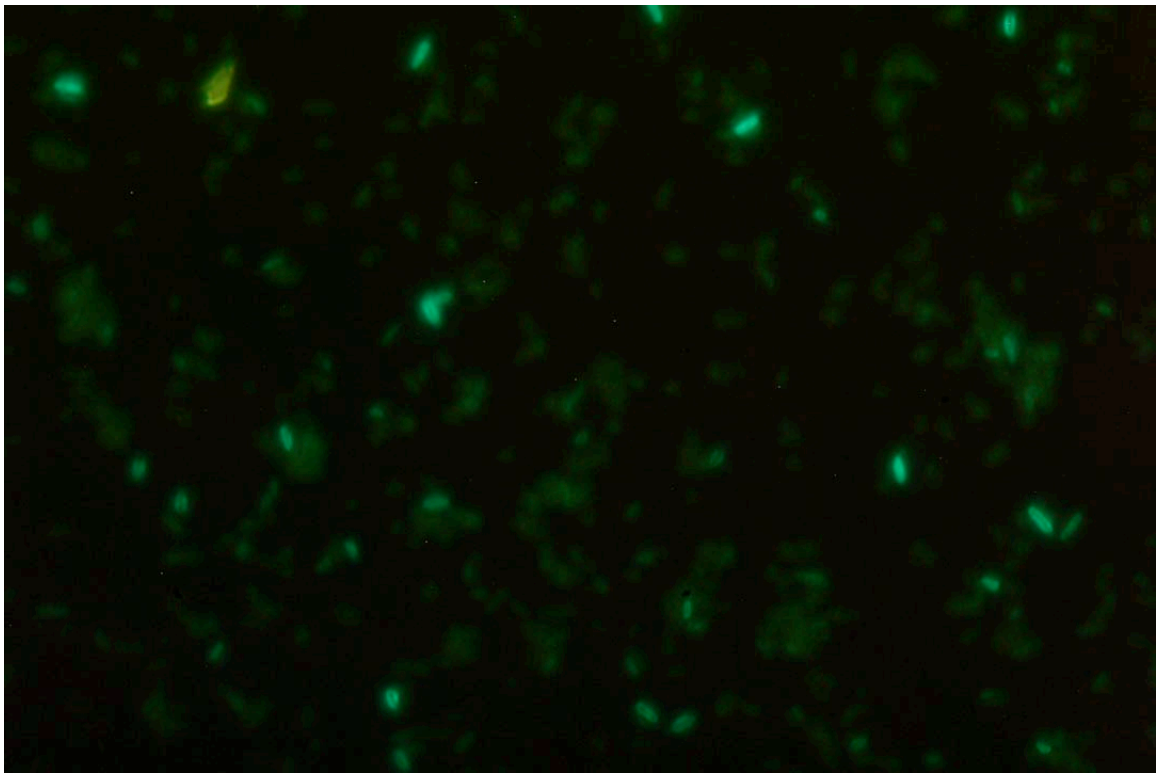

Figure 27: W3110(pCT6+pET200GFP) UV 5 hour

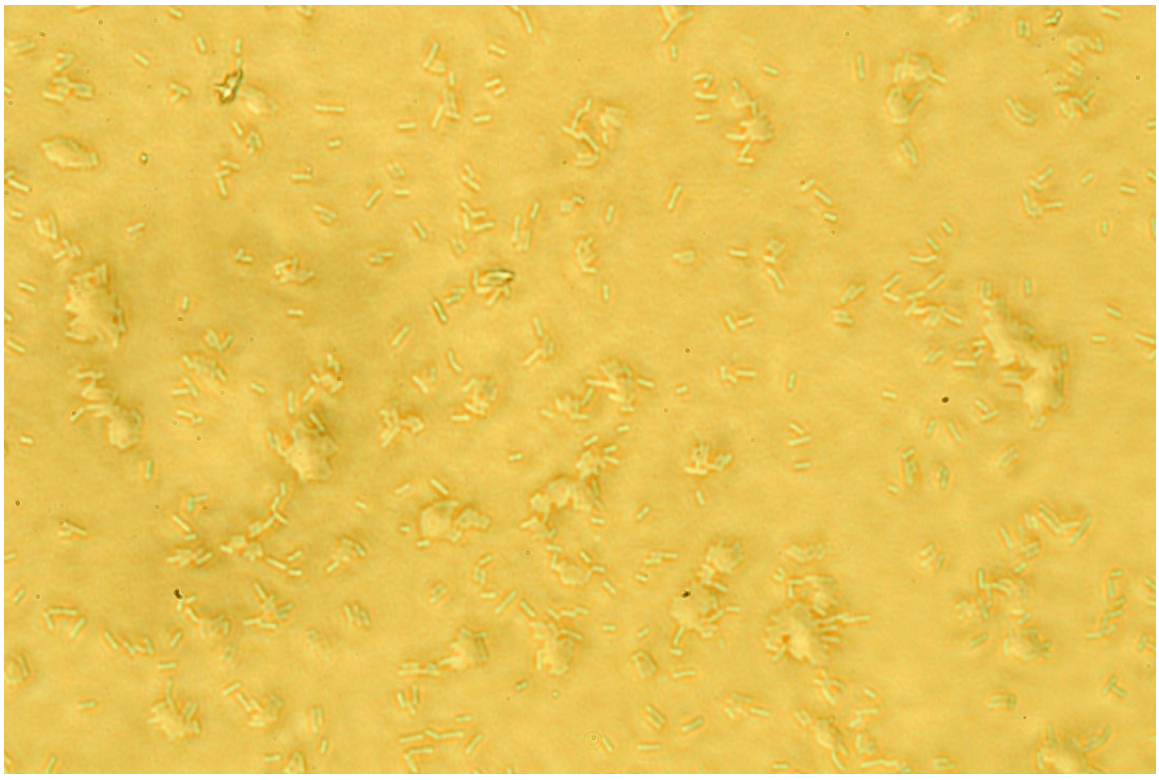

Figure 28: W3110(pCT6+pET200GFP) white 5 hour

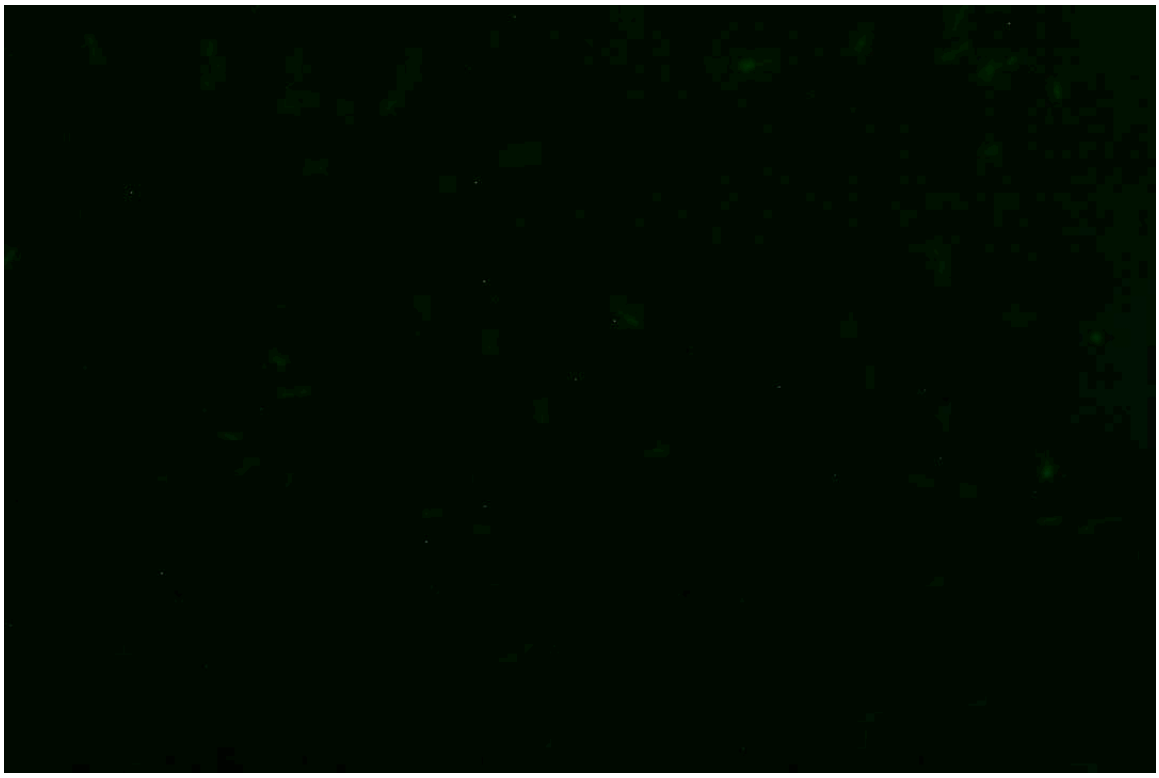

Figure 29: MDAI2(pCT6+pET200GFP) UV 1 hour

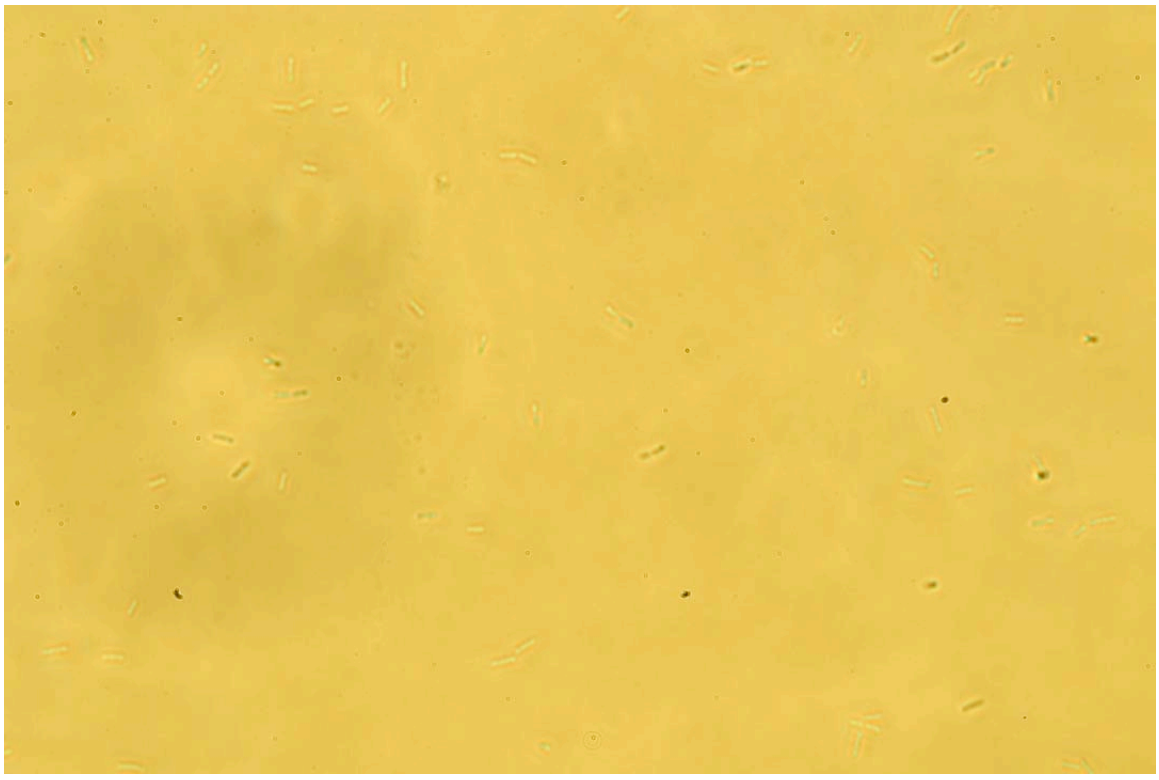

Figure 30: MDAI2(pCT6+pET200GFP) white 1 hour

Figure 31: MDAI2(pCT6+pET200GFP) UV 2 hour

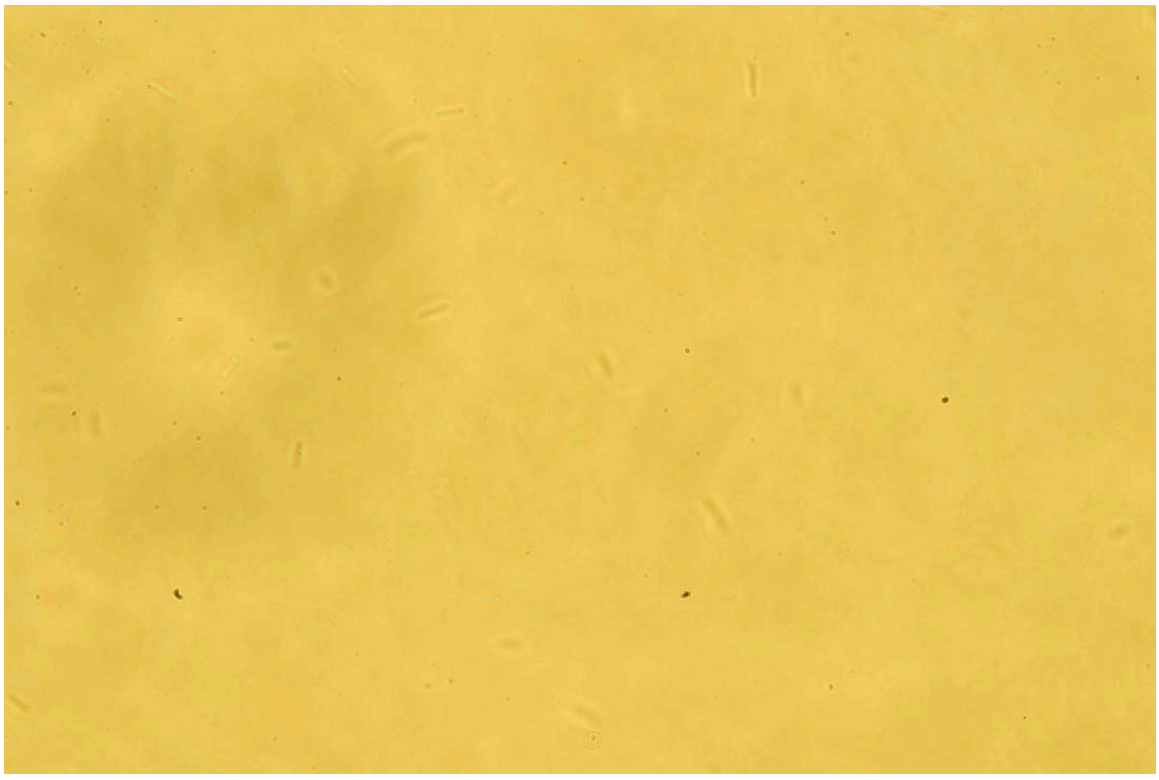

Figure 32: MDAI2(pCT6+pET200GFP) white 2 hour

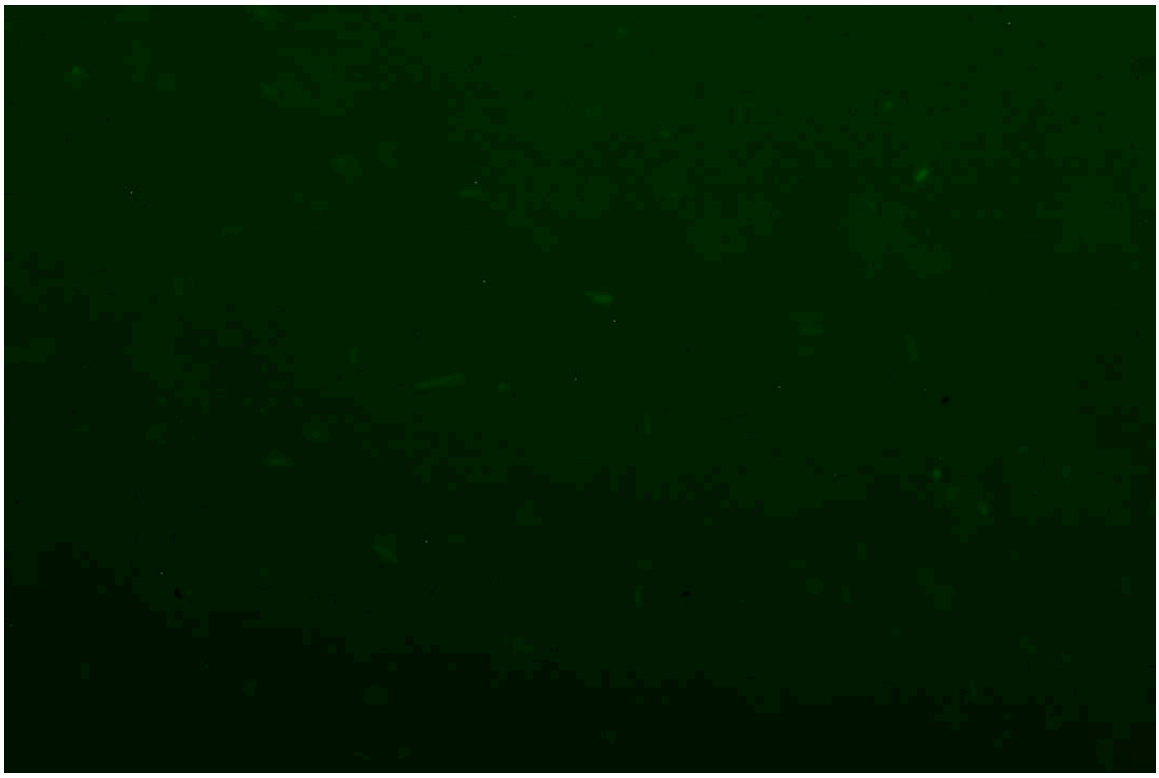

Figure 33: MDAI2(pCT6+pET200GFP) UV 3 hour

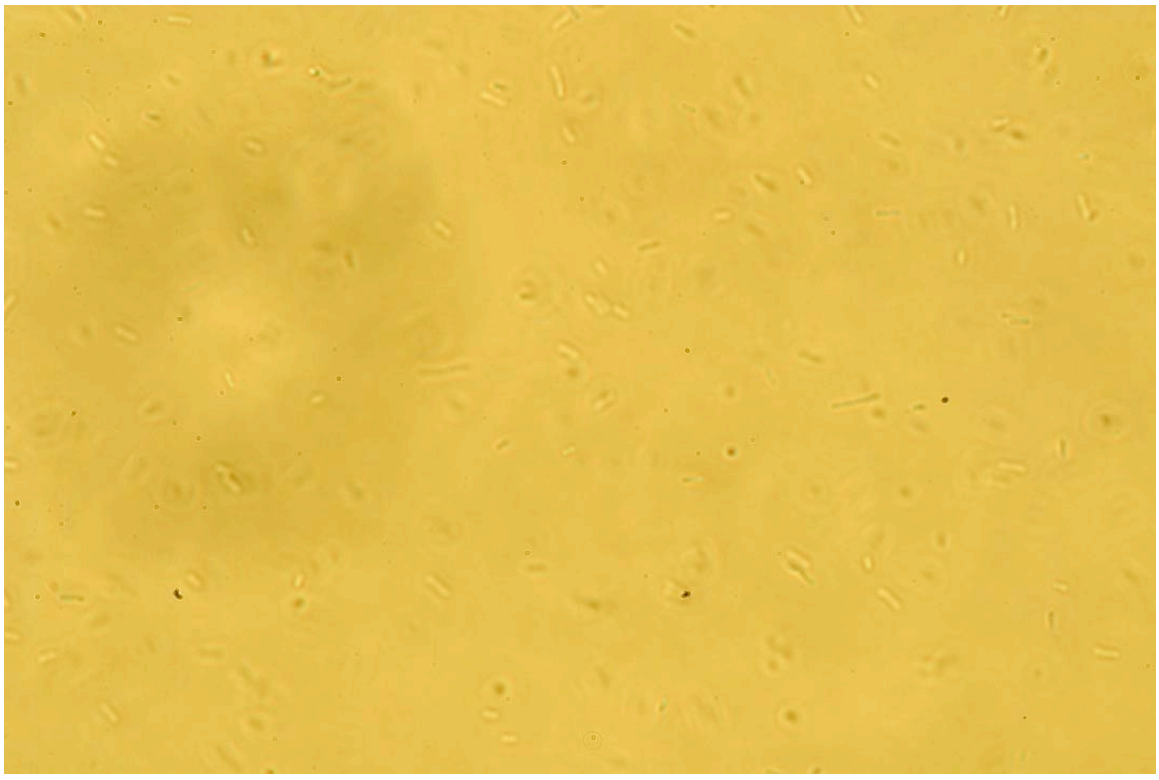

Figure 34: MDAI2(pCT6+pET200GFP) white 3 hour

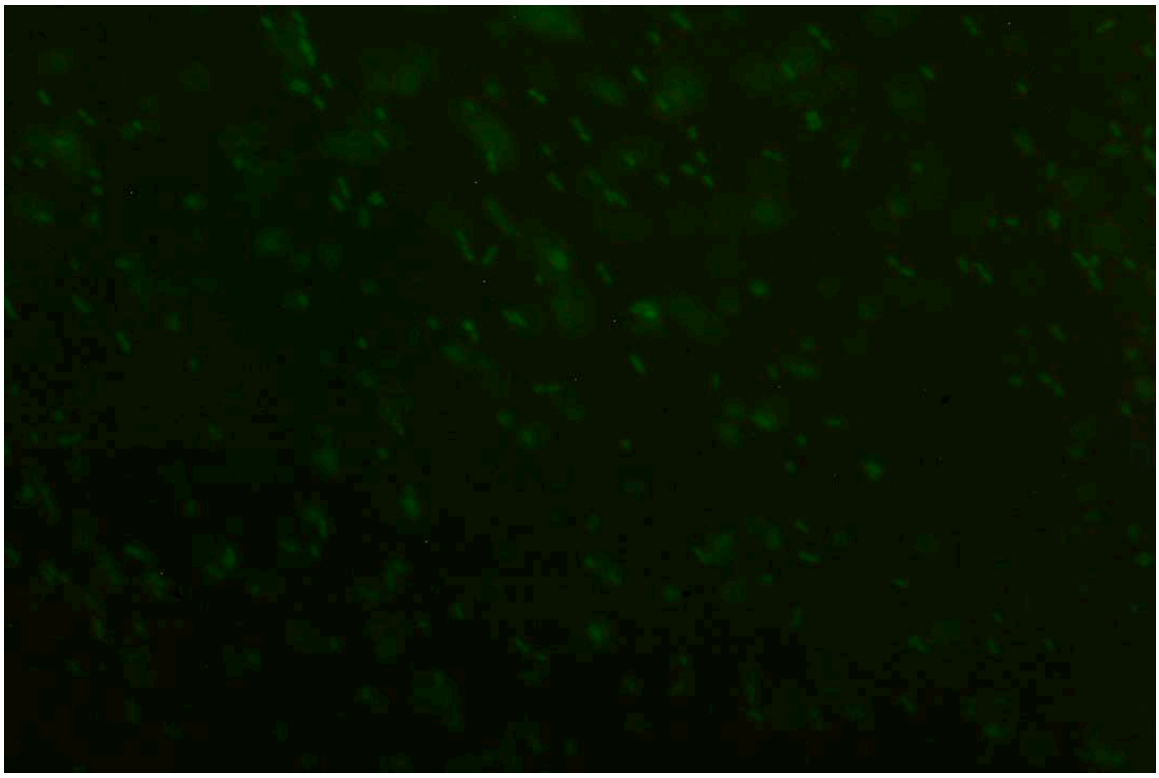

Figure 35: MDAI2(pCT6+pET200GFP) UV 4 hour

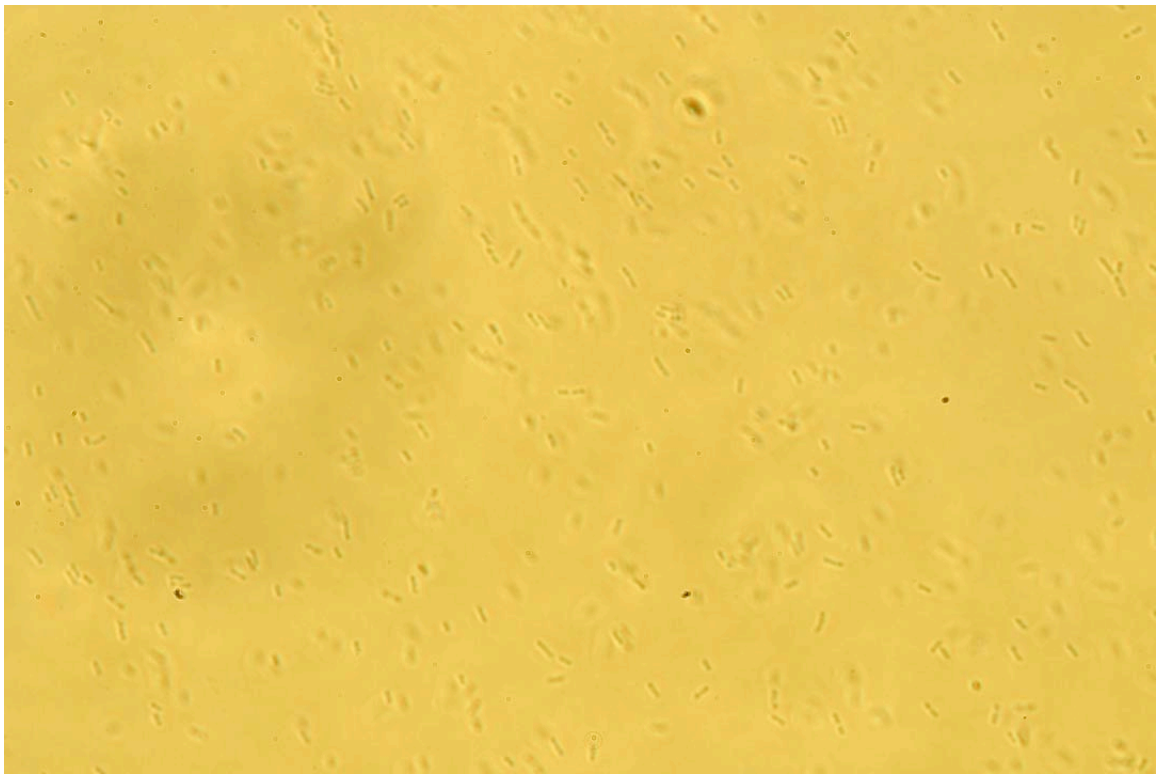

Figure 36: MDAI2(pCT6+pET200GFP) white 4 hour

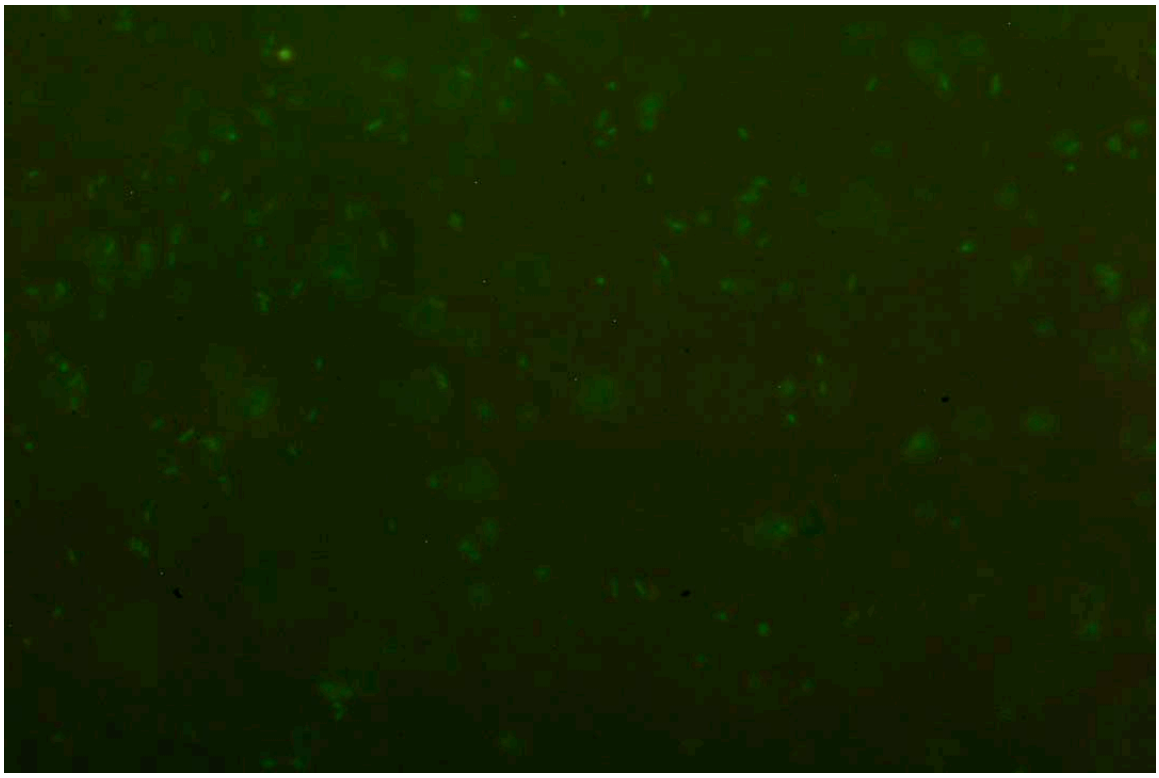

Figure 37: MDAI2(pCT6+pET200GFP) UV 5 hour

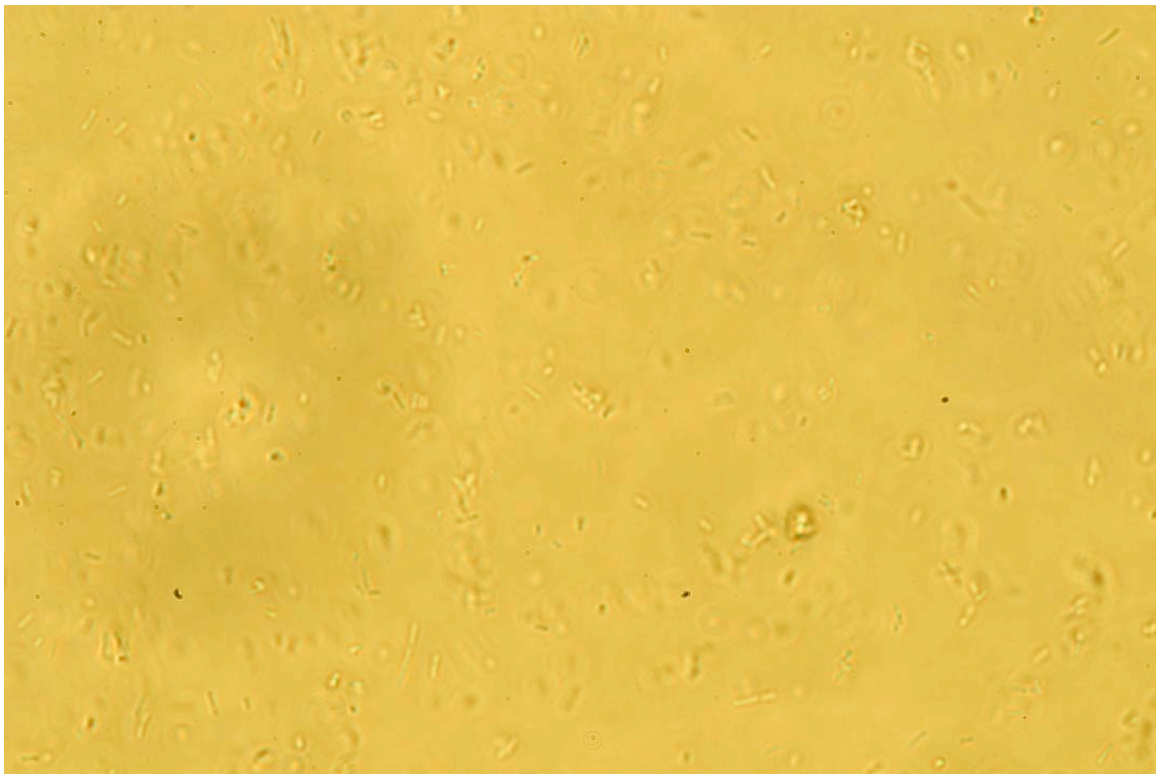

Figure 38: MDAI2(pCT6+pET200GFP) white 5 hour

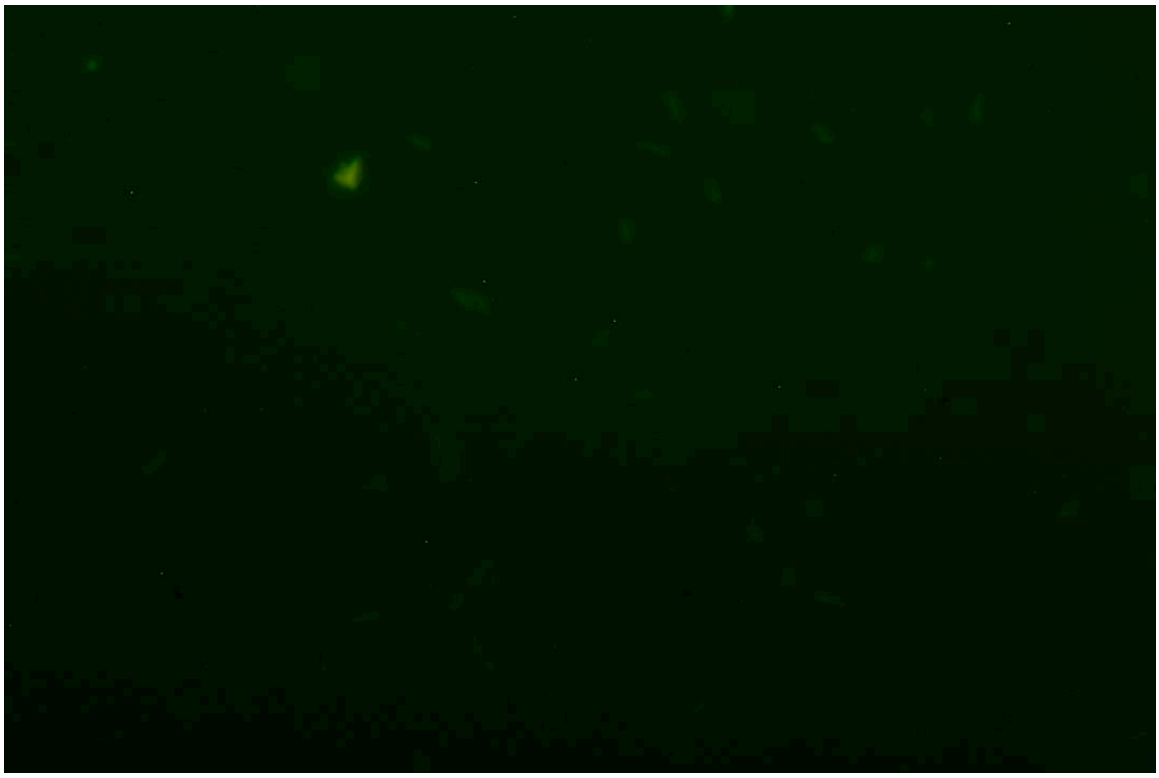

Figure 39: MDAI2(pCT6+pET200GFP) + AI2 UV 1 hour

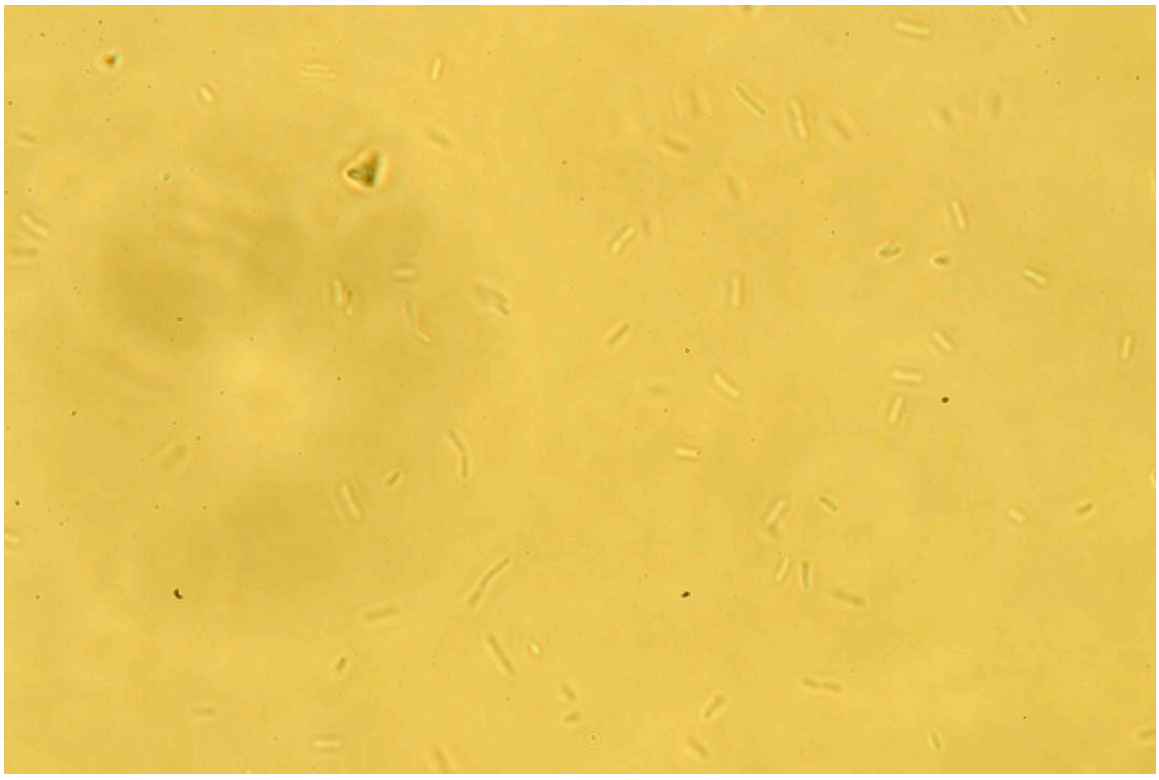

Figure 40: MDAI2(pCT6+pET200GFP) + AI2 white 1 hour

Figure 41: MDAI2(pCT6+pET200GFP) + AI2 UV 2 hour

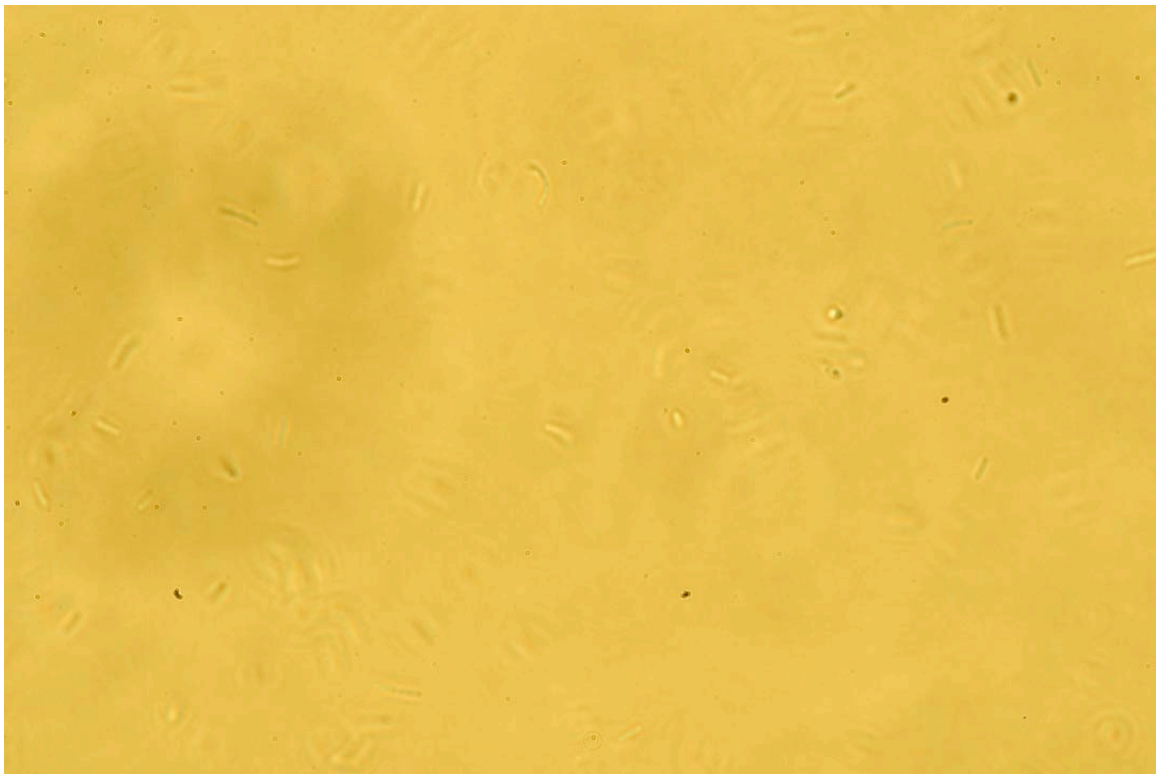

Figure 42: MDAI2(pCT6+pET200GFP) + AI2 white 2 hour

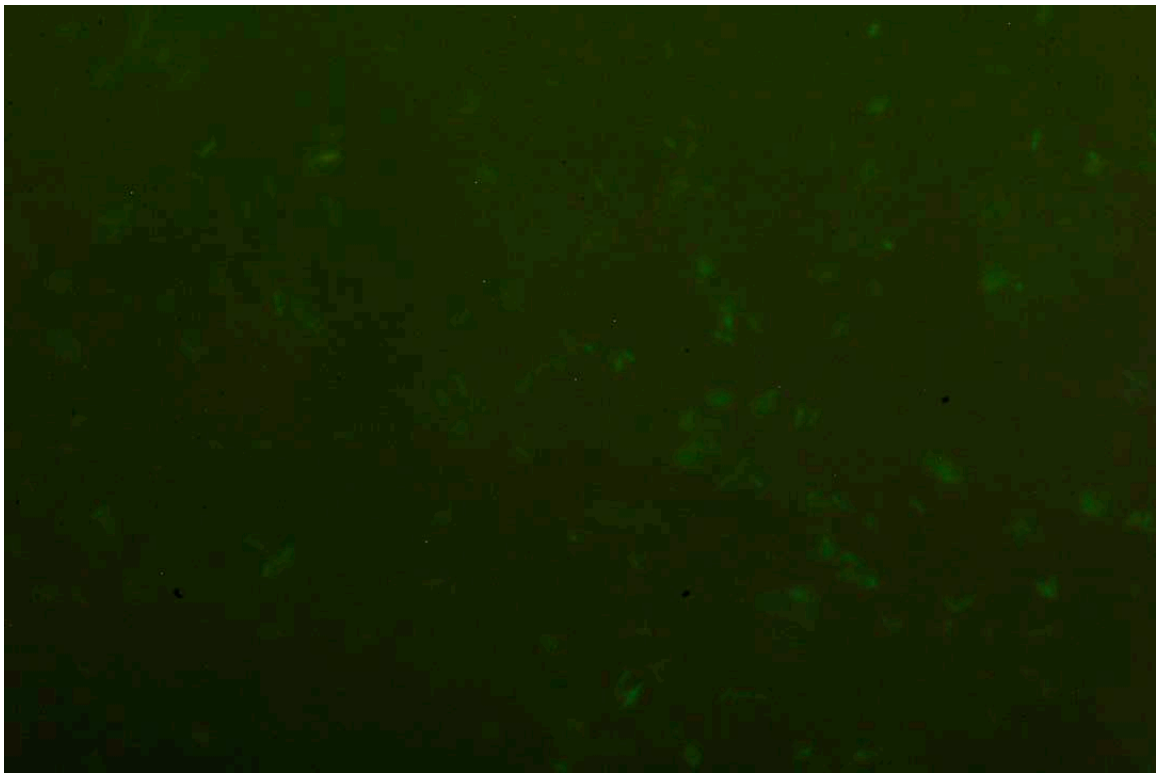

Figure 43: MDAI2(pCT6+pET200GFP) + AI2 UV 3 hour

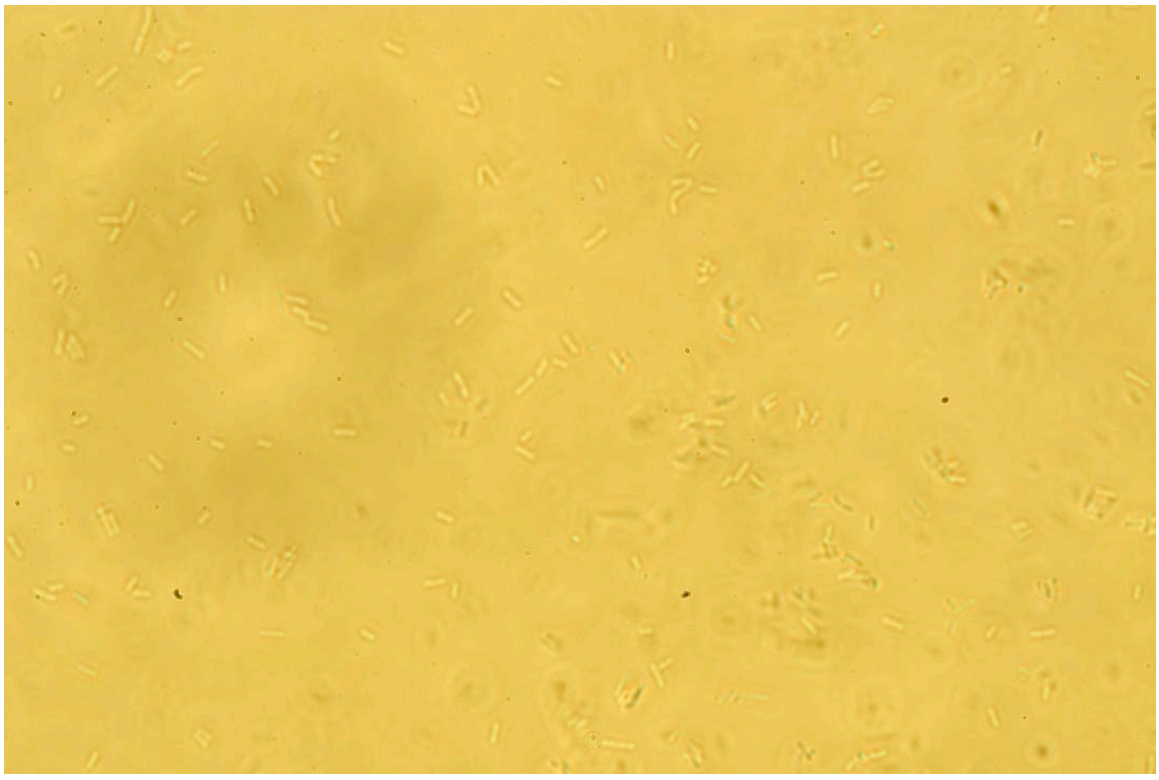

Figure 44: MDAI2(pCT6+pET200GFP) + AI2 white 3 hour

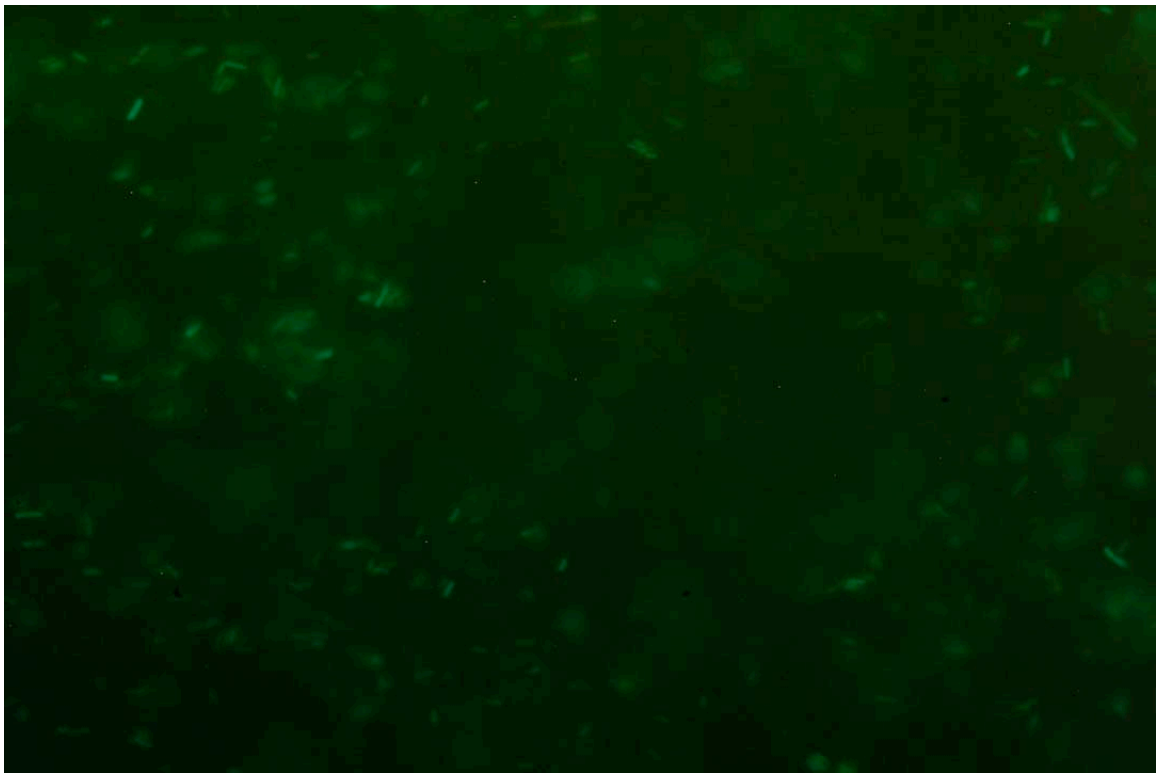

Figure 45: MDAI2(pCT6+pET200GFP) + AI2 UV 4 hour

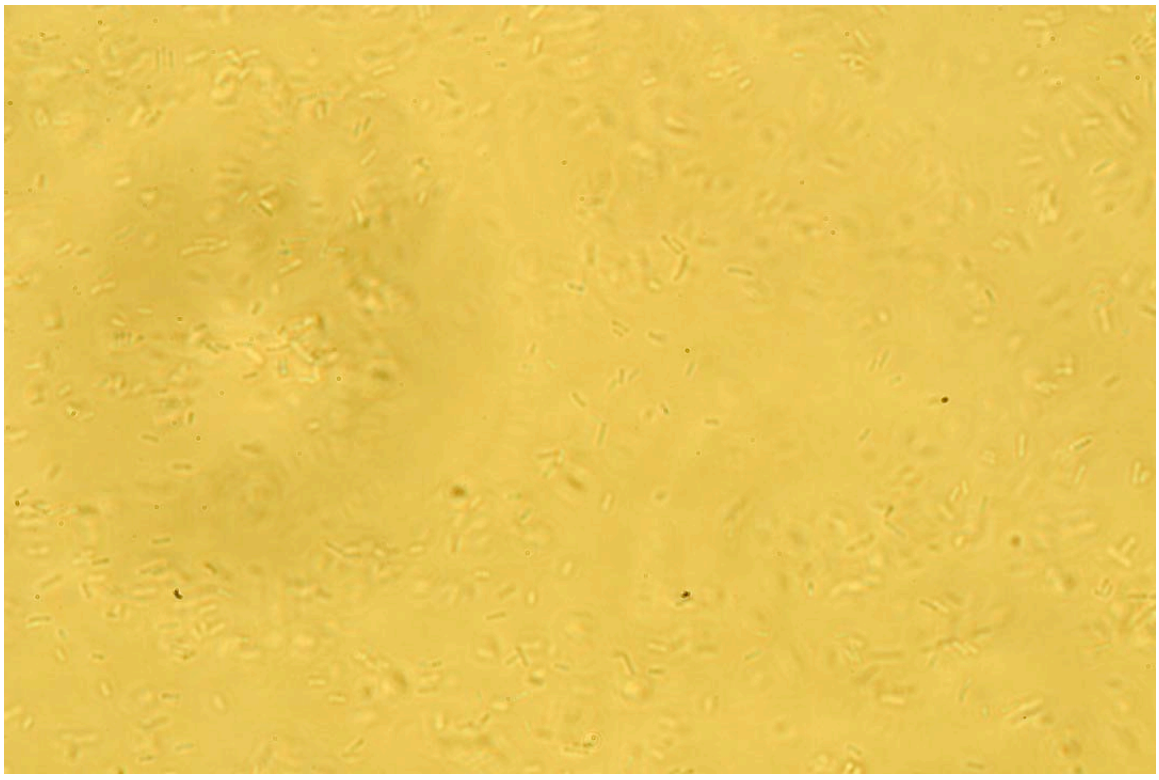

Figure 46: MDAI2(pCT6+pET200GFP) + AI2 white 4 hour

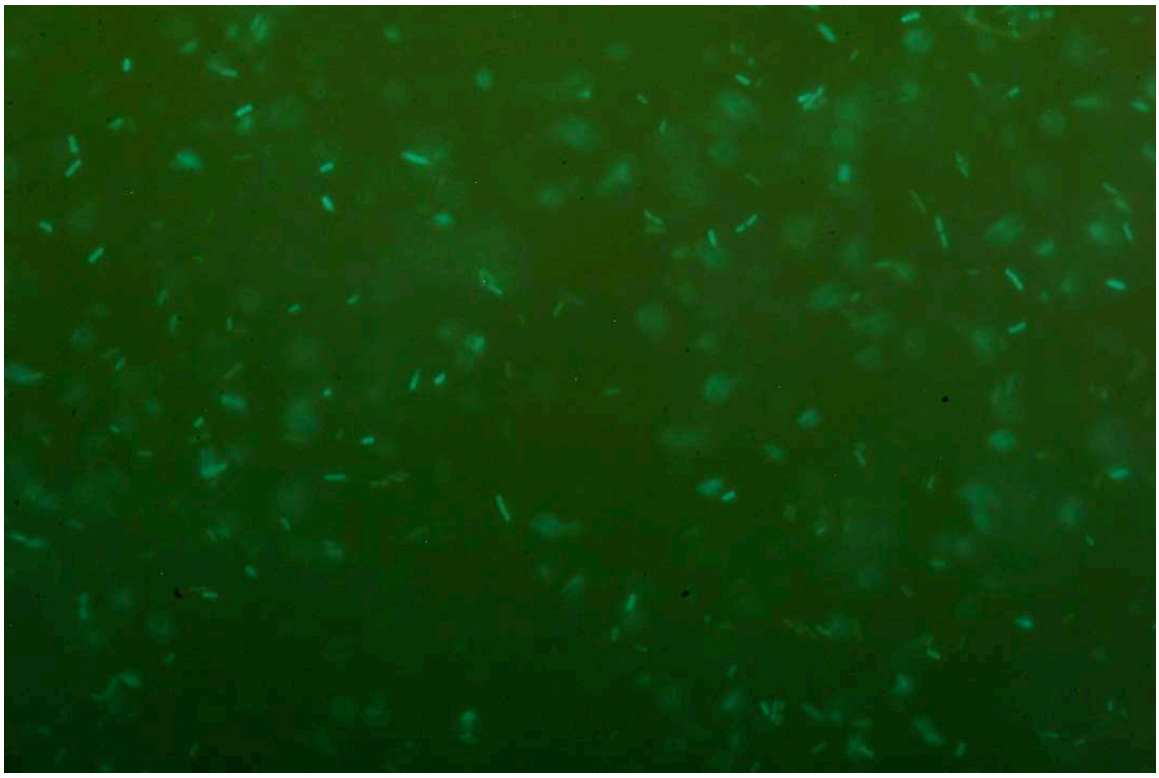

Figure 47: MDAI2(pCT6+pET200GFP) + AI2 UV 5 hour

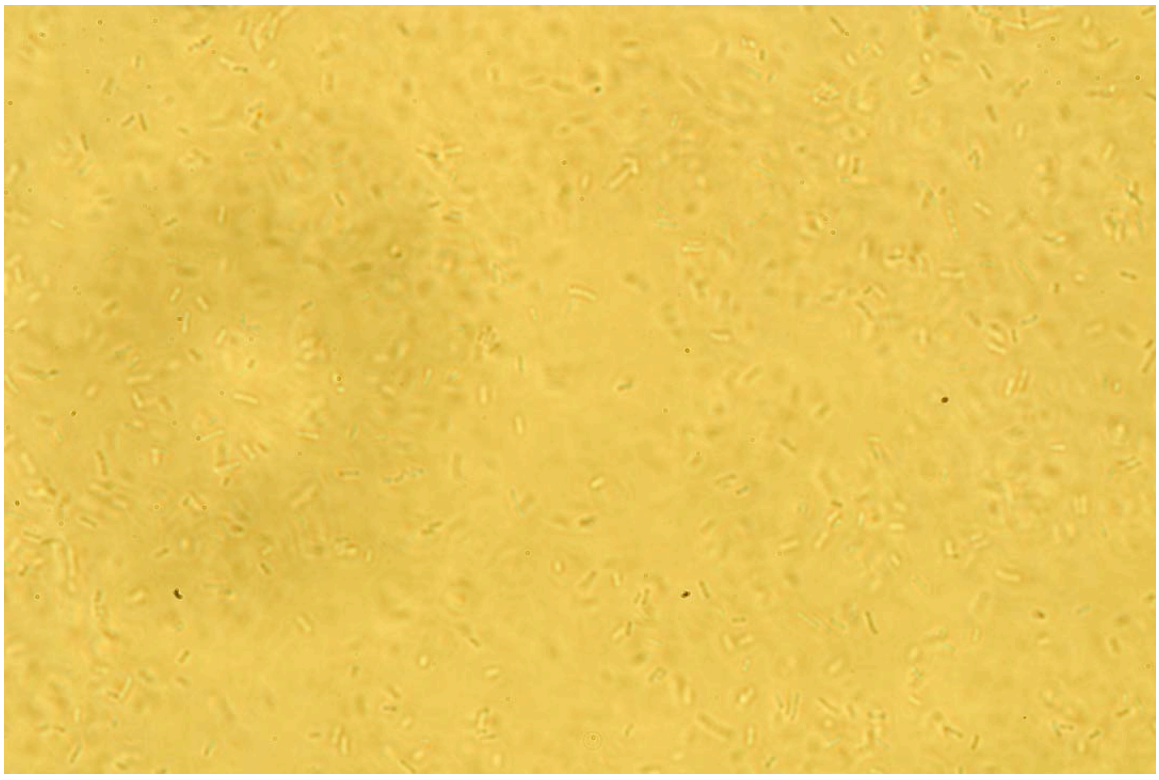

Figure 48: MDAI2(pCT6+pET200GFP) + AI2 white 5 hour

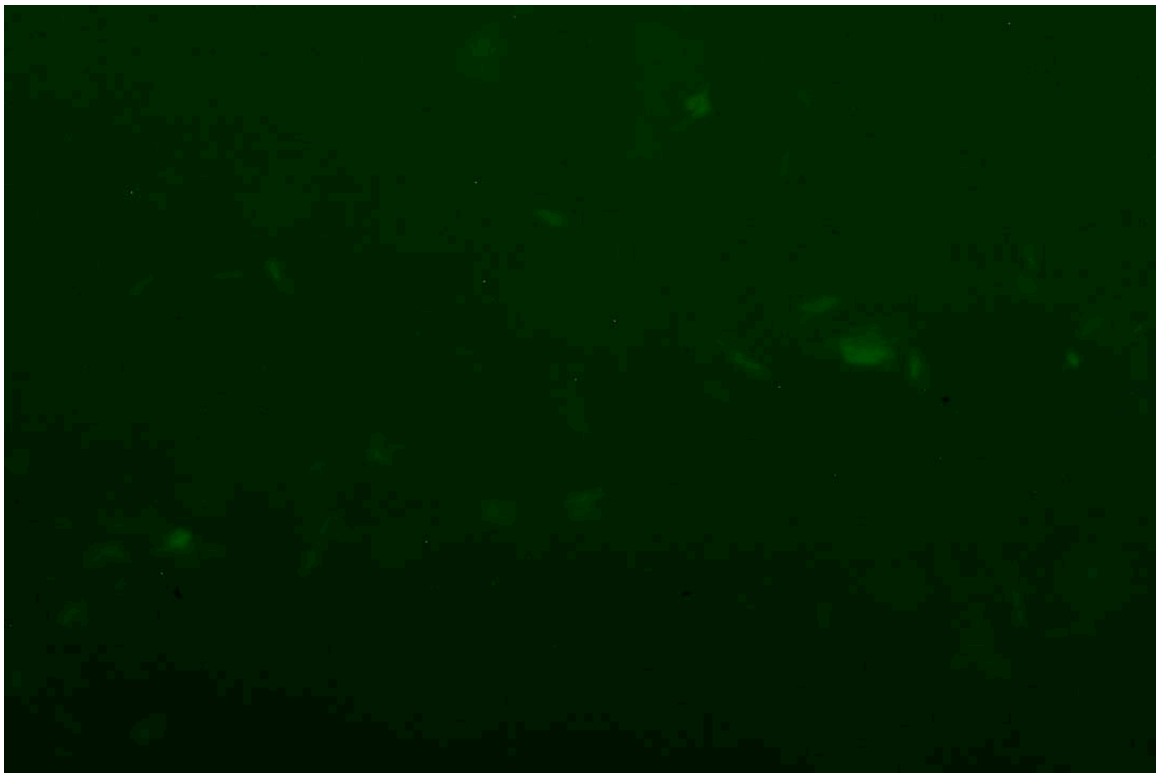

Figure 49: MDAI2(pCT6+pET200GFP) + MDAI2 UV 1 hour

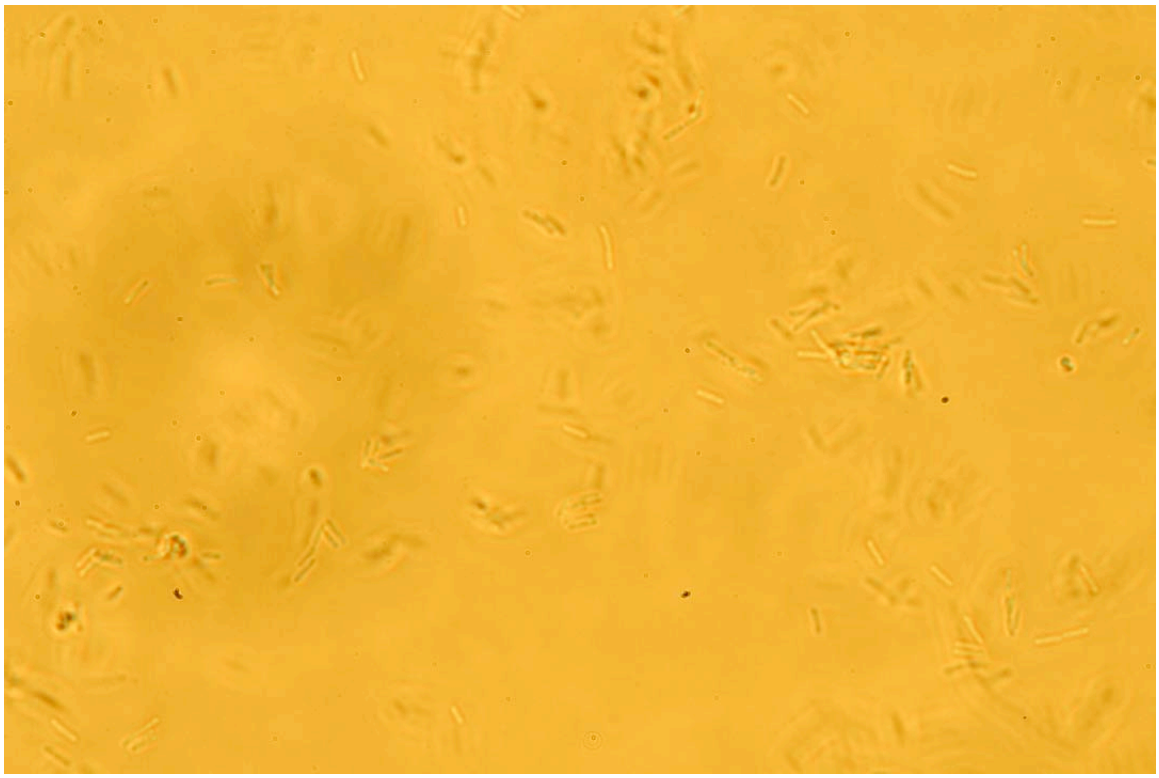

Figure 50: MDAI2(pCT6+pET200GFP) + MDAI2 white 1 hour

Figure 51: MDAI2(pCT6+pET200GFP) + MDAI2 UV 2 hour

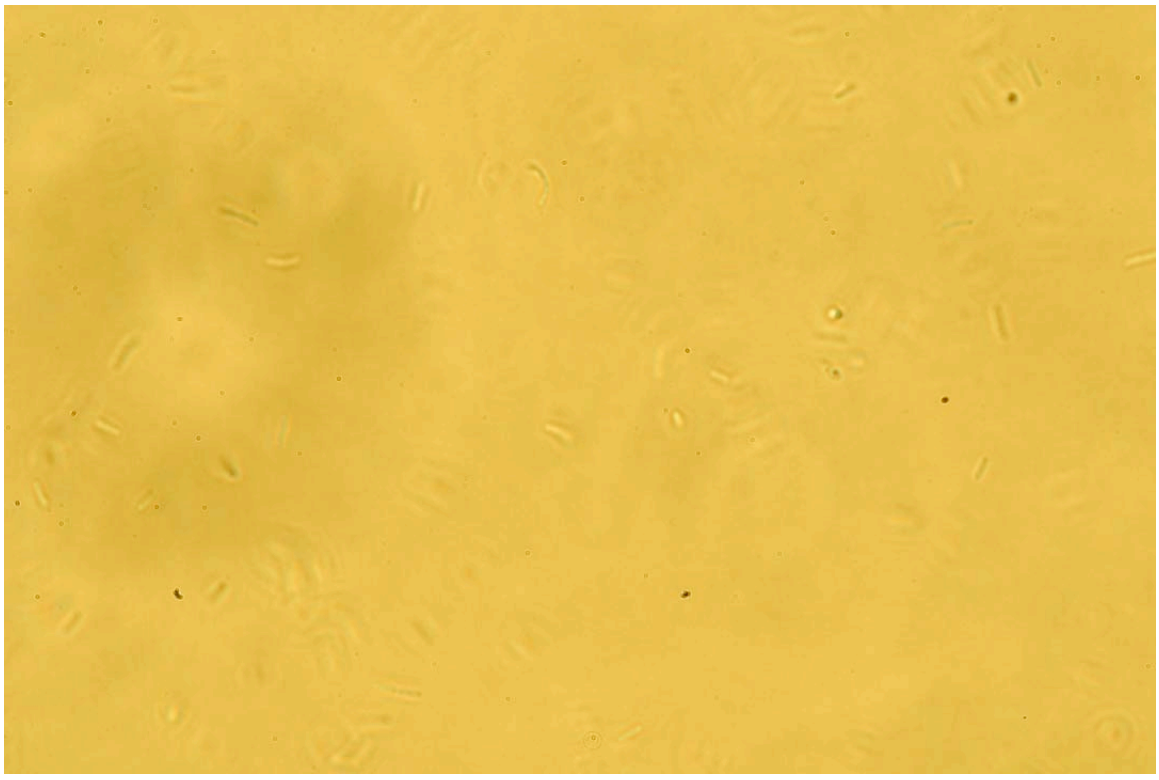

Figure 52: MDAI2(pCT6+pET200GFP) + MDAI2 white 2 hour

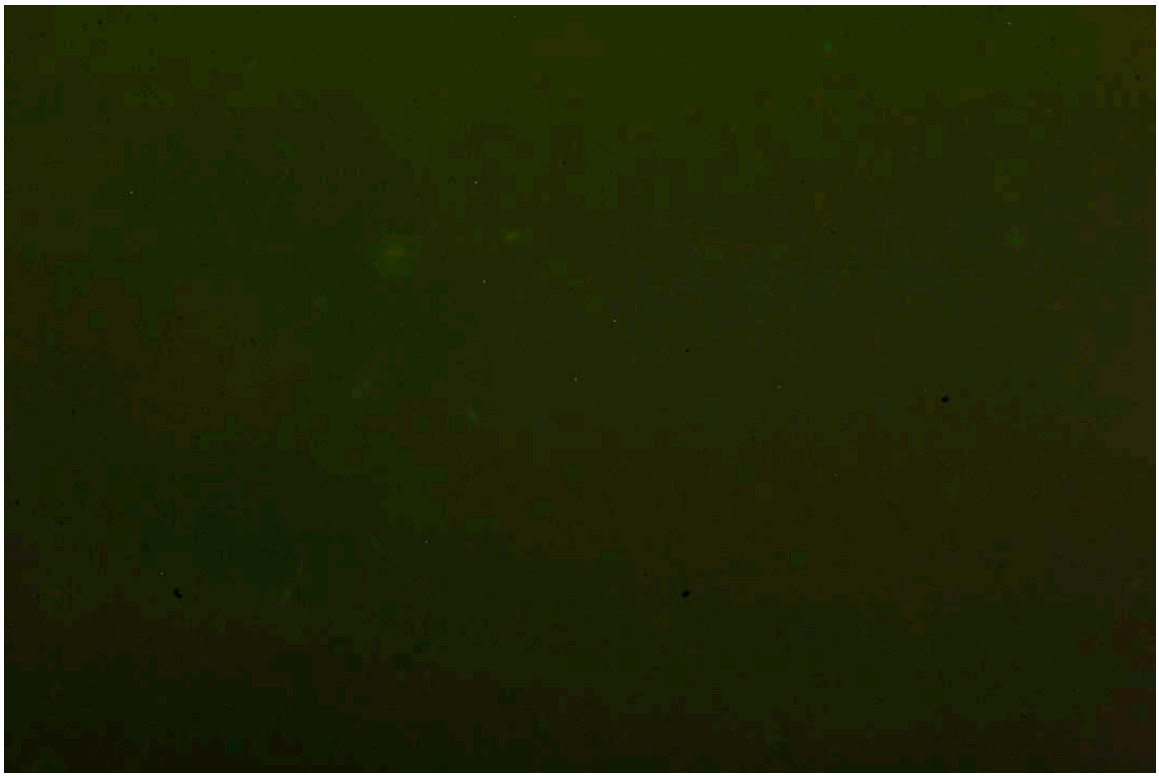

Figure 53: MDAI2(pCT6+pET200GFP) + MDAI2 UV 3 hour

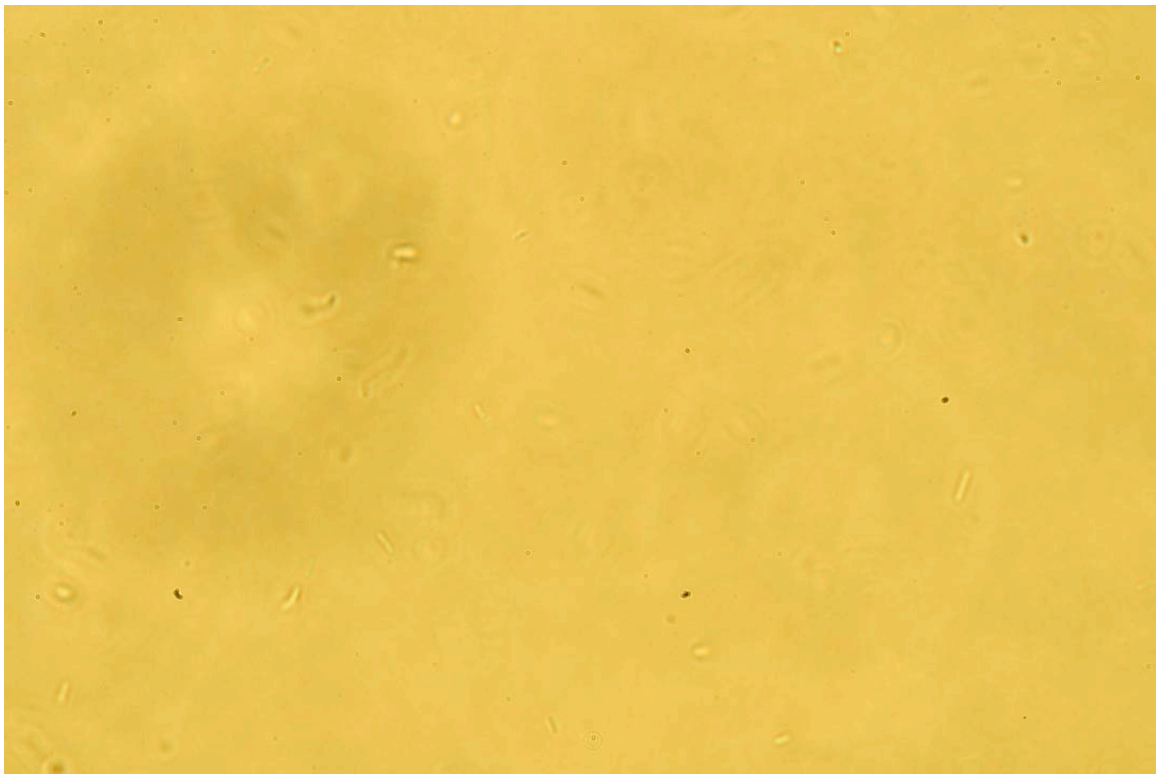

Figure 54: MDAI2(pCT6+pET200GFP) + MDAI2 white 3 hour

Figure 55: MDAI2(pCT6+pET200GFP) + MDAI2 UV 4 hour

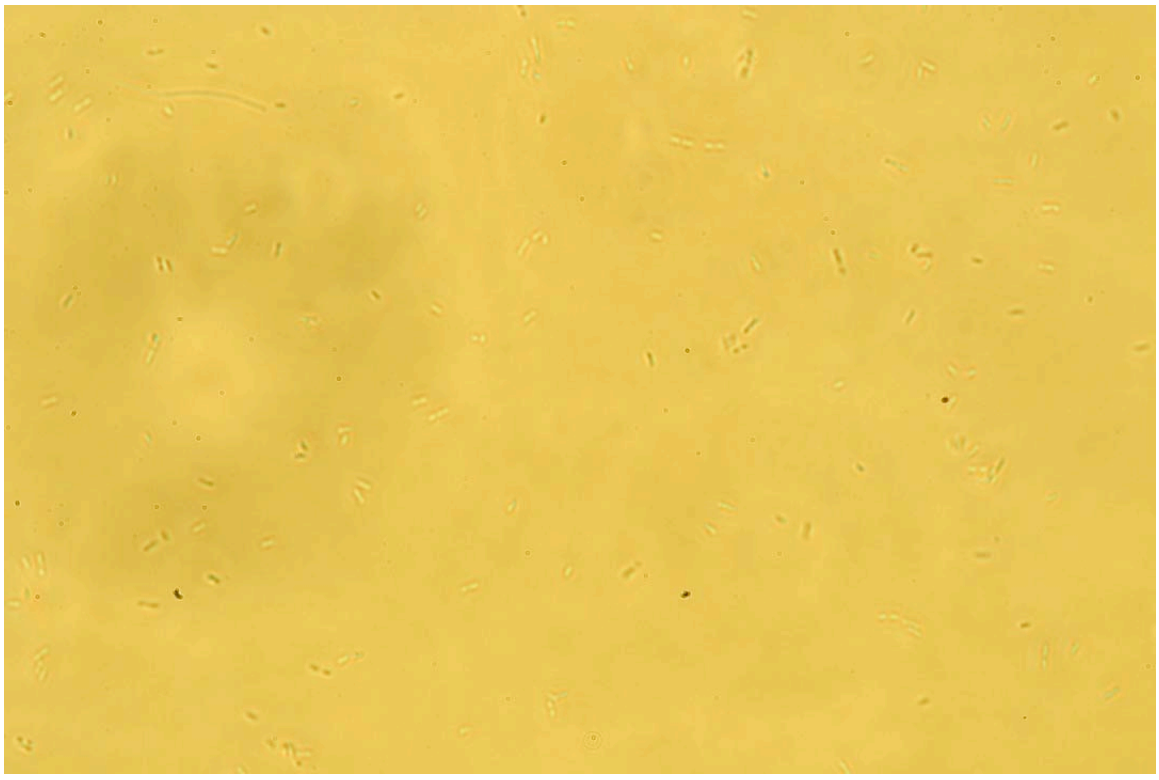

Figure 56: MDAI2(pCT6+pET200GFP) + MDAI2 white 4 hour

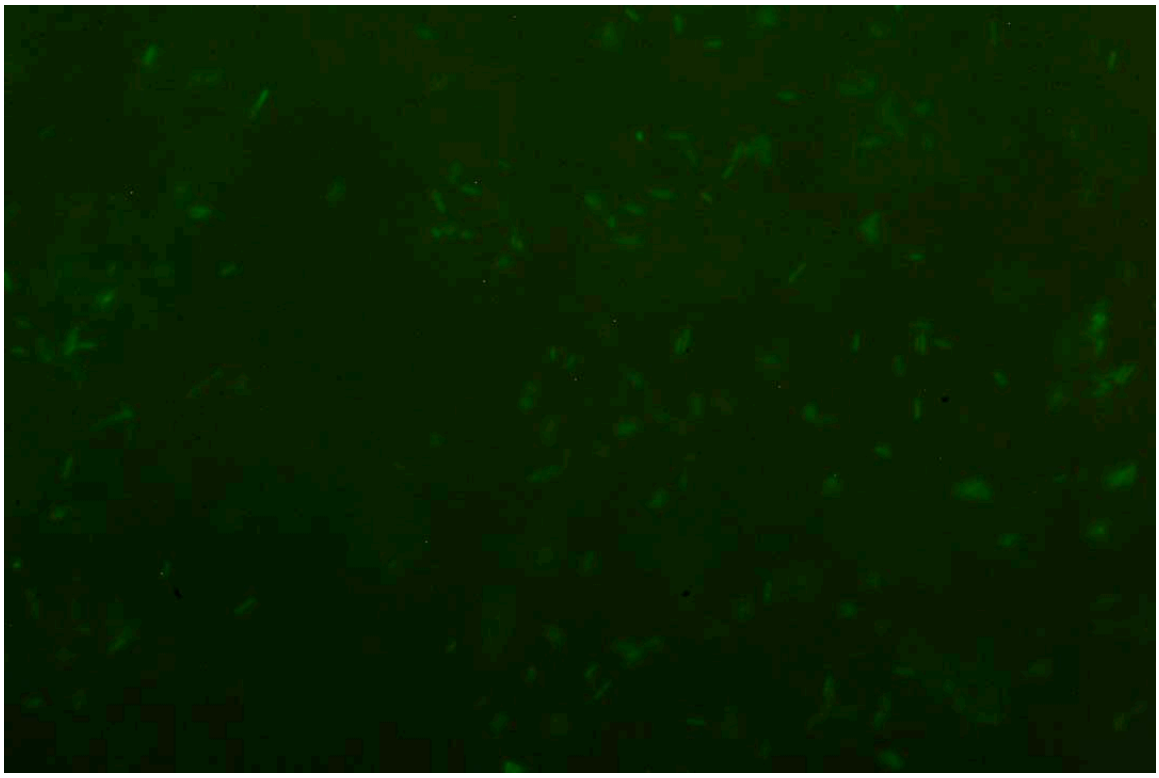

Figure 57: MDAI2(pCT6+pET200GFP) + MDAI2 UV 5 hour

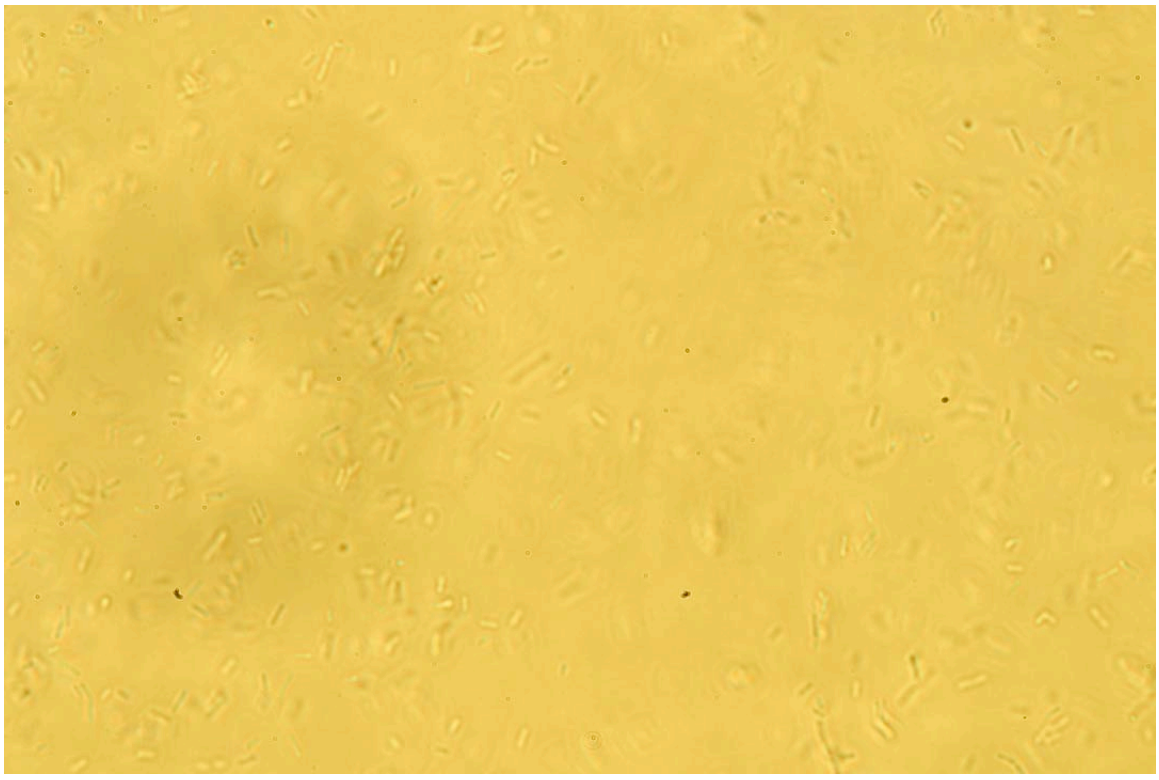

Figure 58: MDAI2(pCT6+pET200GFP) + MDAI2 white 5 hour

Figure 59: MDAI2(pCT6+pET200GFP)+W3110 UV 1 hour

Figure 60: MDAI2(pCT6+pET200GFP)+W3110 white 1 hour

Figure 61: MDAI2(pCT6+pET200GFP)+W3110 UV 2 hour

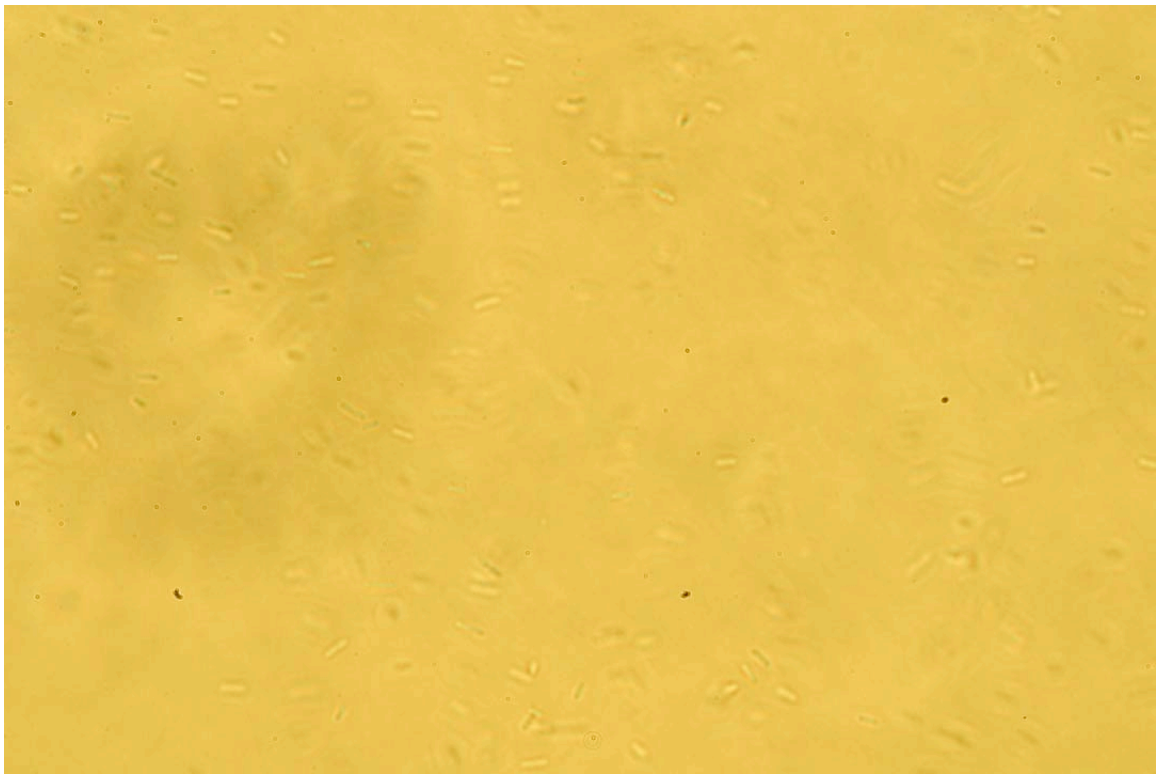

Figure 62: MDAI2(pCT6+pET200GFP)+W3110 white 2 hour

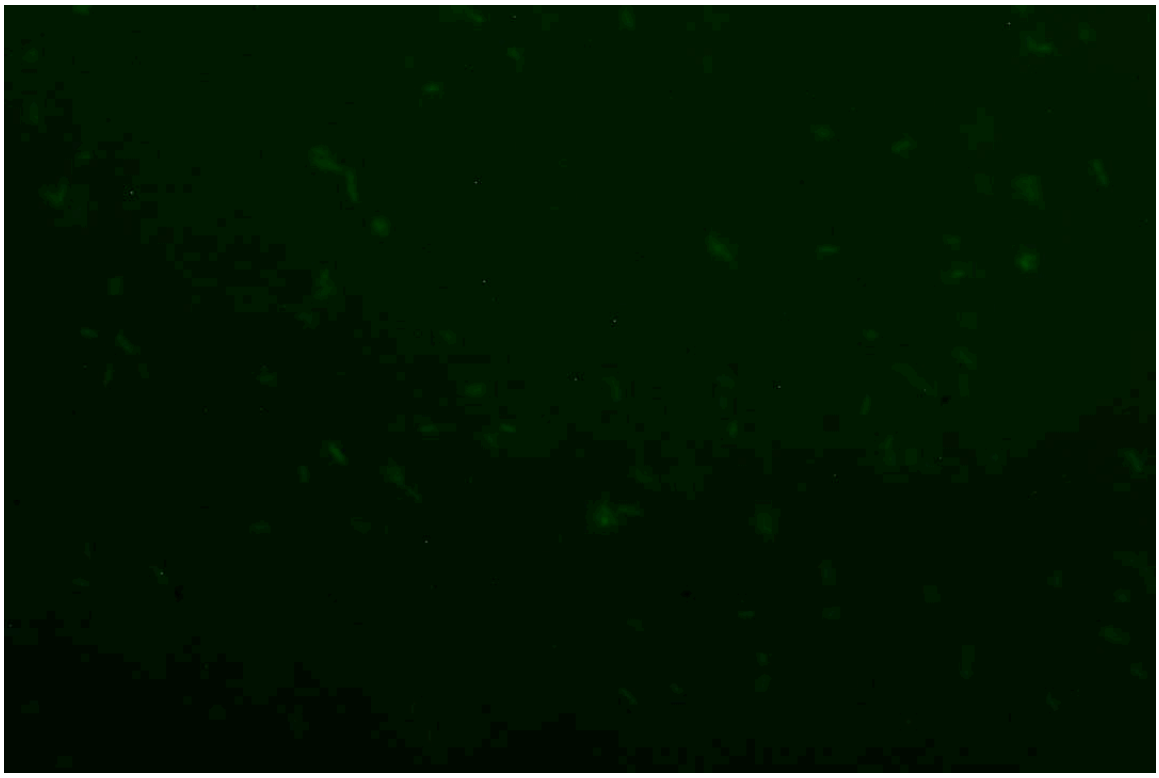

Figure 63: MDAI2(pCT6+pET200GFP)+W3110 UV 3 hour

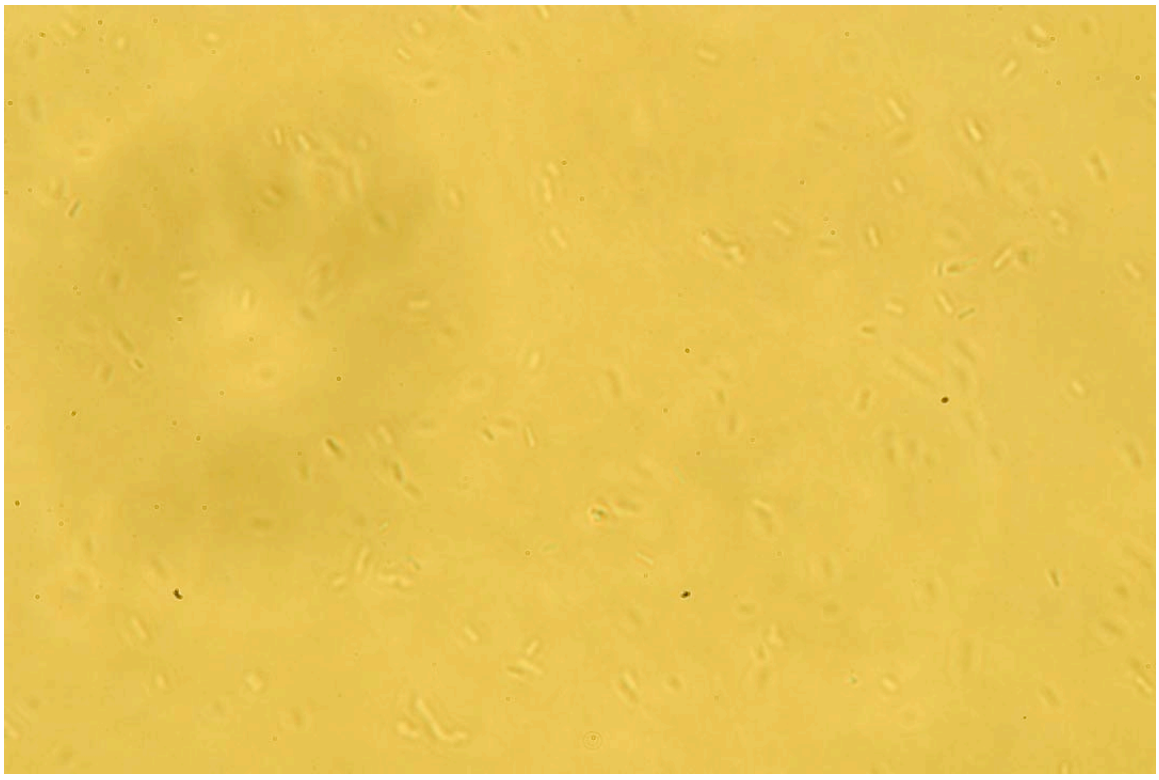

Figure 64: MDAI2(pCT6+pET200GFP)+W3110 white 3 hour

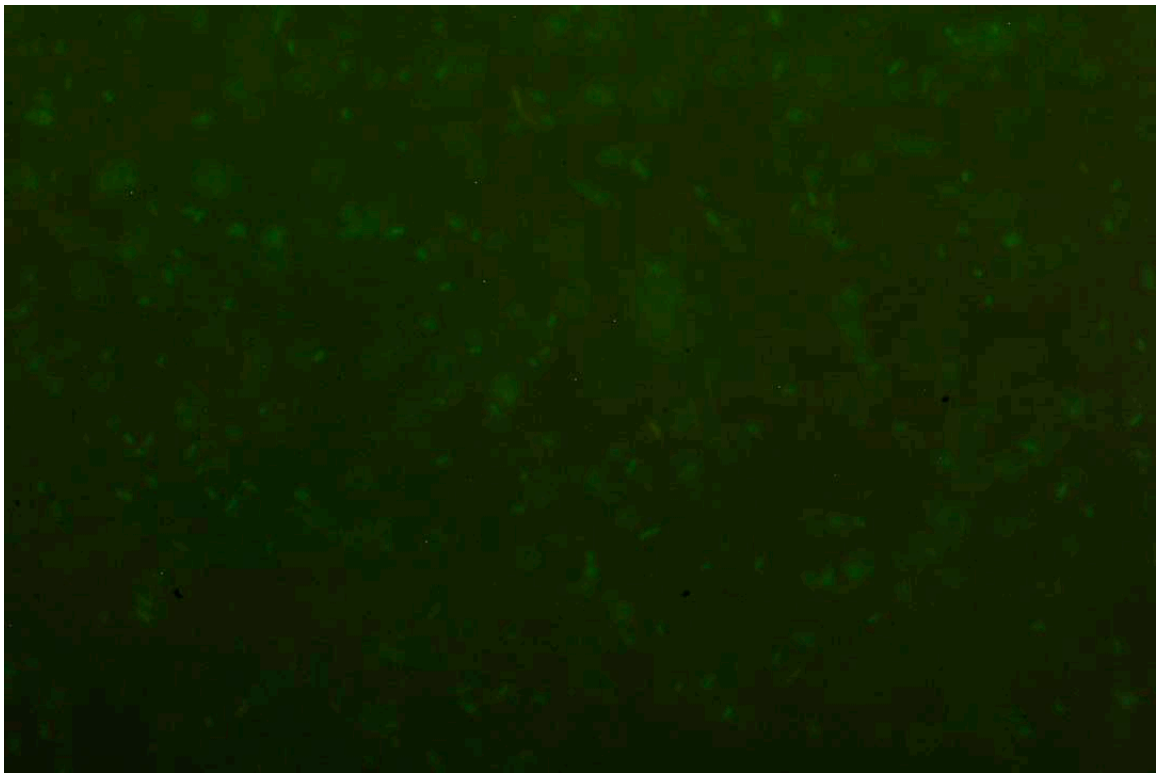

Figure 65: MDAI2(pCT6+pET200GFP)+W3110 UV 4 hour

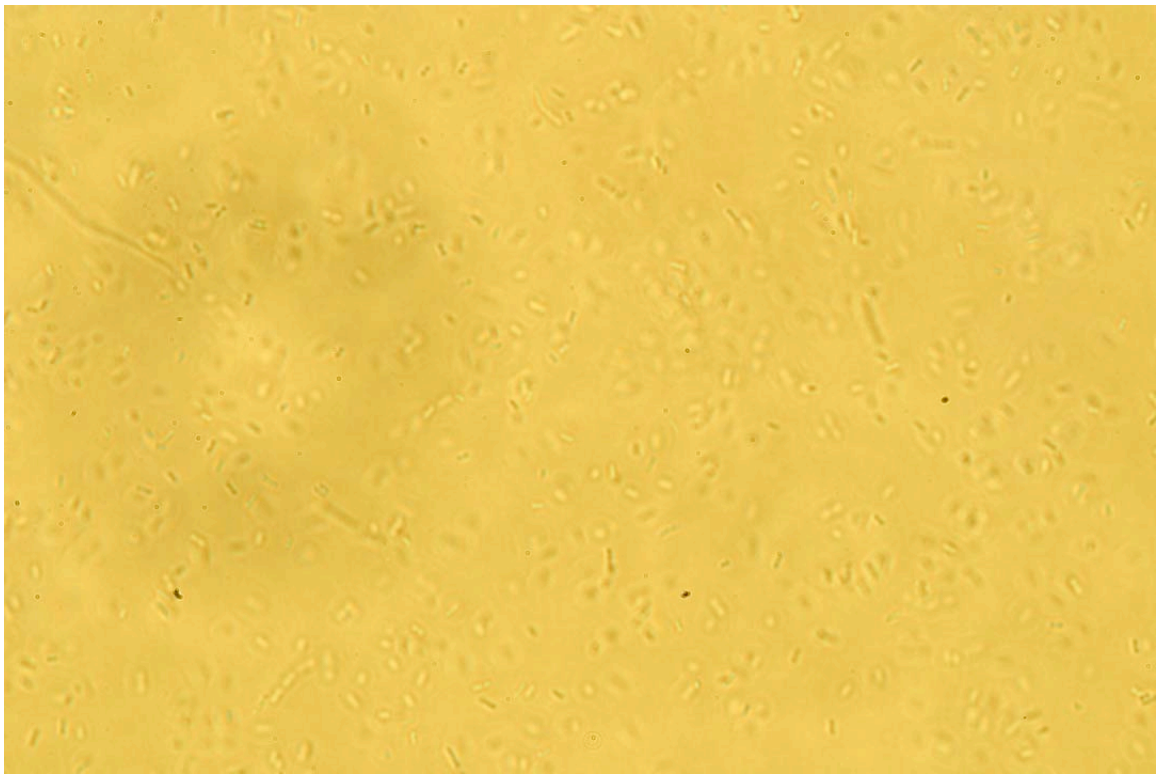

Figure 66: MDAI2(pCT6+pET200GFP)+W3110 white 4 hour

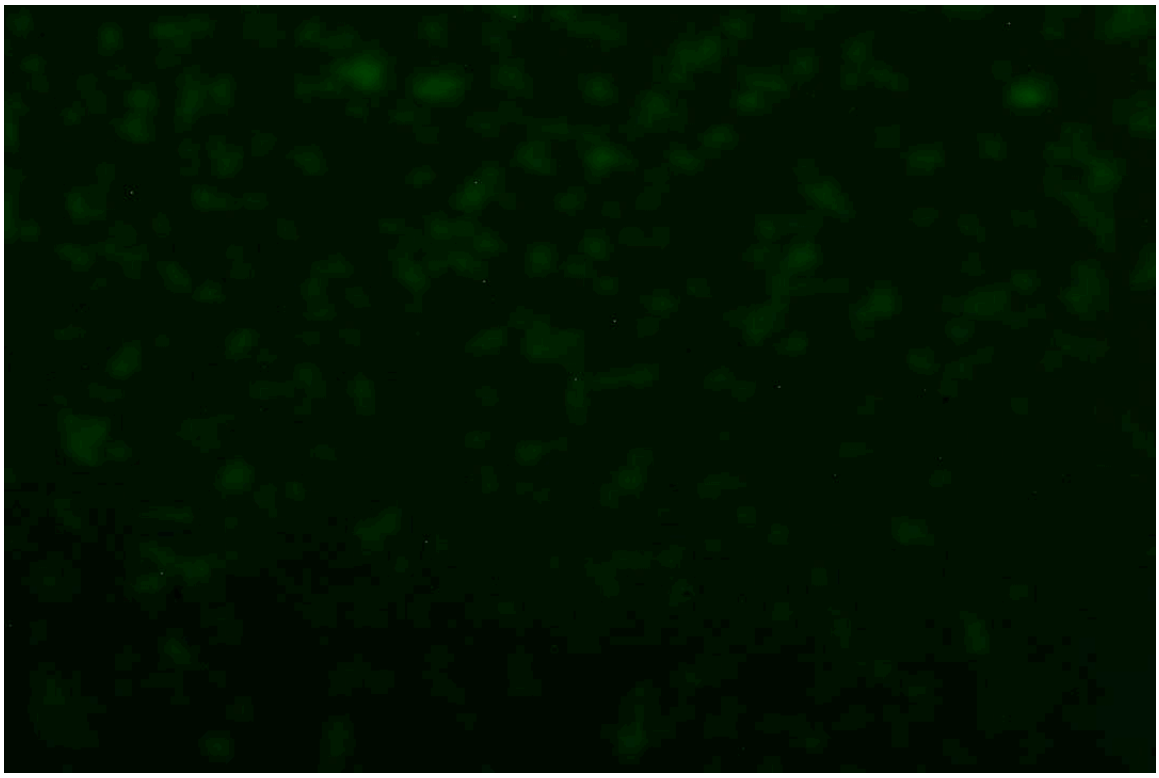

Figure 67: MDAI2(pCT6+pET200GFP)+W3110 UV 5 hour

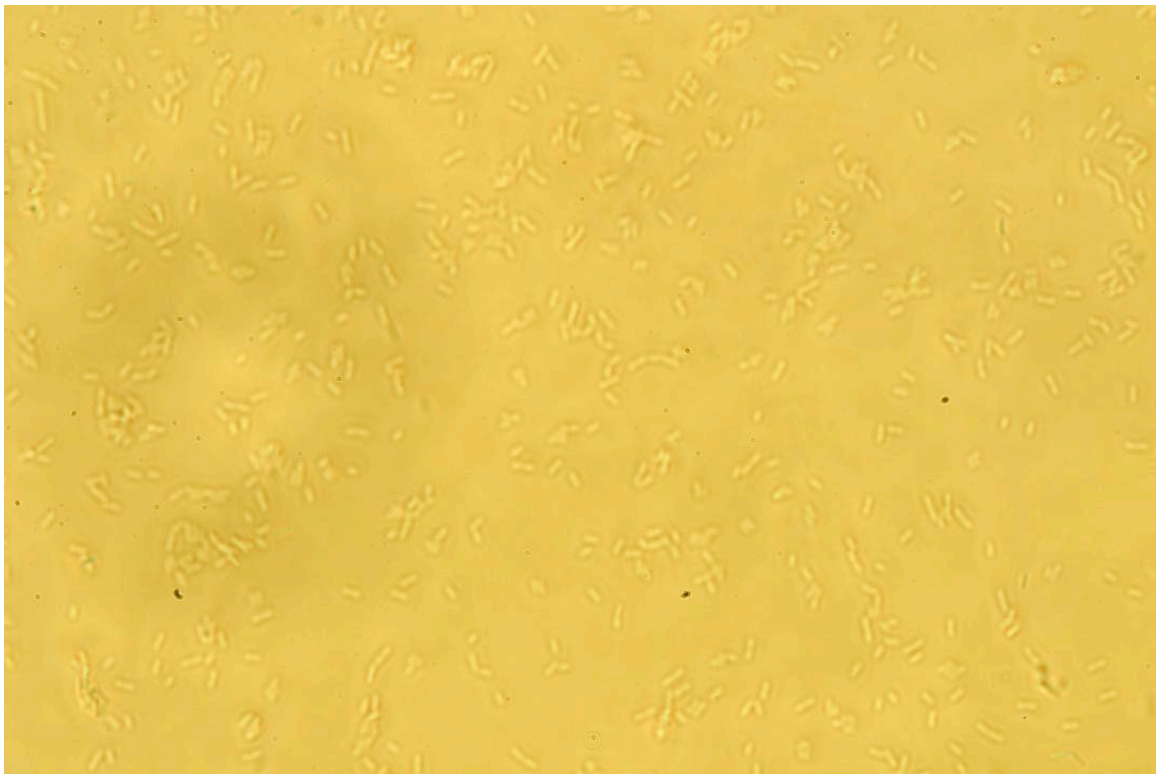

Figure 68: MDAI2(pCT6+pET200GFP)+W3110 white 5 hour