Engineering microbes to sense and eradicate a human pathogen

Saeidi, Nazanin

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TECHNOLOGICAL
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**ENGINEERING MICROBES TO SENSE AND
ERADICATE A HUMAN PATHOGEN**

NAZANIN SAEIDI

**SCHOOL OF CHEMICAL AND BIOMEDICAL
ENGINEERING**

2013

**ENGINEERING MICROBES TO SENSE AND
ERADICATE A HUMAN PATHOGEN**

NAZANIN SAEIDI

School of Chemical and Biomedical Engineering

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Abstract

Synthetic biology has allowed us to design and construct new biological systems that have the potential to resolve important issues related to healthcare. Considering the stalled development of advanced antibiotics and the emergence of antibiotic-resistant pathogens, we must now strive to exploit synthetic biology approaches for designing a new treatment regimen for infectious diseases. In this study we engineer microbes to detect and kill a pathogen using synthetic biology principles. We synthesize a genetic system comprised of quorum sensing, killing, and lysing devices. This system enables *Escherichia coli* (*E. coli*) to sense and kill a pathogenic strain of *Pseudomonas aeruginosa* (*P. aeruginosa*) through the production and release of pyocin S5. To reach our objective, we first design and characterize individual devices to understand their functionalities, which help us to construct the final system and verify its behavior. In the following steps, we show that our engineered *E. coli* detects and kills planktonic state *P. Aeruginosa*, evidenced by a >99% reduction in viable cells. Moreover, we confirm that our engineered *E. coli* inhibits the formation of *P. aeruginosa* biofilm by 90%, leading to much sparser and thinner biofilm matrices. Finally, to further optimize our system, we develop mathematical models using Computer Aided Design (CAD) tools that simulate both the dynamic and static performance of standard parts. We model the pathogen-sensing device of our sensing-killing system that produces Green Fluorescent Protein (GFP) as reporter in the presence of Acyl Homoserine

Lactone (AHL). The parameters of the model are based on experimental results. Since it is important that these models are searchable and readable by machines, standard SBML (System Biology Markup Language) format is used to store the model. All parts and reactions are fully annotated to enable easy searching, and the model follows the Minimum Information Requested In the Annotation of Models (MIRIAM) compliance as well as the Minimum Information About a Simulation Experiment (MIASE). The model accurately simulates experimental results.

In this thesis, we demonstrate that *E. coli* carrying the genetic circuit to sense and kill *P. aeruginosa* may provide a novel synthetic biology-based antimicrobial strategy. This approach could potentially be applied to struggle *P. aeruginosa* as well as other infectious pathogens. With the help of CAD tools we also show that the sensing device behaves as expected. This method can be used to model other parts of our system in order to make the entire system fully predictable.

1- Introduction

1-1- Infectious disease caused by drug resistance bacteria

Infectious diseases are one of the leading causes of death worldwide. Although many efforts have been made to control infectious pathogens, one upsetting fact remains: Wherever antimicrobial agents have been used for long period of time, resistant pathogens have evolved (zur Wiesch, Kouyos et al. 2011). Emergence of pathogenic bacterial resistance to multiple antimicrobial agents has become a major public health threat. Evolution of antibiotic-resistant bacteria has made it difficult to control many diseases (Palumbi 2001). The mortality rate of patients infected with drug-resistant bacteria is increasing, bringing the golden age of antibiotics to an end. For example, about six years after the introduction of penicillin (the first antibiotic) in 1943, more than half of the *Staphylococcus aureus* isolates became resistant to penicillin. This also occurred with chloroquine, the antibiotic that controls malaria (Barber and Whitehead 1949; Wongsrichanalai, Pickard et al. 2002).

Seventeen million people died of infectious disease in 1993. Approximately 70% of these deaths were due to diarrhea and pneumonia. Nowadays the top four infectious disease killers are diarrhea, pneumonia, tuberculosis and malaria. These top killers have not changed since 1900 (Cohen 2000). It has been estimated by the United States Centers for Disease Control and Prevention that 65,000 to 90,000 people die in United States annually from healthcare-associated (nosocomial) infections. Nosocomial infections are associated with treatment from hospitals or other healthcare units. *Staphylococcus*

aureus, *Enterococcus spp.*, *Enterobacteriaceae*, *Pseudomonas aeruginosa*, and *Acinetobacter spp.* are all examples of nosocomial infections that are prone to multi-drug resistance (Magiorakos, Srinivasan et al. 2012). The mortality rate of Intensive Care Unit (ICU) patients with bacteremia can be as high as 50%, especially in children and infants.

1-2- *Pseudomonas aeruginosa* as an opportunistic pathogen

Pseudomonas aeruginosa (*P. aeruginosa*) is a motile Gram-negative bacterium known for its environmental versatility. These rod-shaped vegetative cells can be found ubiquitously in soil, water, and moist places. Due to its impressive ability to adhere to surfaces of plants or animals (Costerton, Lewandowski et al. 1995), *P. aeruginosa* is capable of colonizing new habitats rapidly. As such, it has been recognized as an emerging pathogen of clinical relevance.

As mentioned in Section 1-1, nosocomial infections have a high mortality rate, and blood stream infection is one of the main killers among nosocomial infections (Armstrong, Conn et al. 1999; Wenzel and Edmond 2001). About 40% of blood stream infections are due to Gram-negative bacteria (Munoz, Cruz et al. 2008). *P. aeruginosa* is one of the most important Gram-negative pathogens, especially among nosocomial infections. The pathogen was estimated to be involved in at least 10% of bloodstream infections (Gordon, Serkey et al. 1998), 8% of surgical wound infections (Kluytmans 1997), 12% of urinary tract infections (Pollack 1995), and 16% of pneumonia infections (Wiblin 1997). Marrow transplant and neutropenic patients with compromised immune systems are particularly vulnerable to *P. aeruginosa*-associated outbreaks of pneumonia and

septicemia. These outbreaks accounted for a mortality rate of 30% (Fergie 1994). Figure 1-1 shows the infections caused by *P. aeruginosa* in different parts of the human body. The importance of colonization and adherence are evident when *P. aeruginosa* is found in the respiratory tract in cystic fibrosis patients. Cystic fibrosis is a genetic disease that results in thick mucus production that clogs the bronchial tubes. The wet and warm environment generated by the mucus allows *P. aeruginosa* to establish a permanent residence in the lungs. Thus, cystic fibrosis patients suffer from chronic *P. aeruginosa* infections, which are responsible for high rates of illness and death (Govan and Deretic 1996).

Figure 1-1. Infections caused by *P. aeruginosa* in different part of human body.

P. aeruginosa is an opportunistic pathogen; it rarely infects healthy people. Instead, it takes advantage of lapses in a host's immune system or infects an immuno-compromised patient. Other patients susceptible to life-threatening *P. aeruginosa* infections include bone marrow transplant and neutropenic cancer patients. In AIDS patients, *P. aeruginosa* bacterium is the main cause of the case fatality rate of 50% (Mendelson, Gurtman et al.

1994). *P. aeruginosa* is also capable of secreting exopolysaccharides such as alginate, which contributes to the formation of surface-associated communities enmeshed in the extracellular matrix (biofilm) (Ma, Conover et al. 2009). Biofilm formation among cystic fibrosis patients leads to breathing difficulties and fatalities.

Currents treatments of *P. aeruginosa* infection include chemotherapy using a combination of potent antibiotics and phage therapy with strain-specific bacteriophages that destroy the cellular integrity of the pathogen. However, such methods are ineffective because *P. aeruginosa* rapidly mutates and becomes resistant to synthetic drugs. Also, phage-directed treatment cannot be employed for a second time after the infected host develops immunity to the introduced virus.

Clinical treatments of infection initiated by *P. aeruginosa* remain a central issue among medical researchers due to the pathogen's enhanced resistance to numerous antibiotics. Several mechanisms have been proposed to elucidate its innate defense against antibiotics. For example, a multi-drug efflux system decreases cell envelope permeability against extracellular antibiotics. This mechanism is encoded by the genes MexAB, MexCD, MexEF, and MexXY for drug rejection, and expression of an antibiotic deactivation protein is encoded by the ampC gene (Lambert 2002). Drug exposure incites mutation in the antibiotic resistance genes over time, allowing *P. aeruginosa* to develop resistance against novel antibiotics and prevent vital metabolic activities from being affected by its introduction. Multi-drug resistant *P. aeruginosa* presents an unparalleled challenge to future medical treatments because its pace of spontaneous mutation far exceeds the rate at which artificial antibiotics are being developed.

P. aeruginosa has the ability to form biofilm. A bacterial community in biofilm is protected by an excreted matrix of extracellular polymeric substance (EPS) composed of polysaccharides and proteins. This biofilm impedes drug delivery to target sites of infection and greatly reduces the efficacy of antibiotics. Three possible mechanisms of antibiotic resistance in biofilms are shown in Figure 1-2 (Stewart PS.; Costerton 2001) . The first defense mechanism (yellow) is slow or incomplete penetration of the antibiotic into the biofilm after being deactivated in the surface layers. The second mechanism (green) proposes that a subpopulation of *P. aeruginosa* in biofilm differentiates into a highly protected phenotypic state that is invulnerable to antibiotics. The third protective system (red), which occurs deep within the biofilm, illustrates a nutrient-depleted zone rich in waste concentrations where antibiotic effects are nullified. These mechanisms allow *P. aeruginosa* to persist on infected surfaces and cause recurrent infections.

P. aeruginosa is highly adaptable to different host environments. In addition to its potential to exploit diverse carbon sources ranging from glucose to diesel for growth and metabolism, it is also able to survive in anaerobic conditions by utilizing nitrate or arginine as a respiratory electron acceptor. These strategies are critical to its survival in challenging conditions such as cystic fibrosis infections where oxygen diffusion is restricted.

Figure 1-2. Antibiotic Resistance in Bacterial Biofilm (Stewart PS.; Costerton 2001)

1-3- Quorum sensing mechanism in *Pseudomonas aeruginosa*

Quorum sensing is the regulation of gene expressions in bacteria in response to the changes in the bacteria's population density (Miller and Bassler 2001). It enables the bacteria to sense their own population density and to communicate with each other and behave as a population instead of individual cells (Gray 1997). To do so, quorum sensing bacteria produce and release a type of chemical signaling molecule known as autoinducers. The concentration of autoinducers is a function of cell density; it increases when the bacteria's population density increases. When the threshold stimulatory concentration of autoinducers is reached, it leads to a shift in gene expression. Both Gram-positive and Gram-negative bacteria use a quorum sensing communication circuit to regulate an array of physiological activities such as symbiosis, virulence, motility, and

biofilm formation (refer to Figure 1-3) (Miller and Bassler 2001). Common examples of autoinducers include acyl homoserine lactones (AHLs) that direct gene expression in Gram-negative bacteria (Greenberg 1999), oligo-peptides in Gram-positive bacteria (Kleerebenzem M 1997) and autoinducer-2 (AI-2), which is recognized by both categories of bacteria (K. Winzer 2002).

Figure 1-3. Diagram of *P. aeruginosa* biofilm maturation pathway

The high density microcolonies differentiate into mature biofilms using AHLs such as 3OC₁₂HSL (Parsek and Greenberg 2000).

In the case of Gram-negative bacteria such as *P. aeruginosa*, acylated homoserine lactones (AHLs) (see Figure 1-4) are used as the chief signaling molecules in the quorum sensing mechanism. AHLs are produced from substrates such as S-Adenosyl Methionine (SAM) and acylated acyl carrier protein (Acyl-ACP) (Greenberg 2000; Miller and Bassler 2001) . These substrates are metabolic products of cellular activities (Moré, Finger et al. 1996). *P. aeruginosa* utilizes the enzyme lasI autoinducer synthase to convert SAM and Acyl-ACP into diffusible N-3-oxododecanoyl-L-homoserine lactone (3OC₁₂HSL) (see Figure 1-4) in the Las quorum sensing system (Parsek and Greenberg 2000).

Figure 1-4. Autoinducer biosynthesis pathway

AI synthase represents luxI homologs; ACP as acyl carrier protein; AdoMet as S-adenosyl methionine; MTA as methylthioadenosine (Parsek and Greenberg 2000).

The Las quorum sensing system is the first quorum sensing system to be discovered in *P. aeruginosa*. Its name stems from its ability to regulate the virulent lasB elastase expression (Gambello and Iglewski 1991; Passador, Cook et al. 1993; Rust, Pesci et al. 1996). The Las quorum sensing system is comprised of lasI, the autoinducer synthase gene responsible for producing 3OC₁₂HSL, and the lasR gene that codes the R-type transcriptional activator protein (Pearson, Gray et al. 1994). The expressions of both of these genes are highly dependent on the cell density. An increase in cell population density leads to an accretion of 3OC₁₂HSL concentration in the effluent (see Figure 1-5). Previous studies have estimated the extracellular concentration of PAI (the first type of

quorum sensing system in *P. aeruginosa*) to be between $1\text{E-}6$ and $1\text{E-}4$ M within close proximity of the site of infection (Givskov 2007). Once the basal concentration of $3\text{OC}_{12}\text{HSL}$ has reached the minimum threshold, it triggers a positive feedback loop whereby $3\text{OC}_{12}\text{HSL}$ is bounded to LasR protein to form a LasR/ $3\text{OC}_{12}\text{HSL}$ complex. After that, the complex in turn activates the expression of *lasI* gene. The preferential *lasI* expression allows an initial rapid rise in autoinducer synthase synthesis, which consequentially increases the amount of $3\text{OC}_{12}\text{HSL}$ available for LasR protein binding.

Figure 1-5. *P. aeruginosa* produces and secretes $3\text{OC}_{12}\text{HSL}$ via enzymatic conversion of SAM and Acyl-ACP. Secreted $3\text{OC}_{12}\text{HSL}$ tends to accumulate in the effluent over time as cell-population density increases.

The positive feedback loop in the Las system contributes to the swift increase in the expression of virulence genes such as *lasB* elastase, exotoxin A, and alkaline protease (Gambello, Kaye et al. 1993). It also positively regulates other quorum sensing systems such as the Rhl system, which relies on N-butanoyl-L-homoserine lactone (C_4HSL) as signaling molecules (Pesci, Pearson et al. 1997; Kiratisin, Tucker et al. 2002; Rampioni, Bertani et al. 2006; Bosgelmez-Tinaz and Ulusoy 2008). The Rhl system is comprised of

rhlR transcriptional regulator and rhlI synthase, which generate C₄HSL with some subtle production of C₆HSL. It is activated after initiation of the Las system and controls the expression of rhlAB operon that encodes for rhamnosyltransferase to synthesize rhamnolipid (Ochsner, Fiechter et al. 1994), a biosurfactant that emulsifies hydrocarbon substrate for utilization by the cells. The Rhl quorum sensing system is known as VSM (Virulence Secondary Metabolites) as it also regulates the expression of virulence factors lasA, lasB and pyocyanin (Brint JM 1995; Latifi A 1995; Pearson, Pesci et al. 1997). Uptake and secretion of C₄HSL and 3OC₁₂HSL differ due to disparate molecular length and hydrophobicity of the substituent groups. C₄HSL is freely diffusible through the cell membrane while 3OC₁₂HSL requires an active efflux system (Pearson JP 1999). Studies have shown that the combination of autoinducer and its respective R-proteins is highly specific in terms of transcriptional activity (Latifi A 1995; Pearson, Pesci et al. 1997), i.e. C₄HSL will not activate lasR protein and vice versa. Additionally, the resultant activator complex is only sensitive to specific promoters. It is, however, important to note that neither system is completely independent of the other. Figure 1-6 illustrates cellular communication cascade in *P. aeruginosa* and the interdependency between LasR/3OC₁₂HSL and RhlR/C₄HSL quorum sensing systems. The Las system not only exerts control as an upstream transcriptional regulator to RhlR expression, but is also able to monitor activity of the Rhl system via post-translational competitive binding of 3OC₁₂HSL to RhlR protein (Pesci, Pearson et al. 1997). This can be seen in Figure 1-6.

These quorum sensing systems are responsible for the production of a plethora of other virulent factors (Ochsner, Fiechter et al. 1994; Pearson, Pesci et al. 1997). As a result, 3OC₁₂HSL alone has been touted as the main contributor to *P. aeruginosa*'s virulence

(Van Delden and Igleswski 1998). The positive feedback in the Las system can also be down-regulated when the inhibitor for lasI transcription, RsaL, is being positively regulated by the LasR/3OC₁₂HSL complex (Rampioni, Bertani et al. 2006). The RsaL protein then competes with the activated LasR complex for the lasI transcriptional binding site or promoter (Kievit, Seed et al. 1999). The entire Las quorum sensing hierarchical system is depicted in Figure 1-7.

Figure 1-6. Cell to cell signaling circuitry of *Pseudomonas aeruginosa* (Pesci, Pearson et al. 1997)

The Las quorum sensing system is also observed to be homologous to the Lux quorum sensing system in *Vibrio fischeri* (*V. fischeri*) (Whiteley and Greenberg 2001). Both systems require an autoinducer synthase (lasI in *P. aeruginosa* and luxI in *V. fischeri*) that generates acylated homoserine lactone signals (3OC₁₂HSL in *P. aeruginosa* and

3OC₆HSL in *V. fischeri*) and an R-type protein that serves as a signal receptor and transcriptional activator (LasR in *P. aeruginosa* and LuxR in *V. fischeri*) (Schuster, Urbanowski et al. 2004). In fact, the Las and Lux systems are so similar that the autoinducer and transcriptional activator from the Las system (3OC₁₂HSL and LasR protein) can be used to activate the Lux system and vice versa (Gray, Passador et al. 1994). This interchangeability trait offers us an opportunity to utilize the promoter of the genetically designed receiver F2620 (Canton, Labno et al. 2008), which was modeled after the Lux system, to detect the signaling molecule (3OC₆HSL) generated by *V. Fischeri*.

Figure 1-7. Schematic of quorum sensing system in *p. aeruginosa*

As extracellular 3OC₁₂HSL attains the required threshold, it diffuses into adjacent cells, and thus triggers the positive feedback loop for 3OC₁₂HSL production, while RsaL inhibitor prevents over-production of 3OC₁₂HSL. LasR/3OC₁₂HSL complex also positively regulates the expression of other virulent factors and quorum sensing systems.

Similar production mechanisms are also present in other Gram-negative bacteria, with each luxI homolog producing AHL that differs in either length or functional groups (hydroxyl group, carbonyl group, or hydrogen atoms) on the acyl side-chain. Thus, with each bacterium possessing a disparate synthase sequence, a high level of specificity can be achieved during intercellular quorum communication. A list of common AHLs with their associated species is shown in Table 1-1.

Two mechanisms have been proposed to explain quorum sensing activation in *V. fischeri*. The first theory suggests that in the absence of an autoinducer, the amino terminus domain of LuxR protein binds to the binding domain of the promoter region and obstructs the entrance of RNA polymerase. Association of LuxR dimer to a molecule of 3OC₆HSL then forms a complex with effective binding affinity greater than that of LuxR to pLux promoter, which releases the promoter and activates transcription (Nealson KH 1970; Moré, Finger et al. 1996). The second theory, which is generally adopted for system biology modeling due to ease of implementation, involves transcriptional activation via the binding of the R-protein/autoinducer complex to the DNA binding domain of promoter (Passador, Cook et al. 1993; Stevens AM 1997).

Table 1-1. Structures of AHLs and its associated bacteria

Full Name	Molecule Abbreviation	Species	Chemical formula
Butanoyl-homoserine lactone	C4-HSL	<i>P. aeruginosa</i>
N-(3-oxododecanoyl) homoserine lactone	3OC12-HSL	<i>P. aeruginosa</i>
3-oxohexanoyl-homoserine lactone	3OC6-HSL	<i>V. fischeri</i>
Hexanoyl-homoserine lactone	C6-HSL	<i>C. violaceum</i>
Heptanoyl-homoserine lactone	C7-HSL	<i>E. psidii</i>
Octanoyl-homoserine lactone	C8-HSL	<i>B. cepacia</i> , <i>V. fischeri</i>
3-oxooctanoyl-homoserine lactone	3OC8-HSL	<i>A. tumefaciens</i>
Decanoyl-homoserine lactone	C10-HSL	<i>B. pseudomallei</i>

1-4- Fundamentals of Synthetic Biology

Synthetic biology is an emerging research field that applies an engineering approach to the design and construction of genetic circuits. What distinguishes this nascent field from traditional genetic engineering is its approach and framework. The traditional genetic engineering approach is an improvised process for which costs, time to completion, and probabilities of success are hard to gauge accurately (Canton, Labno et al. 2008). To

overcome such problems and to make the process of engineering biological systems easier, synthetic biology has developed a design and construction framework that routinely incorporates biological functions into a genetic system. This system is comprised of many integrated components that behave as expected. Its emphasis is on engineering principles such as abstraction, decoupling, and standardization (Endy 2005). Genes from different organisms can be assembled to create new metabolic pathways.

In order to survive and proliferate, bacteria must detect a wide range of environmental signals with high sensitivity. These signals include pH, temperature, light, and chemical substances. The genetic information encoding many such mechanisms has been unveiled, making bacteria great candidates for biosensors. The receiver device in engineered bio-sensing bacteria, which has been taken from the wild strain, is ligated upstream of a reporter gene such as Green Fluorescence Protein (GFP). The amount of input signals can then be inferred from the concentration of reporter proteins being produced, which is measurable. Examples include engineered bacteria that can sense poisonous arsenic in drinking water (J. Aleksic 2007), red light (Levskaya A 2005), or various types of AHL produced by pathogenic bacteria (Canton, Labno et al. 2008).

Another promising area for applications of synthetic biology is the field of metabolic engineering. Natural metabolic pathways in microorganisms can be employed and modified to provide cost-effective chemical and drug synthesis. One medical application that has developed from synthetic biology is the control of falciparum malaria caused by a protozoan of the genus *Plasmodium*. This disease is estimated to cause deaths of one to three million people per year, with worldwide contagion of 300 to 500 million (Jacobs-

Lorena 2003; Al-Jabri 2004). Although the drug artemisinin has proven effective in the treatment of malaria, current methods of production are too expensive. Therefore it cannot realistically the malaria situation in developing Sub-Saharan Africa. In 2006, Dr. Jay Keasling, one of the leading authorities in the field of synthetic biology, achieved success with the production of artemisinin precursors in metabolically engineered *E. coli*. This breakthrough allows the production cost of artemisinin to be drastically lowered from US\$2.40 per dose to US\$0.25 per dose (DK Ro 2006). Other groups, including the Silver group at Harvard and the Liao group at UCLA, are focusing on engineering microorganisms for biofuel production (Hanai, Atsumi et al. 2007; Atsumi, Cann et al. 2008; Savage, Way et al. 2008). Great strides have also been made to create minimal genomes required to support life (Murtas 2007). Cells with minimized genomes will serve as ideal chassis for production of pharmaceuticals or biofuels because they do not waste energy on unnecessary processes such as motility.

The third application involves the combination of sensing and manufacturing abilities in microorganisms. They can be engineered to be intelligent carriers for targeted drug delivery. One example is the design of tumor-killing bacteria (see Figure 1-8) (Anderson 2006). Upon simultaneously detecting a hypoxic environment and high bacterial population density, engineered bacteria will invade and produce toxins to destroy cancerous cells. The AND gate is included in the detection part to enhance the specificity of sensing the environment surrounding tumors (Anderson 2007).

Figure 1-8. Tumor-Killing Bacteria

1) Engineered bacteria are injected into the bloodstream; polysaccharide molecules on their surfaces allow them to evade the immune system; 2) When they detect the low-oxygen environment of a tumor, the bacteria produce invasion, a protein that allows them to infiltrate the cancer cells; 3) The invasion binds to the cancer cells, prompting the cells to engulf the bacteria; 4) The cancer cell bursts the bacterium, releasing a toxic enzyme that kills the cell (Pak, Janssen et al. 2010).

Having drawn lessons from mature engineering disciplines, synthetic biology has used the concept of abstraction to reduce the complexity of biological processes and components. Using the concept of abstraction, biological components can be viewed as simple modules (see Figure 1-9). In addition to abstraction, synthetic biology has also

introduced the element of standardization. This element helps to create sets of standardized and functional biological modules that can be reused in combination and coupled with composition rules that define how such modules can be assembled (Andrianantoandro, Basu et al. 2006). Implementing standardization of biological components will not only allow re-usability but also bring about inter-operability of the components in different devices or systems. Both advantages translate to economic benefits; time-to-completion and costs can be reduced significantly. For every new innovation to be relevant in the practical world, it is paramount that such inventions are standardized and characterized by their inherent properties. While standardization allows for the architecture of intricate systems from modular units, characterization provides designers with critical information to optimize operational performance, durability, and safety. Characterization of standard biological parts also aims to describe the intrinsic properties of discrete biological parts appropriately so as to enable controlled and predictable performance of synthetic biological systems (Endy 2005). Recent advances in synthetic biology have provided a unified foundational methodology for conducting characterization experiments of biological parts (Canton, Labno et al. 2008). Published works at present include the measurement of promoter activity for constitutive and inducible promoters and functional biological logic gates and quorum sensing elements. These are strategic tools in either transcriptional control or intercellular communication.

Figure 1-9. Abstraction hierarchy of Synthetic Biology, in comparison to that of computer engineering, similarities are observed (Andrianantoandro, Basu et al. 2006).

One popular technical standard that has been widely adopted by synthetic biologists is the BioBrick standard, an idea that was proposed by Dr. Tom Knight (Massachusetts Institute of Technology, USA) and later developed by Dr. Drew Endy (Dr. Knight's former colleague at MIT). The BioBrick standard defines the physical composition of the biological deoxyribonucleic acid (DNA) parts. Any biological genetic component that conforms to the BioBrick standard is known as a BioBrick part. A BioBrick part is a strand of DNA that codes for a biological function (e.g. promoter / ribosome binding site / gene / terminator). The promoter allows RNA polymerase to bind and initiate transcription upon detection of the transcription start site. The ribosomal binding site encodes genetic information for the binding of ribosomal subunits on mRNA transcript to activate translation. Protein coding sequences code for functional proteins that are largely

associated with the application of interest. Terminator sequences stop transcription by forming a hairpin secondary structure that disrupts the DNA-mRNA-RNA polymerase ternary complex. BioBricks have universal connectors that allow them to be linked together to form higher level components such as devices, or systems to perform user-defined functions such as switches, pulse generators, amplifiers, oscillators, or logic gates (see Figure 1-10). These devices can be introduced into the host cells, where they can initiate new metabolic processes or control the host's cellular activities. Ongoing research by Dr. Chris Anderson involves programming harmless bacteria cells to 'swim' towards oxygen-deficient cancerous tissues, after which anti-cancer peptides are synthesized and released within locus of such tissues. These highly systemic tasks occur by means of several biological devices: a device that detects the location of cancerous tissues, a device that orientates the mobility of the microbe towards cancerous tissues, a device that produces anti-cancer peptides, and a device that enables the release of anti-cancer peptides by apoptosis (JC Anderson 2005).

Figure 1-10. A hierarchy of BioBrick parts to systems.

Multiple BioBrick parts form a device, and multiple devices form a system. A synthetic biological construct may consist of multiple systems (Rouilly 2007).

To create a BioBrick part, universal connectors have to be added to both ends of the DNA sequence. The universal connectors defined by the BioBrick standard are the endonuclease sites: EcoRI/NotI/XbaI for the upstream end of DNA sequence termed as prefix and SpeI/NotI/PstI for the downstream end of the DNA sequence termed as suffix (see Figure 1-11). XbaI and SpeI are isocaudomeric, meaning that they possess different restriction enzyme recognition sites but generate similar termini upon cleavage. The introduction of XbaI and SpeI in prefix and suffix sequences enables BioBricks to be cleaved individually in order to anneal and form longer composites and devices (Shetty R 2008). Once one BioBrick part is ligated to another BioBrick part, the composite object forms a new BioBrick part. It can then be combined with any other BioBrick part. As shown in Figure 1-11, a ‘scar’ will develop once the two endonuclease sites, XbaI and SpeI, are joined together.

Figure 1-11. BioBricks assembly schematic

Two BioBrick parts, A & B can be joined together to form a new BioBrick part due to the presence of universal prefix (EcoRI/NotI/XbaI restriction sites) and suffix (SpeI/NotI/PstI restriction sites).

Based on this methodology, BioBrick parts can be assembled to form a multiple-component synthetic biological device / system. The behavior of the newly constructed BioBrick device or system can be analyzed by introducing the device or system into host cells such as *E. coli*. Thus, by utilizing the BioBrick standard and the BioBrick library that contains a wide and diverse range of BioBrick parts, a designed synthetic system can be constructed.

However, before the construction can begin, the design of the genetic circuitry has to be verified via modeling. The model is created by using the transfer function of each BioBrick part (derived by previous users) to predict the outcome. Once the design has been validated by the deterministic equations of the simulation, the design is can then be

implemented in the wet lab. The wet lab results are analyzed, and refinements to the design are made accordingly. The flow chart for the synthetic biology approach is illustrated in Figure 1-12.

Figure 1-12. Flow chart of the Synthetic Biology approach

Considering the synthetic biology framework, we aim to use this promising approach to tackle *P. aeruginosa* pathogenicity. We have equipped our host cell *E. coli* to be able to detect and attack *P. aeruginosa*. To do so, three genetic devices were designed and characterized accordingly. The first device senses the presence of *P. aeruginosa* in an infected environment. Once the *P. aeruginosa* is detected, the second device is triggered. This device produces bacteriocin as the antimicrobial peptide to kill *P. aeruginosa*.

Finally, to release the accumulated bacteriocin into the environment, the third device is introduced. This device lyses *E. coli* and releases the bacteriocin to attack *P. aeruginosa*.

1-5- Bacteriocins and their role to preserve the dominance of bacteria

Bacteriocins are proteins produced by bacteria to elicit bactericidal activities against other strains of bacteria. Bacteriocins are named according to the bacterial species that produces them, such as colicins from *E. coli*, cloacins from *Enterobacter cloacae*, pesticins from *Yersinia pestis*, and klebicins from *Klebsiella*. Bacteriocins produced in *P. aeruginosa* are termed pyocins. Pyocins are narrow-spectrum bacteriocins produced by clinical isolates of *P. aeruginosa* and named after the species' former name: *Pseudomonas pyocyanea* (AM. 2001). They were first discovered by Francois Jacob (Jacob 1954). Ecologically, the main purpose for pyocin production is to preserve the dominance of a given strain in a bacterial niche upon exposure to mutagenic agents such as mitomycin C, UV, and H₂O₂ that provoke DNA damage (Michel-Briand and Baysse 2002; Chang, Small et al. 2005; Waite and Curtis 2009). Another reason for pyocin production is to defend a bacteria's ecological niche against rival bacteria strains in mixed populations (Waite and Curtis 2009). Most of the *P. aeruginosa* strains (around 90%) produce pyocins, and each strain may synthesize more than one type of pyocin (Michel-Briand and Baysse 2002). *P. aeruginosa* strains that produce pyocins are insensitive to their own pyocins, but their secreted pyocins act against other bacteria of the same species or other species. The killing mechanism of pyocin is initiated upon binding of pyocins to the specific receptors in the outer membrane of the susceptible cells. Because of immunity proteins, most pyocinogenic strains are insensitive to their

own pyocins, which helps to preserve the initial predominance of pyocinogenic bacteria against pyocin-sensitive cells (Goodwin, Levin et al. 1972). Three main types of pyocins are produced by *P. aeruginosa*: R-type, F-type and S-type. R-type pyocins (rod like) resemble bacteriophage (P2 phage) tails that are non-flexible and contractile. Their killing mechanism lies in binding their tail fibers to targeted bacterial surface receptors and then killing by forming pores in the target cell membrane (Scholl and Martin 2008). F-type pyocins also resemble bacteriophage (λ phage) tails, but they are flexible and non-contractile. S-type pyocins are colicin-like and Protease-sensitive proteins (Waite and Curtis 2008), the S-type was first discovered by Ito and Kageyama with an isolate of *P. aeruginosa* that was known to produce R-type pyocin (S.I. Ishii 1965). These pyocins, which have a range of 498 to 776 amino acids, consist of three main parts: 1) N-terminal binding domain, 2) a translocation domain, and 3) C-terminal killing domain (Michel-Briand and Baysse 2002). The killing mechanism of most S-type pyocins (AP41, S1, S2, and S3) is through DNA breakdown because of an endonuclease C-terminal domain (Michel-Briand and Baysse 2002). Genomic analysis of the S4 coding sequence illustrated ternary structures analogous to tRNase proteins (Michel-Briand and Baysse 2002; Parret and De Mot 2002). The immunity component of S-type pyocins, which have 87 to 153 amino acids, renders pyocinogenic strains immune to their produced pyocins. Table 1-2 summarizes S-type pyocins.

Table 1-2. S-Type Pyocin

S-Type Pyocin	Mode of Killing	Reference
S1	DNase	(S. Ito 1970) S. Ito, M. Kageyama, F. Egami
S2	DNase	(I. Ohkawa 1973) I. Ohkawa, M. Kageyama, F. Egami
S3	DNase	(Govan 1986) J.R. Govan
S4	tRNase	(Parret and De Mot 2000) A. Parret, R. De Mot
S5	Pore forming	(Parret and De Mot 2000) A. Parret, R. De Mot
AP41	DNase	(Y. Sano 1981) Y. Sano, M. Kageyama

1-5-1- Pyocin S5 and its antimicrobial efficiency

We are interested in the S-type pyocins because they are mainly responsible for bactericidal activities. Also, they are soluble. More importantly, only 70% of *P. aeruginosa* strains produce S-type pyocins (in contrast with more than 90% producing R- and F- type pyocins) (Smith, Hirst et al. 1992). Hence the remaining 30% of *P. aeruginosa* strains may be susceptible to S-type pyocins.

The availability of *P. aeruginosa* genome sequences enables the prediction of a sub-type of additional pyocin gene. This predicted pyocin, known as S5, contains translocation-receptor binding-killing domains. The killing domain shares 46 % homology with that of colicin Ia, a pore-forming bacteriocins (see Figure 1-13) (Parret and De Mot 2000).

Pyocin S5 kills susceptible cells by forming a voltage-gated, ion-conducting channel across the plasma membrane (Wiener, Freymann et al. 1997). Pyocin S5's bactericidal activity closely resembles that of the pore-forming colicin Ia protein produced by *E. coli*.

For the first time, we experimentally demonstrated the antimicrobial spectrum, activity, and mechanism of pyocin S5.

(A) Pyocin S5 domains

(B) Secondary structure

Figure 1-13. The pyocin S5 domains, and the secondary structure analysis of pyocin S5 and colicin Ia.

(A) A schematic diagram three main domains of pyocin S5. (B) The secondary structure of pyocin S5 (498 amino acids) and colicin Ia from *E. coli* (626 amino acids, GenBank ID: ZP_02783400.1), analyzed using the Hierarchical Neural Network (HNN) program (Ling, Saeidi et al. 2010) .

1-5-1-1- Antimicrobial efficiency of pyocin S5 against *P. aeruginosa*

Pyocin S5 is proven to successfully kill various strains of *P. aeruginosa*. Minimum Inhibitory Concentrations (MICs) were measured to evaluate the antimicrobial activity of pyocin S5 against different sensitive strains. As shown in the work done by Ling et al.

DWW3 (isolated from cystic fibrosis patients) possessed the highest sensitivity to pyocin S5 (MIC of 12.6 µg/ml) (Ling, Saeidi et al. 2010).

P. aeruginosa PAO1, *E. coli*, and *Staphylococcus aureus* were insensitive to pyocin S5 even at the highest available pyocin S5 concentration (2.16 mg/ml). There are two possible explanations for this insensitivity: (1) These strains have no specific receptor on the cell surface for pyocin S5 and/or there is no translocation system for pyocin S5 on these strains, or (2) Pyocin S5 immunity protein might be expressed in these strains, which would make it resistant to pyocin S5 by binding to the C terminal of pyocin S5. The S5 immunity protein (108 amino acids encoded by PA0984) potentially resides in the inner membrane and its immunity mechanism is through specific intramembrane helix-helix interactions with pyocin S5. For strains sensitive to pyocin S5, the antimicrobial activity is initiated by binding of pyocin S5 to its receptor on the cell membrane. Even though there are several known receptors for pore-forming colicins and other types of pyocins like Cir, BtuB, FpvA, and lipopolysaccharide (Lazdunski, Bouveret et al. 1998; Cascales, Buchanan et al. 2007; Barreteau, Bouhss et al. 2009; Kohler, Donner et al. 2010), to date there has not been any characterized receptor for pyocin S5.

1-5-1-2-Pyocin S5 mode of action

As mentioned before, pyocin S5 shows 46% homology to colicin Ia in the killing domain. For colicin Ia it is proposed that it binds to specific receptors on the outer cell membrane and is finally translocated to the cytoplasmic membrane (Tokuda and Konisky 1978). Therefore it was hypothesized that the killing mechanism of pyocin S5 might be through

cytoplasmic membrane damage, which leads to the leakage of intracellular materials and the increase of membrane permeability.

To investigate the leakage of nucleic acids and Adenosine-5'-TriPhosphate (ATP) from DWW3 cells after exposure to pyocin S5, absorbance at 260 nm and ATP release in cell-free supernatant were measured by Ling *et al.* (Ling, Saeidi et al. 2010). Figure 1-14A shows a threefold increase in absorbance at 260 after treatment with pyocin S5. This increase confirms the leakage of nucleic acids from DWW3 cells after exposure to pyocin S5. Similarly, Figure 1-14B shows that the relative luminescence unit (RLU) in the supernatant after treatment with pyocin S5 for one hour is about two times higher than the control cells. The increase in the luminescence signal represents the ATP release from the cells through the membrane damage. This phenomenon is due to the ATP-luciferin reaction, which is catalyzed by luciferase in the presence of Mg^{2+} and molecular oxygen.

To confirm the membrane permeability of DWW3 cells after exposure to pyocin S5 as another characteristic of membrane damage, live and dead kits were performed. Figure 1-14C and 1-14D show that DWW3 cells without treatment are mostly stained with SYTO9 (green) while the majority of DWW3 cells after exposure to pyocin S5 are stained with PI (Red). Fluorescent nucleic acid dye SYTO 9 penetrates and labels the bacteria with intact and damaged membranes; on the other hand, PI can only penetrate damaged membranes. Therefore, the DWW3 cells stained with PI (red) represent the cells with damaged membranes.

To visually characterize membrane damage caused by pyocin S5, the cell morphology of DWW3 cells with and without exposure to pyocin S5 was observed under Field Emission

Scanning Electron Microscopy (FESEM). Figure 1-14E and 1-14F show that more than half of the cells after treatment with pyocin S5 for one hour decreased in size and became irregular and distorted. This might be due to the leakage of intercellular materials. The differences between cell morphology with and without treatment with pyocin S5 confirm the damage to DWW3 cells' membranes after exposure to pyocin S5 (Ling, Saeidi et al. 2010).

Figure 1-14. Membrane damage of *P. aeruginosa* DWW3 upon exposure to pyocin S5.

(A) Absorbance at 260 nm; (B) ATP assay; (C,D) live and dead kit assay; (E,F) cell morphology test under FESEM . C and E show cells without treatment with pyocin S5 (Ling, Saeidi et al. 2010) .

1-6- Mathematical modeling and synthetic biology

As mentioned in section 1-5, synthetic biology uses engineering techniques and concepts such as standardization, modularity, and Computer Aided Design (CAD). Taking advantage of engineering principles enables us to build our view of molecular biology to equip microbes with new human-defined functions. Such systematic and predictable manipulation of microbes needs both experimental and modeling approaches. Modeling of biological parts allows us to design complex genetic circuits and analyze their behavior before wet-lab implementation (Chandran, Copeland et al. 2008). Models that can properly predict the behavior of biological devices allow us to design and program new biological systems without doing a large number of trial-and-error wet-lab experiments. Schematics of biological pathways are a simple example of qualitative models. These models have some limitations because they cannot be analyzed by CAD tools (Arkin 2001). For this reason, quantitative models are an important synthetic biology tool.

In synthetic biology, models are used to predict the behavior of a network after some modifications. Simpler models with fewer parameters that can reproduce the behavior of biological systems are generally preferred over complicated models because they are much easier to analyze (Bornholdt 2005). To simplify a model, some assumptions should be considered. One of the most common assumptions in modeling biological networks is that the molecular components are homogeneously distributed inside the cell. By making this assumption, the concentration gradient of species can be ignored. Although this is the most common assumption, it may not be valid in some cases (Kholodenko 2006). For

models that involve transcriptional regulation and enzymatic reactions, an additional assumption must be considered. This assumption is that some of the reactions are steady state or are at equilibrium. All of these assumptions simplify the model without much effect on the observed output. It is worth mentioning that if a model cannot reproduce expected behavior, then all of the assumptions should be revisited and modified.

The most common approach to modeling a biological system is to consider it as a system with molecular components and reactions. Each reaction is based on the production and consumption of components and the reaction rate, which depends on the component concentration. After construction of the reactions for all the components, the behavior of the system can be observed through either deterministic or stochastic simulations. Figure 1-15 shows a classical example of how to model a chemical reaction (Chandran, Copeland et al. 2008). Enzyme (E) converts substrate (S) to product (P) through an intermediate substrate-enzyme (ES). This reaction has four components, and the reactions are as follow:

Figure 1-15. Schematic of enzyme-catalyzed reaction (Chandran, Copeland et al. 2008).

As mentioned earlier, models can be simulated deterministically or stochastically. Deterministic models do not include the randomness that exists in natural systems, while stochastic models do. Differential equations are the most common way to present deterministic models. After defining the rate equations for each reaction, deterministic models can be constructed based on the changing rate for each component:

$$\frac{dX}{dt} = \text{production rate} - \text{degradation rate} \quad (1-1)$$

In Equation 1-1, X represents each component in a biological system. For example, for the substrate (S) in Figure 1-15, the differential equation would be:

$$\frac{dS}{dt} = (k_1 \times ES) - (k_2 \times E \times S) \quad (1-2)$$

k_1 and k_2 are the rate constants for the respective reactions

The complete differential equation consists of four differential equations for each individual component. The other three equations are as follow.

$$\frac{dE}{dT} = (k_1 \times ES) + (k_3 \times E \times S) - (k_2 \times E \times S) \quad (1-3)$$

$$\frac{dP}{dt} = (k_3 \times ES) \quad (1-4)$$

$$\frac{dES}{dt} = (k_2 \times E \times S) - (k_1 \times ES) - (k_3 \times ES) \quad (1-5)$$

It should be noted that k_3 is the constant rate for the following reaction

A biological model that has been constructed using a differential equation can be analyzed numerically. Time series of the component concentrations can be generated using numerical simulation. Researchers are usually interested in steady state behavior of a system and how each parameter can affect the steady state values. Steady state analysis can help find the sensitive parameters (Heinrich, Rapoport et al. 1977). In cases when there are multiple steady state points like oscillators, bifurcation analysis can determine the range of parameters in which a system can show such behaviors (Pajevic 1995; Tyson, Chen et al. 2001). These analyses and techniques for differential equations can help us adjust parameters in order to model a specific behavior. After building a

mathematical model, experiments with quantitative data can be used to find parameter values and validate the proposed model.

1-7- Overview of the workflow to create sensing-killing device

As mentioned in the previous sections, infectious diseases are a leading cause of death worldwide. Due to the increase in the rate of multi-drug-resistant pathogens, there is an urgent need for a new approach to tackle these infections. Despite the recent encouraging advances in synthetic biology (see section 1-5), this technique has not been used to develop novel strategies to deal with infectious disease to date. Thus, we have decided to use this emerging approach to design new treatment regimens for infectious disease. Therefore, in this study, we have made a step forward in developing a novel antimicrobial strategy based on an engineered microbial system, using the synthetic biology framework. A genetic system has been designed and constructed based on standardization, decoupling, and abstraction. This system allows sensing and killing of *P. aeruginosa*, a human pathogen, in a nonpathogenic chassis, *E. coli*, utilizing BioBrick parts from the BioBrick library (<http://partsregistry.org>). To fulfill these objectives, a synthetic biological system comprised of multiple genomic functions from diverse origins was integrated to create a novel and effective treatment for *P. aeruginosa* infection. We have designed our system to contain three devices: (1) the sensing device for detection of the 3OC₁₂HSL (pTetR-LasR-pLuxR), (2) the pyocin S5 producing device, and (3) the E7 lysis device. The genetic sequences of the three devices were assembled using Informax Inc.'s Vector NTI Suite 8 (Lu and Moriyama 2004). The final sequence (pTetR-LasR-pLuxR-S5-pLuxR-E7) was also screened by Vector NTI for any EcoRI / XbaI / SpeI /

PstI restriction sites (see Appendix Figure 1). Figure 1-16 shows an overview of the workflow. Details are explained in the following chapters.

Figure 1-16. Overview of the work flow to fulfill the objective of sensing and killing a human pathogen

2- Characterization of individual Sensing, Killing and Lysing devices to target *Pseudomonas aeruginosa*, a human pathogen

2-1-Introduction

Synthetic biology aims to engineer genetically modified biological systems that perform novel functions that do not exist in nature, with reusable, standard interchangeable biological parts. The use of these standard biological parts enables the exploitation of common engineering principles such as standardization, decoupling, and abstraction for synthetic biology (Endy 2005). Furthermore, well-characterized standard biological parts, whose behaviors including inputs and outputs are defined, can allow reliable physical and functional composition and facilitate biological engineering through abstraction hierarchies. With this engineering framework in place, synthetic biology has the potential to make the construction of novel biological systems a predictable, reliable and systematic process rather than an *ad hoc* process. While most synthetic biological systems are still being developed based on the latter, recent efforts to implement the engineering framework in synthetic biology provided long-awaited evidences that the engineering principles can facilitate the construction of novel biological systems. Synthetic biology has demonstrated that its framework can be applied to a wide range of areas such as energy, environment and healthcare. For example, novel biological systems have been constructed to produce drugs (Ro, Paradise et al. 2006) and biofuels (Steen,

Kang et al. 2010), to degrade containments in water (Sinha, Reyes et al. 2010), and to kill cancer cells (Anderson, Clarke et al. 2006).

Despite such great potential of synthetic biology, few studies have exploited the rewiring of genetic circuits to offer a new strategy of tackling infectious disease, a leading cause of death worldwide especially in poor countries. Given the stalled development of new antibiotics and the increasing emergence of multi-drug-resistant pathogens, there is an urgent need for expanding synthetic biology capacity to designing a new treatment regimen for infectious disease. Consequently, in this study, we aimed to develop a novel antimicrobial strategy of engineering a microbial system that senses and eradicates a human pathogen using the synthetic biology framework. Towards this aim, we designed and constructed the genetic system, based on standardization, decoupling, and abstraction, that allows sensing and killing of *P. aeruginosa*, a human pathogen, in a non-pathogenic chassis *E. coli*. The biological parts of our devices were designed and synthesized in compliance with the BioBrick assembly standards (Canton, Labno et al. 2008). Each of the biological devices was characterized to understand its behavior. Furthermore, the correlation between the input and output of one key biological device was established for functional composition.

P. aeruginosa colonizes the respiratory and gastrointestinal tract (Fujitani, Sun et al. 2011), and causes life-threatening infections to the patients with immunodeficiency such as cystic fibrosis and cancer (Chang, Small et al. 2005; Chang, Small et al. 2005) . Despite a wide range of antibiotics available in the market, *P. aeruginosa* is still among the leading causes of nosocomial infection. The primary reason attributed to the fact that

it is intrinsically resistant to many antibiotics and antimicrobials, contributed to by its effective efflux systems (Chang, Small et al. 2005). Contemporary treatments against *P. aeruginosa* infection include antibiotic chemotherapy and bacteriophage therapy. In antibiotic chemotherapy, a combinatorial treatment involving multiple antimicrobial agents is usually preferred over monotherapy due to the rapid acquisition of drug tolerance in *P. aeruginosa*. However, this approach promotes unspecific killing of bacteria and upsets a healthy human microbiome. Phage therapy involves strain-specific bacteriophages that invade and destroy the cellular integrity of pathogens (Wright, Hawkins et al. 2009). The therapeutic potential of employing virus in bacterial infection, however, is limited as a directed treatment cannot be re-employed after the infected host develops specific antibodies against the introduced virus (Häusler 2006).

Certainly, combating *P. aeruginosa* infections now requires the development of novel, unconventional antimicrobial strategies that do not entirely rely on current antibiotics. For this, in this study, we exploited *P. aeruginosa*'s own strategy for survival in a competitive environment by means of bacteriocins, ribosomally synthesized antimicrobial peptides. Bacteriocins are specific and effective against closely related species and thus have garnered attention as a new generation antibacterial agent (Baba and Schneewind 1998). Pyocins are narrow-spectrum bacteriocins produced by *P. aeruginosa*. Contrary to traditional antibiotics, the acquisition of pyocin resistance by lateral gene transfer between bacteria has not yet been encountered, thus advocating for the use of pyocins in targeting *P. aeruginosa* infection (Scholl and Martin 2008). In general, pyocins are classified into 3 types: R, F and S. An earlier study estimated that R and F- type pyocins are synthesized by 90% of all *P. aeruginosa* strains and S-type by

70% (Smith, Hirst et al. 1992). S-type pyocins are soluble and comprise two protein molecules associated in complex, with parallel structural and functional features similar to DNase-based colicins. The larger protein has been identified as the functional protein for killing while the smaller protein is regarded as the immunity protein that confers the host cell with defense mechanism against its own pyocin (Seo and Galloway 1990). Our group has recently reported that pyocin S5 exhibits strong bactericidal activity against *P. aeruginosa* clinical isolates through membrane damage but is ineffective against *E. coli* (Ling, Saeidi et al. 2010). To fulfill my project's primary objective, which was to destroy *P. aeruginosa* or inhibit its growth, the gene encoding this pyocin was used in our final construct. Since S-type pyocins lack a lysis gene (Parret and De Mot 2002), a lysis protein is required to release the accumulated pyocins. The 144-bp lysis gene was taken from the colicin E7 (Soong BW 1992).

Pyocin S5 is a small protein consisting of 498 amino acids or 1497 nucleotides, which is short enough to be included in our plasmid construct. At the downstream end of the pyocin S5 gene, there is an open reading frame that encodes an immunity protein (108 amino acids). As mentioned earlier, in section 1-5 this immunity protein is necessary as it renders the pyocinogenic bacteria resistant to pyocin S5. However, in our engineered system we have ignored this immunity protein because soluble (S-type) pyocins' mechanism for killing is through specific receptors that do not exist in *E. coli*. Using NCBI's reference sequence NC_002516.2 of *P. aeruginosa* PAO1, the pyocin S5 genetic sequence was derived from genomic position 1,066,321 to 1,067,817 (anti-sense strand) (NCBI). The gene was then directly synthesized by Geneart Company.

Further, to enable our engineered microbes to produce pyocin S5 only in response to the presence of *P. aeruginosa*, we exploited the quorum sensing mechanisms of *P. aeruginosa*. Quorum sensing is an organic chemical signaling cascade that regulates a myriad of physiological activities such as cell motility, virulence, biofilm formation and growth (Chang, Small et al. 2005). This sensing mechanism is mediated by various diffusible, chemical signals known as autoinducers that are produced by the synthase genes of the bacteria. The extracellular concentration of signaling molecules increases as a function of cell density and is permeable to cell membrane. Upon attaining a threshold concentration of the chemical signals, the quorum sensing cascade is activated to elicit expressions or repressions of multiple genes, including those that are functional for production of autoinducers such as acyl homoserine lactones (AHLs) (Asad and Opal 2008). Although similar production mechanisms are present in some Gram-negative bacteria, each synthase homolog producing AHLs differs in either length or functional groups (e.g. hydroxyl and carbonyl groups) on the acyl side-chain. Thus, with each bacterium possessing disparate synthase sequence, a high level of specificity can be achieved during intercellular quorum communication (Schuster, Urbanowski et al. 2004; Smith, Song et al. 2008).

To enable an effective release of pyocin S5, we utilized lysis E7 protein to lyse the *E. coli* chassis. The E7 lysis protein is a key component of the SOS response system in colicin-producing cells and functions to export bacteriocins into the extracellular space under stressful environmental conditions (Chak, Kuo et al. 1991). Recent studies have shown that the E7 lysis protein is effective in causing inner membrane damage and maybe associated with the activation of Outer Membrane PhosphoLipase A (OMPLA) for outer

membrane modification (Lin 2009). In addition to being specific to *E. coli*, the E7 lysis protein is small at 47 amino acids and can be easily utilized as a modular part in the assembly of novel genetic circuits.

2-2-Methodology

2-2-1-Strains and media

All cells involved in cloning and characterization experiments are *E. coli* TOP10 (Invitrogen) unless otherwise stated. Commercial Luria-Bertani (LB) and Muller Hinton (MHB) were used as the medium for cloning and inhibition studies unless otherwise stated. Supplemented M9 (M9 salts, 1 mM thiamine hydrochloride, 0.4% glycerol, 0.2% casamino acids, 0.1 M MgSO_4 , 0.5M CaCl_2) was used as the medium for the characterization. Ampicillin (100 $\mu\text{g/mL}$) was added to the culture media for antibiotic selection where appropriate. Homoserine lactone (3OC₁₂HSL; Sigma Aldrich) was used for characterization experiments. All restriction and ligation enzymes were purchased from New England Biolabs (NEB). Table 2-1 summarizes all plasmids, BioBrick parts and devices used in this study. Genetic mapping of representative engineered constructs are illustrated in Appendix Figure 1.

Table 2-1. List of all genetically encoded parts, devices and systems used in this work.

The part number, functional description and symbol used are listed for each component. Descriptions of all BBa parts can be found in the Registry of Standard Biological Parts while the rest are explained in text.

Part Number	Description	Symbol
BBa-R0040	TetR repressible promoter
BBa-R0062	LuxR & AHL inducible promoter
BBa-B0032	Ribosome Binding Site (medium)
BBa-B0034	Ribosome Binding Site (strong)
BBa-C0179	LasR coding region
BBa-E0040	Green Fluorescence Protein
BBa-K117000	Lysis Protein
BBa-B0015	Terminator
pTetR-LasR-pluxR	Sensing device
pTetR-LasR-pluxR-GFP	Sensing device with reporter protein
pTetR-LasR-pluxR-S5	Sensing device with pyocin S5
pTetR-LasR-pluxR-E7	Sensing device with ColicinE7 lysis protein
pTetR-LasR-pluxR-S5-pluxR-E7	Sensing/killing system construct

2-2-2-System assembly

The genetic constructs developed in this study were assembled using standard synthetic biology protocols as described before (Shetty, Endy et al. 2008). Briefly, for front insertion of BioBrick parts, purified insert and vector plasmids were digested with EcoRI / SpeI and EcoRI / XbaI respectively. For back insertion to upstream vector, the insert and vector plasmids were digested with XbaI / PstI and SpeI / PstI in that order. Digested

fragments were separated by DNA gel electrophoresis and ligated with NEB Quick Ligase in accordance with the manufacturer's instructions. Plasmids from chemically transformed cells were purified by affinity columns and verified by DNA sequencing.

2-2-3-Characterization of quorum sensing device (pTetR-LasR-pLuxR-GFP with 3OC₁₂HSL)

Single colonies of pTetR-LasR-pLuxR-GFP (Top10) were each inoculated into 5 mL of pre-warmed supplemented M9 ampicillin for overnight culture in a shaking incubator at 37°C. After overnight growth, the cultures were diluted to OD₆₀₀ of 0.002 and allowed to incubate further to OD₆₀₀ of 0.5 or 5.0×10E-7 cfu/mL under the same condition. Cultures were then transferred into a transparent, flat-bottom 96 well plate in triplicate aliquots of 200 µL for induction with 3OC₁₂HSL at varying molar concentrations (0, 5E-10, 1E-9, 5E-9, 1E-8, 5E-8, 1E-7, 2.5E-7, 5E-7, 1E-6, 5E-6, 1E-5, 5E-5 and 1E-4 M). The plate was incubated at 37°C with rapid shaking in a microplate reader (Biotek) and assayed for green fluorescence. Time-series fluorescence and OD₆₀₀ data were obtained at intervals of 10 minutes for a total run time of 3 hours. The result was zeroed with supplemented M9 to remove background fluorescence and OD₆₀₀. A relative GFP production rate was derived as a ratio of background subtracted green fluorescence to OD₆₀₀ value. The averages of experimental results between 20 and 80 minutes, when the fluorescence readings were in exponential phase, were fitted using an empirical mathematical model (Hill-like Equation),

$$y = A + \frac{B[AHL]^n}{C^n + [AHL]^n} \quad (2-1)$$

Equation 2-1 models GFP synthesis rate (y) as a function of input concentration of 3OC₁₂HSL ([AHL]), the four parameters (A , B , C , n) were estimated to obtain the best fit curve by performing a nonlinear curve fitting using the experimental results. This curve fitting was performed using MATLAB Curve Fitting Toolbox (The Mathworks, Natwick, MA, USA).

2-2-4- Characterization of killing device with 3OC₁₂HSL

One single colony of *E. coli* Top10 containing pTetR-LasR-pLuxR-S5 was inoculated in 5 ml of LB broth with 100 µg/ml ampicillin resistances in a shaking incubator at 37°C with 250 rpm for overnight. At the same time one single colony of normal Top10 cell was grown at the same condition with expectation of using LB broth without any antibiotic resistance. Then the overnight culture for both samples was diluted 100 times in 50 ml of pre-warmed fresh media in 250 ml canonical flasks for an average of 2.5 hours to reach OD₆₀₀ 0.7. Then culture containing S5 producing part was aliquot in eight 50 ml centrifuge tubes, each containing 5 ml of cell culture. After that normal cells were also aliquot in to three, 50 ml tubes each contained of 5 ml culture. Then tubes containing S5 construct were induced with 50 µl of 3OC₁₂HSL (Sigma Aldrich, cat# 09139) to reach four different final concentrations (0, 1E-8, 1E-6 and 1E-4 M). Two tubes for each final concentration were considered for two different time points with interval of 1 hour. At the same time normal *E. coli* Top10 was induced with 200 µl of 1E-6 M AHL in three replicates as well.

After one and two hours of induction, all the 5 samples for each time-point were centrifuged at 6000 g, 4°C for 10 minutes. Then cell pellets were re-suspended again in 5ml of sterilized lysis buffer which contains 1mL 1M Tris, 200uL 0.5M EDTA , 15g sucrose, 200mg lysozyme and 10mg BSA. Bring up to 100mL with water, total PH should be 7.4. Samples for each time-point were aliquot in 2ml tubes with 0.3-0.5 mg of glass beads (Sigma cat# O79K5310) to be lysed by using of Qiagen's tissue lyser with 50 HZ and for 5min. lysed samples were centrifuged at 10000 g for 10 minutes to collect supernatant.

Ten µl of filtered supernatant was used to pour on LB agar plates, dried for around 1 hour. Then an overlay of 2.5 ml of soft agar (1% peptone, 0.5% agar) containing 0.1 ml of DWW3 culture grown in Mueller-Hinton broth (**MHB**) that is in mid-exponential growth phase ($OD_{600}=0.8$), was poured on The agar surface. After that the plates were incubated in 37°C incubator for overnight (Iwatani, Zendo et al. 2007) and the results were taken by Bio-Rad's molecular imager ChemiDoc XRS+.

Using induced normal cells is to verify that pyocin S5 is causing inhibition in the *P. aeruginosa*. This experiment was done to check the expression of pyocin S5 protein in mentioned quorum sensing (QS) gene circuits by using the total protein concentration in supernatant with manual lysis and in different concentration of AHL before adding collicinE7 lysis gene to this circuit. This experiment has been repeated in two separate days, with two colonies from a single plate.

2-2-5- Detection of the native autoinducer produced by *P. aeruginosa*

Extracellular homoserine lactone (3OC₁₂HSL) from six *P. aeruginosa* isolates: In3, In4, In7, In, DWW3 and InB (Waite and Curtis 2009) were measured with *E. coli* Top10 containing pTetR-LasR-pLuxR-GFP. Table 2-2 shows the minimum inhibitory concentrations (MIC) of six different strains (Ling, Saeidi et al. 2010).

Table 2-2. list of sensitive *P. aeruginosa* strains to pyocin S5 and their MIC results (Ling, Saeidi et al. 2010) (with permission).

	<i>DWW3</i>	<i>In7</i>	<i>InB</i>	<i>In4</i>	<i>In3</i>	<i>In8</i>
MIC (µg/ml)	12.6	50.5	151.6	202.2	404.4	808.8
MIC (µM)	0.20	0.81	2.43	3.24	6.49	12.97

One single colony of *E. coli* Top10 containing pTetR-LasR-pLuxR-GFP was inoculated in 5ml of pre-warmed supplemented M9 medium containing 100 µg/ml ampicillin, for overnight in a shaking incubator at 37°C and 250 rpm. *P. aeruginosa* isolates were also cultured in the same condition with the exception of using supplemented M9 instead of this medium with antibiotic resistance.

All cell cultures were diluted 100 times in pre-warmed fresh medium and grown in same condition. Re-inoculated cultures were grown to $OD_{600}=1.0$ and $OD_{600}=0.8$ for *P. aeruginosa* and *E. coli* respectively. Cultures of *P. aeruginosa* isolates were filtered with a filter membrane (0.22 μm) to obtain extracellular homoserine lactone. Clear filtrates obtained from each isolate were then mixed with *E. coli* Top10 containing GFP producing part with a ration of 1:10 and 1:100. GFP reporter culture and the resultant solutions were transferred into a flat-bottomed 96 well plate. To obtain fluorescence background normal *E. coli* Top10 cells were also mixed with *P. aeruginosa* strains.

The plate was incubated in a BioTek microplate reader at 37°C and assayed with an automatically repeating protocol. This process was arranged for 3 hours of absorbance measurements (600 nm absorbance filter), fluorescence measurements (485 nm excitation filter, 520 nm emission filter) and shaking with medium speed for 3 minutes for each reading. Time between repeated measurements was 10 minutes.

The absorbance and GFP intensity results were zeroed with supplemented M9 and normal *E. coli* Top10 mixed with *P. aeruginosa* isolates respectively. GFP production rate was derived as a ratio of background subtracted green fluorescence to OD_{600} value. This experiment has been repeated in three different days with three colonies. The rates obtained were then compared with the Hill function mathematical model derived earlier using 3OC₁₂HSL to estimate the native 3OC₁₂HSL concentration from *P. aeruginosa* strains.

2-2-6-Characterization of lysis device with 3OC₁₂HSL

Overnight cultures of pTetR-LasR-pLuxR-E7 (Top10) were diluted in supplemented M9 and harvested at an OD₆₀₀ of 0.5. The resultant cultures were transferred into a transparent, flat-bottom 96 well plate in triplicate aliquots of 200 µL for induction with 3OC₁₂HSL at varying concentrations (0, 1E-8, 1E-6 and 1E-4 M). The plate was incubated at 37°C with rapid shaking in a microplate reader (Biotek) and assayed for cell turbidity. Time-series absorbance at OD₆₀₀ was obtained at intervals of 10 minutes for a total run time of 6 hours. The result was zeroed with supplemented M9 to remove background absorbance.

2-2-7-FESEM assay

To examine the effect of E7 lysis protein on cell morphology, re-inoculated cultures of pTetR-LasR-pLuxR-E7 (Top10) and pTetR-LasR-pLuxR-S5-pLuxR-E7 (Top10) were induced with 1E-6 M 3OC₁₂HSL at OD₆₀₀ of 0.5 and cultured for 2 hours. Cell pellets collected after centrifugation at 4000 RPM for 15 minutes were washed with 0.1 M sodium cacodylate (pH 7.4) three times before fixation with 2.5% glutaraldehyde in 0.1 M sodium cacodylate for 2 hours of incubation at 4°C. Cell pellets were further washed three times with sodium cacodylate after fixation and re-suspended in 0.1 M sodium cacodylate (volume depends on cell amount). 2 µL of sample was loaded onto PEI coated silicon slide followed by incubation at 25°C for 30 minutes. The loaded silicon slide was fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate at 25°C for 90 minutes. Silicon slide was then dehydrated in serial concentrations of absolute ethanol (37%, 67%,

95% and three times of 100%) for 15 minutes each before drying in a vacuum evaporator overnight. Coating of silicon slide was performed with 20 nm of gold-palladium alloy (60:40) and examined using a field-emission scanning electron microscope (JSM-6700F FESEM) at 10kV

2-3-Results

2-3-1-Design of the sensing and killing genetic system

Figure 2-1 shows a schematic of our sensing and killing genetic system. The sensing device was designed based on the Type I quorum sensing mechanism of *P. aeruginosa*. The *tetR* promoter, which is constitutively on, was coupled to LasR, a transcriptional activator, which binds to AHL 3OC₁₂HSL. In our sensing device, instead of employing the *las* promoter present in *P. aeruginosa*, the *luxR* promoter was used as the inducible promoter in congruent to the activator complex LasR-3OC₁₂HSL. This inter-switchability was possible due to the similar genetic homology shared by both the *lux* and *las* quorum sensing proteins (Gray, Passador et al. 1994). Next, the formation of the LasR-3OC₁₂HSL complex, which binds to the *luxR* promoter, activates the killing and lysing devices, leading to the production of pyocin S5 and lysis E7 proteins (Chak, Kuo et al. 1991) within the *E. coli* chassis. Upon reaching a threshold concentration, the lysis E7 protein perforates membrane of the *E. coli* host and releases the accumulated pyocin S5. Pyocin S5, a soluble protein, then diffuses into the target pathogen and damages its cellular integrity, thereby killing it. Appendix Figure 1A shows the plasmid map of the final system.

Figure 2-1. Schematic of ‘Pathogen Sensing and Killing’ system.

luxR promoter is activated by LasR-3OC₁₂HSL complex, leading to production of E7 lysis protein and S5 pyocin within *E. coli* chassis. After the E7 protein attains the threshold concentration that causes the chassis to lyse, accumulated S5 is released into the exogenous environment and kills *P. aeruginosa*.

2-3-2-Characterization of the sensing device

To evaluate and characterize the sensing device, the gene encoding the green fluorescent protein (GFP) was fused to the sensing device (i.e. pTetR-LasR-pLuxR-GFP; the plasmid map is shown in Appendix Figure 1B) and the GFP expression was monitored at a range of concentrations of 3OC₁₂HSL. From the measured GFP synthesis rates (Figure 2-2), we observed a basal expression level of 0.1 RFU OD⁻¹min⁻¹ without induction, followed by a sharp increase in GFP production rate as the concentration of 3OC₁₂HSL was increased

beyond $1\text{E-}7\text{ M}$. This transition peaked at $1\text{E-}6\text{ M}$ of $3\text{OC}_{12}\text{HSL}$ and exhibited a sharp decline afterward. These results suggest that the optimal detection range of the sensing device was between $1\text{E-}7\text{ M}$ and $1\text{E-}6\text{ M}$ $3\text{OC}_{12}\text{HSL}$. Note that previous studies estimated extracellular concentration of $3\text{OC}_{12}\text{HSL}$ to be in the range of $1\text{E-}6\text{ M}$ to $1\text{E-}4\text{ M}$ within proximity to the site of *P. aeruginosa* infection (Pearson, Passador et al. 1995; Charlton, de Nys et al. 2000).

Figure 2-2. Characterization results of sensing device coupled with GFP reporter.

GFP production rates per cell over time at different $3\text{OC}_{12}\text{HSL}$ inducer concentrations; as explained in section 2-2-3, time-series fluorescence and OD_{600} data were obtained at intervals of 10 minutes for a total run time of 3 hours. The result was zeroed with supplemented M9 to remove background fluorescence and OD_{600} . A relative GFP production rate was derived as a ratio of background subtracted green fluorescence to OD_{600} values. High GFP production rates per cell were observed when the input $3\text{OC}_{12}\text{HSL}$ concentration was between $10\text{E-}7\text{ M}$ to $10\text{E-}6\text{ M}$.

2-3-3-Transfer function of the sensing device

One important characteristic of the sensing device was the transfer function that describes the static relationship between the input ($3\text{OC}_{12}\text{HSL}$) and output (GFP production rate) of the sensing device. The transfer function was determined by fitting an empirical

mathematical model (Hill-like equation) to the experimental data where the input 3OC₁₂HSL concentration is smaller than 1E-6 M. The resulting best fit model demonstrated that the static performance of the sensing device follows a Hill-like equation below the input concentration of 1E-6 M 3OC₁₂HSL (Figure 2-3). The model showed that the sensing device saturated at a maximum output of 1.96 RFU OD⁻¹ min⁻¹ at input concentration greater than 3.3×1E-7 M but smaller than 1E-6 M 3OC₁₂HSL, and the switch point for the sensing device was 1.2×1E-7 M 3OC₁₂HSL, the input concentration at which output is at half-maximal. Since this switch point concentration is smaller than the concentration of 3OC₁₂HSL presence (1E-6 M to 1E-4 M) within proximity to the site of *P. aeruginosa* infection as earlier reported in the literature (Pearson, Passador et al. 1995; Charlton, de Nys et al. 2000), the sensing device would be sensitive enough to detect the amount of 3OC₁₂HSL natively produced by *P. aeruginosa*.

Figure 2-3. GFP production rates per cell at different input 3OC₁₂HSL concentrations.

Data are the average between 20 and 80 minutes after induction, when the fluorescence reading was in an exponential phase before reaching a stationary phase. Details have been explained in section 2-2-3. Showing that the optimal operating concentrations for the sensing device range from 10E-7 to 10E-6 M 3OC₁₂HSL

2-3-4-Characterization of killing device with 3OC12HSL

In this experiment, we seek to determine whether pyocin S5 is expressed in test construct pTetR-LasR-pLuxR-S5 (Appendix Figure 1D), and pTetR-LasR-pLuxR-S5-pLuxR-E7. To achieve this purpose, an inhibition experiment was done on DWW3, the most sensitive strain to Pyocin S5 (Ling, Saeidi et al. 2010), to check whether pyocin S5 is expressed and whether the concentration is enough to inhibit DWW3 growth by commercial AHL induction (0M, 1E-8M, 1E-6M and 1E-4M). For the test construct (e.g.

pTetR-LasR-pLuxR-S5), manual lysis was done using lysis buffer as described in section 2-2-4, and for the final construct (e.g. pTetR-LasR-pLuxR-S5-pLuxR-E7) the lysis gene was used to damage cells and enable the release of pyocin S5 from the cell. As explained before, the experiment was done at two different time points (e.g. 1 and 2 hours after induction) with 4 different AHL concentrations (e.g. 0, 1E-8, 1E-6 and 1E-4 M). Induction by 1E-6M of commercial 3OC₁₂HSL (optimal inducer concentration) would lead to maximal expression of pyocin S5 (see section 2-3-3). Figure 2-4 has shown the results for 1E-6M of AHL and after 2 hours from induction as the most significant contrast.

Figure 2-4. Pyocin-detecting agar overlay assay using DWW3 as the overlay.

Circular shaded regions were inhibition zones containing pyocin S5. As described in section 2-2-4, this experiment was done on DWW3, a sensitive strain to pyocin S5 (Ling, Saeidi et al. 2010). For the test construct pTetR-LasR-pLuxR-S5, manual lysis was done in order to release the accumulated pyocin S5 to the extracellular environment. a) *E. coli* carrying pTetR-LasR-pLuxR-S5 without induction to see the basal expression of pyocin S5, b) *E. coli* carrying pTetR-LasR-pLuxR-S5 after 2 hours of induction, c) *E. coli* carrying pTetR-LasR-pLuxR-S5-pLuxR-E7 without induction to see the basal expression of pyocin S5 in final construct, d) *E. coli* carrying pTetR-LasR-pLuxR-S5-pLuxR-E7 after 2 hours of induction.

The same growth inhibition experiment has been performed on different *P. aeruginosa* isolates using commercial 3OC₁₂HSL. The result indicates that among the clinical isolates of *P. aeruginosa* we used for our experiments DWW3, In7, In4 and InB may be susceptible to the amount of pyocin S5 produced by pTetR-LasR-pLuxR-S5 (for last two strains data not shown). However the native 3OC₁₂HSL production assay in section 2-3-4, has proved that all the *P. aeruginosa* strains (with exception of DWW3) used in this project are capable of producing sufficient 3OC₁₂HSL molecules to induce transcriptional activity in sensing device, pTetR-LasR-pLuxR. DWW3 did bring forth some activity in pTetR-LasR-pLuxR, however the activity was deemed too low.

2-3-5-Detection of the native autoinducer produced by *P. aeruginosa*

As stated above, the characterization of the sensing device indicated that it produces an optimal output at 1E-7 M to 1E-6 M 3OC₁₂HSL. To verify that the sensing device is able to sense the amount of 3OC₁₂HSL natively produced by *P. aeruginosa* strains, filtered culture of *P. aeruginosa* clinical isolates that are sensitive to pyocin S5 (Ling, Saeidi et al. 2010) were used to induce the sensing device coupled with a GFP reporter (i.e. pTetR-LasR-pLuxR-GFP) at the ratio 1:100 and 1:10.

This was necessary as characterization results with synthetic 3OC₁₂HSL implied that a certain GFP production rate per cell could correspond to inducer concentration either below or above 1E-6M. The average production rate between 20-80 minutes were input into Equation 2.1 to estimate inducer concentration of various *P. aeruginosa* isolates as shown in Figure 2-5 below.

Figure 2-5. GFP production rate of different *P. aeruginosa* isolates.

As described in section 2-2-5, in order to find the native inducer concentration of various *P. aeruginosa* isolates, filtered *P. aeruginosa* culture was diluted to (a) 1:100 and (b) 1:10 times with *E. coli* carrying pTetR-LasR-pLuxR-GFP. Average GFP production rate between 20 ~ 80 minute after induction with 1:100 and 1:10 filtered supernatant is shown in the bar graph.

Results show that GFP synthesis rate measured for the isolate In7 was 1.375 RFU OD⁻¹ min⁻¹. This value was above the minimum synthesis rate and greater than the half-maximal of the sensing device and would be further tested for co-culturing with engineered treatment system. This result confirmed that the sensing device was able to detect the natively produced 3OC₁₂HSL. Further, to have an insight into the amount of 3OC₁₂HSL natively produced by the isolate, we used the GFP synthesis rate measured and the model (Equation 2-1) derived in earlier experiment. The average concentration of

3OC₁₂HSL in the liquid culture of the *P. aeruginosa* strain was estimated to be approximately 1E-6 M 3OC₁₂HSL. This result which has been shown in Figure 2-6 was coherent with previous studies that had estimated that the extracellular concentration of 3OC₁₂HSL is between 1E-6 and 1E-4 M (Pearson, Passador et al. 1995; Charlton, de Nys et al. 2000).

Figure 2-6. Predicted concentration of 3OC₁₂HSL inducer in *P. aeruginosa* culture

The data were obtained using the methodology explained in section 2-2-5, average GFP production rate between 20 ~ 80 minute after induction with 1:100 and 1:10 filtered supernatant was put into equation 2-1 to estimate the amount of native 3OC₁₂HSL produced by *P. aeruginosa*. As the results suggest, the extracellular concentration of 3OC₁₂HSL is between 1E-6 and 1E-4 M.

2-3-6-Characterization of lysis device

As stated above, our system was designed to release pyocin S5 through lysis upon detection of *P. aeruginosa*. To determine the lysis activity of the system, we characterized the behavior of the E7 lysis protein under the transcriptional control of the sensing device before integrating both the pyocin S5 and E7 genes into the system. The E7 lysis gene was ligated downstream to the sensing device (i.e. pTetR-LasR-pLuxR-E7; the plasmid map is shown in Appendix Figure 1C) and its performance was evaluated in the chassis *E. coli* over time by measuring absorbance at OD₆₀₀ at a range of concentrations of 3OC₁₂HSL. Figure 2-7 shows that at 0 and 1E-8 M 3OC₁₂HSL, the growth rates of *E. coli* underwent no noticeable transition into a lysis state. However, at higher concentrations of 3OC₁₂HSL (i.e., 1E-6 and 1E-4 M), the cells exhibited a significant reduction in optical density, likely due to lysis activity.

To verify the effect of lysis, cell integrity was examined with and without 1E-6 M 3OC₁₂HSL using Field Emission Scanning Electron Microscopy (FESEM). Figure 2-8A shows that *E. coli* containing pTetR-LasR-pLuxR-E7 induced with 3OC₁₂HSL appeared 120 minutes. In perspective, this value is close to the delay reported earlier by another group using a different inducible promoter system. It may imply that transcriptional kinetics is not rate limiting for the onset of lysis in strongly induced systems (Lin 2009).

To further confirm that the lysis activity was sustained in the final system including pyocin S5, the morphology of *E. coli* containing the sensing and killing devices (i.e. pTetR-LasR-pLuxR-S5-pLuxR-E7) was monitored using FESEM. Figure 2-8B shows that

these *E. coli* cells, which included the final system, underwent the similar changes in cell surface morphology with 3OC₁₂HSL. This result suggests that 3OC₁₂HSL induced the lysis of the *E. coli* containing the final system (i.e. pTetR-LasR-pLuxR-S5-pLuxR-E7; Figure 2-1).

Figure 2-7. Characterization results of lysis device using 3OC₁₂HSL.

As stated in section 2-2-6 time-series absorbance at OD₆₀₀ was obtained at varying concentrations (0, 1E-8, 1E-6 and 1E-4 M). The graph shows the Growth curve of *E. coli* expressing E7 lysis protein after induction with different concentrations of 3OC₁₂HSL. The significant reduction in cell density at higher concentrations (i.e., 1E-6 and 1E-4 M) is most likely due to the lysis activity.

Figure 2-8. Characterization results of lysis device using 3OC₁₂HSL.

(A and B) Effects of lysis protein on *E. coli* surface morphology as observed using FESEM. As explained in section 2-2-7, cell integrity was examined with and without 1E-6 M of 3OC₁₂HSL. The results show that the surface of the *E. coli* was damaged when *E. coli* carrying pTetR-LasR-pLuxR-E7 and *E. coli* carrying pTetR-LasR-pLuxR-S5-pLuxR-E7 (the final system) were induced with 3OC₁₂HSL. Scale bar: 1 μ m.

2-4-Conclusion

In summary, we engineered a novel biological system, which comprises sensing, killing, and lysing devices, that enables *E. coli* to sense and eradicate pathogenic *P. aeruginosa* strains by exploiting the synthetic biology framework. We designed and synthesized all the biological parts in compliance with the BioBrick assembly standards. The sensing,

killing, and lysing devices were characterized to elucidate their functionalities. Based on the characterization and modeling results, we verified that the sensing device had an optimal activity approximately in the range of $10\text{E-}7$ to $10\text{E-}6$ M $3\text{OC}_{12}\text{HSL}$, which was also in line with the concentration of $3\text{OC}_{12}\text{HSL}$ secreted by *P. aeruginosa*. We further confirmed the activity of the killing and lysing devices, designed based on the pyocin S5 and E7 lysis genes, respectively, in response to this range of $3\text{OC}_{12}\text{HSL}$ concentration. Next piece of work which has been explained in the following chapter would be to test efficiency of lysis device in the final sensing-killing system by measuring the amount protein release. Lastly to proof the concept of our proposed approach we will show you the ability of our novel biological system to kill pathogenic strains of *P. aeruginosa* in planktonic state as well as inhibition of biofilm formation.

3- Characterization of final sensing-killing system in mixed microbial community to target *Pseudomonas aeruginosa*, a human pathogen

3-1- Introduction

As mentioned earlier in the previous chapter, using synthetic biology approach we have designed a genetic circuit to be transformed in to the host cell *E. coli* to target pathogenic strains of *P. aeruginosa* as one of the leading cause of death worldwide. *P. aeruginosa* with the ability to cause versatile infection types is the second most common cause of pneumonia (18.1%), the third most common cause of urinary tract infection (16.3%) and the eighth most frequently isolated pathogen from the blood stream (3.4%) (Gaynes and Edwards 2005). Our genetic circuit has consisted of three genetic devices; first device is sensing device to enable our *E. coli* to detect the presence of *P. aeruginosa*. Once the cells sense the presence of *P. aeruginosa* it would trigger the second device which is the killing device, killing device is to produce pyocin S5 as the antimicrobial peptide, the purpose of pyocin is to kill the *P. aeruginosa* however the pyocin S5 produced are being trapped within host cell. Therefore we need to introduce third device which is the lysis device, so the *E. coli* would be lysed to release the Pyocin S5 in to the external environment.

Creating a sensing mechanism for *E. coli* to detect *P. aeruginosa* will make our host system to be truly specific to *P. aeruginosa* and not targeting other cells that are not of our interest. After sensing the presence of *P. aeruginosa*, the sensing device will trigger to produce toxic protein in order to attack *P. aeruginosa*. In a way our cells will exhibit

control release of the drug to *P. aeruginosa* so that there won't be any waste of cellular resources in case there is no infections.

Before exposing the *E. coli* carrying the final sensing-killing system to the *P. aeruginosa* in planktonic and biofilm state, all three devices (e.g. sensing, killing and lysis device) were characterized individually. As mentioned in the previous chapter we could succeed to confirm the efficiency of all these devices, which leads to the design of our final system. Taken together, our final system was designed to (i) detect AHLs produced by *P. aeruginosa*; (ii) produce pyocin S5 upon the detection and (iii) lyse the *E. coli* cells so that the produced pyocin S5 is released from the cells, leading to the killing of *P. aeruginosa*. In this chapter, we first demonstrate the effect of lysis device in mediating the release of pyocin S5 to the environment and secondly we show that our engineered *E. coli* effectively senses and kills *P. aeruginosa*. This approach provides a novel synthetic biology-based antimicrobial strategy that could be applied to eradicating other infectious pathogens.

3-2- Methodology

3-2-1- Characterization of lysis device by protein release in engineered *E. coli*.

To characterize the efficiency of lysis device in mediating pyocin release, pTetR-LasR-pLuxR-S5-pLuxR-E7 and pTetR-LasR-pLuxR-S5 plasmids were PCR-modified with the reverse primer carrying the sequence for hexahistidine (5'-TCTAGAAAGCTTTTAGTGGTGGTGGTGGTGGTGGTGAATGCTAATCACCAGAT TAT-3') on the 3' terminus of S5 gene. PCR modification was performed using *pfu*

polymerase (Promega, U.S.A.) these genetic constructs were then transformed into *E. coli* Top10. Overnight cultures of the His-tag version of pTetR-LasR-pLuxR-S5-pLuxR-E7 and pTetR-LasR-pLuxR-S5 were then diluted in LB and harvested at an OD₆₀₀ of 0.7. The collected cultures were induced with 1E-6 M 3OC₁₂HSL and incubated for 6 hours in a shaking flask culture set at 37°C and 170rpm. At regular intervals of 2 hours, cell cultures were drawn and filter sterilized (0.22µm). The filtered culture were mixed with 1/10 volume of 100% (w/v) trichloroacetic acid (Sigma Aldrich) and incubated on ice for an hour to allow protein precipitation, before being washed with an equal volume of acetone. Precipitated proteins were reconstituted in 1ml of reconstitution solvent (1x PBS, 30 mM imidazole and 4 M urea; pH 6.0) and purified by immobilized metal affinity chromatography using Vivapure miniprep MC (Sartorius Stedim Biotech GmbH) in accordance to the manufacturer's instruction. Finally, purified pyocin proteins were analyzed by SDS PAGE and Bradford assay (Bradford 1976).

3-2-2- Overlay inhibition assay with 3OC₁₂HSL and the final system

Overnight cultures of pTetR-LasR-pLuxR-S5-pLuxR-E7 (Top10), *P. aeruginosa* ln7 and PAO1 were diluted in LB and harvested at OD₆₀₀ of 0.7 and 0.2 separately. Collected cultures of pTetR-LasR-pLuxR-S5-pLuxR-E7 (Top10) were induced with varying molar concentrations of 3OC₁₂HSL (0, 1E-8, 1E-6 and 10E-4 M) and incubated for two hours before being filtered with a filter membrane (0.22 µm). 30 µL of sterile filtrate from each induced sample containing soluble S5 were spotted onto trypticase soy agar (TSA) plate in triplicates. Upon drying of spots, 0.1 mL of ln7 at OD₆₀₀ of 0.2 in soft agar (1% peptone, 0.5% agar) pre-warmed at 55°C was thinly filmed over the spotted TSA and

allowed to dry completely. Resultant TSA plate was then incubated for 6 hours at 37°C before image analysis with Bio-Rad ChemiDoc XRS. To evaluate the effectiveness of the engineered system coupled with the sensing function, overnight culture of *P. aeruginosa* ln7 was also harvested at OD₆₀₀ of 1.0 after redilution. The culture was filtered with a filter membrane (0.22 µm) and the sterile filtrate obtained, containing planktonic 3OC₁₂HSL was used to induce pTetR-LasR-pLuxR-S5-pLuxR-E7 (Top10). These procedures were repeated to capture inhibitory images for the engineered system that was activated by 3OC₁₂HSL natively produced from *P. aeruginosa*.

3-2-3- Co-culturing of the engineered *E. coli* and *P. aeruginosa*

GFP reporter plasmid pMRP9-1 was transformed into *P. aeruginosa* ln7 and PAO1 using a method described before (Choi, Kumar et al. 2006). Overnight cultures of *P. aeruginosa* (ln7/PAO1 with pMRP9-1), pTetR-LasR-pLuxR-S5 (Top10), pTetR-LasR-pLuxR-E7 (Top10) and pTetR-LasR-pLuxR-S5-pLuxR-E7 (Top10) were diluted and harvested at an OD₆₀₀ of 1.0. pTetR-LasR-pLuxR-S5-pLuxR-E7 (Top10) was added to ln7 or PAO1 in the ratio 4:1 to obtain a mixed culture with an overall cell density of 10E-8 cfu/mL in 25mL of MHB. The resultant mixture was grown for 15 hours in a shaking flask culture set at 37°C and 170rpm. At regular intervals of 3 hours, the mixed culture was transferred into a transparent, flat-bottom 96 well plates in aliquots of 200 µL at and assayed for background subtracted green fluorescence in a microplate reader (Biotek). The same procedures were repeated for pTetR-LasR-pLuxR-S5 and pTetR-LasR-pLuxR-E7 as negative controls.

3-2-4- Live and dead fluorescent microscopy

Overnight cultures of ln7 and pTetR-LasR-pLuxR-S5-pLuxR-E7 (Top10) were diluted in LB and harvested at an OD₆₀₀ of 0.5 and 1.0 respectively. 3OC₁₂HSL from ln7 were obtained after passing ln7 culture through a filter membrane (0.22 µm) and the sterile filtrate was used to induce expression of engineered system by mixing it with pTetR-LasR-pLuxR-S5-pLuxR-E7 (Top10) in 1:1 mixing ratio to a total volume of 2 mL. The resultant culture was grown for 3 hours and filtered with a similar membrane to obtain sterile S5 filtrate. The filtrate was mixed with ln7 at OD₆₀₀ of 1.0 in 1:1 mixing ratio to a total volume of 2 mL and incubated for 3 hours. One ml of the final culture was stained with bacterial viability kit (Invitrogen) according to the manufacturer's instruction and analyzed with a fluorescent microscope (Zeiss Axio Scope A1).

3-2-5- Biofilm inhibition assay

P. aeruginosa (ln7/PAO1 with pAWG1-1) conferred with chloramphenicol resistance was mixed with pTetR-LasR-pLuxR-S5-pLuxR-E7 (Top10) in the ratio 1:4 to obtain a mixed culture with an overall cell density of 10E-8 cfu/mL in 6mL of MHB. The resultant mixture was transferred to the wells of a polystyrene microtiter plate (Iwaki) in aliquots of 1mL each and grown at 37°C and 150rpm. After 18 hours of growth, biofilm on the microtiter plate were rinsed and recovered in fresh MHB by sonication and quantified by CFU count on chloramphenicol-selective plate (100 µg/ml). The same procedures were repeated for pTetR-LasR-pLuxR-S5 (Top10) and pTetR-LasR-pLuxR-E7 (Top10) as negative controls.

3-2-6- Confocal Microscopy of Biofilm

Mixed bacteria cultures of *P. aeruginosa* (ln7 with pMRP9-1) and engineered *E. coli* systems were grown in MHB in 50mL tubes containing sterile glass slide. Biofilm developed on the glass slides after 18 hours of growth were rinsed in PBS, dried and visualized by confocal laser scanning microscopy (Zeiss LSM 510). Collected Z-stack biofilm images were reconstructed using Zeiss 2.5D software.

3-3- Results and discussion

3-3-1- Characterization of lysis device by protein release in engineered *E. coli*

In line with the overall objective of E7 lysis device in mediating the export of pyocin, we studied the efficiency of lysis device in the final system by measuring the amount of protein release. After induction with 1E-6 M 3OC₁₂HSL, histidine-tagged S5 protein was purified by immobilized metal affinity chromatography from filtered supernatant and analyzed by SDS PAGE and Bradford assay. Distinct bands that corresponded to pyocin S5 were observed on the SDS PAGE of the final system in Figure 3-1 (i.e. pTetR-LasR-pLuxR-S5-pLuxR-E7) while no bands was seen in lanes without the lysis device (i.e. pTetR-LasR-pLuxR-S5; the plasmid map is shown in Appendix Figure 1D). We further validated the results by estimating the protein concentrations in the supernatant with Bradford assay and showed that the amount of pyocin released by our final system was eight times higher than system without the lysis device (Figure 3-2). The dynamic performance of lysis device in the final system was characterized by an impulse release

of protein two hours after induction, followed by a steady state response. We also observed that co-expression of both pyocin S5 and E7 lysis proteins causes a decrease in lytic activity as evident from the V shape response of cell turbidity. Likely, this result is a consequence of reduced transcription in the final system brought about by lesser availability of activated LasR factors split between two *luxR* promoters. On the whole, the reduced lytic activity presents an advantageous situation whereby engineered *E. coli* could outgrow and inhibit *P. aeruginosa* while maintaining a substantial steady state release of pyocin.

Figure 3-1. Characterization results of lysis device in final system using 3OC₁₂HSL, by SDS page.

SDS PAGE of (i - ii) total extracellular proteins and (iii - viii) IMAC purified His-tagged S5 protein sampled from extracellular supernatant. Extracellular proteins exported from (i) *E. coli* carrying pTetR-LasR-pLuxR-S5 (without lysis device) was significantly lesser than that exported from (ii) *E. coli* carrying pTetR-LasR-pLuxR-S5-pLuxR-E7 (the final system) as indicated in darker lanes of (ii) relative to (i). (iii - v): *E. coli* carrying pTetR-LasR-pLuxR-S5 (without lysis device) in 0, 2 and 4 hours after induction. (vi - viii) *E. coli* carrying pTetR-LasR-pLuxR-S5-pLuxR-E7 (the final system) in 0, 2 and 4 hours after induction; the results show that pyocin S5 (57 kDa; arrowed) was only detectable in lanes that corresponded to *E. coli* carrying

the final system and not in lanes of *E. coli* without the lysis device. Ladder used was Bio-Rad's Precision plus Protein standards.

Figure 3-2. Characterization results of lysis device in final system using 3OC₁₂HSL, by OD₆₀₀ and concentration measurement.

Characterization of lysis device in final system by optical density (bar graphs) and concentration of pyocin released (lines) after induction. The results show an impulse release of pyocin 2 hours after induction, followed by a sustained steady-state release in the final system (solid lines). Optical density of the final system followed a V shape response, implying lower lytic activity and a regrowth of engineered *E. coli* after 4 hours (unshaded bar). Correspondingly, the concentration of pyocin released in *E. coli* without the lysis device was 1/8 that of the final system with a continually increasing optical density (shaded bar).

3-3-2- Verification of the final system with the sensing, killing and lysing devices

We have so far demonstrated that our engineered microbes are able to sense natively produced AHL 3OC₁₂HSL, which subsequently triggers cell lysis. To further determine whether the sensing of 3OC₁₂HSL also leads to killing of *P. aeruginosa* as designed, the growth of *P. aeruginosa* was monitored in the presence of the engineered *E. coli* containing the final system. First, to determine the concentration of 3OC₁₂HSL that causes a significant growth inhibition and confirm that the concentration falls within the range of concentrations of 3OC₁₂HSL naturally produced by *P. aeruginosa*, the engineered *E. coli* was exposed to 3OC₁₂HSL at 0, 1E-8, 1E-6, and 1E-4 M, and the filtered supernatants were added onto *P. aeruginosa*-grown agars. We observed that the growth of *P. aeruginosa* was clearly inhibited by the filtered supernatants of the *E. coli* cultures exposed to 1E-6 and 1E-4 M 3OC₁₂HSL, whereas very faint inhibition zones were observed at 0 and 1E-8 M, likely due to the basal expression of pyocin S5 and E7 (Figure 3-3).

Figure 3-3. Inhibition of *P. aeruginosa* by the engineered *E. coli* induced with 3OC₁₂HSL.

Agar overlay assay of *P. aeruginosa* after exposure to supernatant of the *E. coli* carrying pTetR-LasR-pLuxR-S5-pLuxR-E7 (the final system) at different 3OC₁₂HSL concentrations. Faint inhibition areas were observed with 0 M and 1E-8 M 3OC₁₂HSL. The results show that supernatant of engineered *E. coli* culture induced by 1E-6 M 3OC₁₂HSL produced wider and clearer inhibition zones relative to other inducer concentrations.

Second, to further confirm the inhibition effects, *P. aeruginosa* was examined upon exposure to the supernatant of the *E. coli* cultures with 1E-6 M 3OC₁₂HSL using the LIVE/DEAD cell viability assay. As seen under many *P. aeruginosa* cells exposed to the supernatant of the engineered *E. coli* were stained with the PI dye, which stains a dead cell, whereas all the cells incubated with the wild-type *E. coli* were stained with the SYTO 9 dye, which stains a live cell (Appendix Figure 2A). This result suggests that our engineered *E. coli* carrying the final system (i.e. pTetR-LasR-pLuxR-S5-pLuxR-E7) can kill *P. aeruginosa* in response to as low as 1E-6 M 3OC₁₂HSL. Since our earlier

estimation indicated that the concentration of 3OC₁₂HSL natively produced by *P. aeruginosa* was approximately 1E-6 M, as described above, this outcome may imply that this killing activity would be sustained against *P. aeruginosa* in response to its producing 3OC₁₂HSL.

Therefore, subsequently, to confirm the killing activity by the native 3OC₁₂HSL produced by *P. aeruginosa*, the filtered supernatant of *P. aeruginosa* cultures was mixed with the *E. coli* cultures, whose supernatant was then added to *P. aeruginosa*-grown agars. Figure 3-4A shows that *P. aeruginosa* growth was significantly inhibited by the engineered *E. coli* cultures exposed to the supernatant of *P. aeruginosa* cultures, while neither the wild-type *E. coli* cells with nor without the *P. aeruginosa* supernatant led to growth inhibition. This result indicates that our final system produces pyocin S5 and E7 in response to the 3OC₁₂HSL natively produced by *P. aeruginosa*, which resulted in the killing of *P. aeruginosa*.

To further visualize the inhibition effects on *P. aeruginosa* by our engineered *E. coli*, *P. aeruginosa* cells were stained using the LIVE/DEAD cell viability assay. Figure 3-4B shows that many *P. aeruginosa* cells exposed to the supernatant of the engineered *E. coli* induced with native 3OC₁₂HSL were stained with the PI dye, whereas all the cells incubated with the wild-type *E. coli* were stained with the SYTO 9 dye (green). This result suggests that our engineered *E. coli* carrying the final system can kill *P. aeruginosa* in the presence of native 3OC₁₂HSL produced by *P. aeruginosa*.

Figure 3-4. Inhibition of *P. aeruginosa* by the engineered *E. coli* induced with native 3OC₁₂HSL produced by *P. aeruginosa*; by using agar overlay assay and live/dead kit.

(A) Agar overlay assay of *P. aeruginosa* after exposure to supernatant of 4 different cultures. First, *P. aeruginosa* exposed to supernatant of wild-type *E. coli* showed no bactericidal activity. Second, *P. aeruginosa* exposed to supernatant of wild-type *E. coli* mixed with *P. aeruginosa* produced no inhibition zones. Third, exposure to supernatant of *E. coli* carrying pTetR-LasR-pLuxR-S5-pLuxR-E7 (final system) did not produce any inhibition as well. Fourth, only *P. aeruginosa* exposed to supernatant of *E. coli* carrying final system with *P. aeruginosa* displayed clear inhibition zones, which suggested that the system produced sufficient pyocin S5 to exhibit bactericidal activity. (B) *P. aeruginosa* cells stained using the LIVE/DEAD cell viability assay. Many *P. aeruginosa* cells were stained with PI dye, which indicate dead cells, when exposed to supernatant of engineered *E. coli* carrying the final system that was induced by native 3OC₁₂HSL produced by *P. aeruginosa*. This result confirms that our system is effective in killing *P. aeruginosa*. Scale bar: 5 μ m.

To verify that our engineered *E. coli* that contains the final system (i.e. pTetR-LasR-pLuxR-S5-pLuxR-E7) exerts a killing activity against *P. aeruginosa* in a mixed culture, we monitored the growth of *P. aeruginosa* co-cultured with the engineered *E. coli* in the ratio 1:4. To determine the growth inhibition of *P. aeruginosa* in the mixed culture, we used *P. aeruginosa* that constitutively expresses GFP (Appendix Figure 3 shows fluorescent microscopy images of In7 transformed with pMRP9-1 which contains GFP reporter) and *E. coli* that is without either the pyocin S5 or E7 lysis gene as negative controls. Figure 3-5A shows that the GFP expression level of the *P. aeruginosa* co-cultured with the *E. coli* that carries the final system remained almost constant, whereas the GFP level underwent an exponentially increase when *P. aeruginosa* was cultured with the negative control *E. coli* systems. To verify the efficiency in growth inhibition, we also performed CFU count on mixed cultures using *P. aeruginosa* that is transformed with chloramphenicol resistant plasmid. Figure 3-5B shows that our engineered *E. coli* inhibited the growth of *P. aeruginosa* by >99% while continues growth were apparent in *P. aeruginosa* co-cultured with incomplete *E. coli* systems missing either the pyocin S5 or E7 lysis devices. The results also imply that our engineered system was activated only after the pathogen entered the late exponential and stationary phase when the autoinducers were released (Appendix Figure 2B).

Figure 3-5. inhibition of *P. aeruginosa* by the engineered *E. coli* induced with native 3OC₁₂HSL produced by *P. aeruginosa* fluorescence measurement and CFU counting.

(A) Fluorescence measurement of *P. aeruginosa* that constitutively expresses GFP in mixed culture with engineered *E. coli*. The result from the mixed culture with the engineered *E. coli* carrying pTetR-LasR-pLuxR-E7 and pTet-LasR-pLuxR-S5 shows an exponential increase in the fluorescence readings, whereas the mixed culture with *E. coli* carrying pTetR-LasR-pLuxR-S5-pLuxR-E7 (the final system) exhibited no increase in the readings. This suggests that the growth of *P. aeruginosa* was significantly inhibited in the mixed culture with engineered *E. coli* carrying the final system. PAO1, which pyocin S5 was derived from, was included as a negative control. (B) Percentage survival of *P. aeruginosa* carrying chloramphenicol-

resistant plasmid in mixed culture with the engineered *E. coli*. *Pseudomonas* in the mixed culture was quantified by viable cell count using chloramphenicol selection. The result shows that our engineered *E. coli* inhibited the growth of *Pseudomonas* by more than 99%. In contrast, inhibition was less observed in *Pseudomonas* co-cultured with incomplete *E. coli* systems missing either the pyocin S5 killing device or E7 lysis device.

For any novel biological system to be of therapeutic relevance, it is important that cells in the biofilm state can be targeted as well. To study the feasibility of our engineered system, static biofilm inhibition assay was performed in a 24 well polystyrene plate by culturing *P. aeruginosa* carrying chloramphenicol resistance plasmid with engineered *E. coli*. Figure 3-6 shows that our engineered *E. coli* inhibited the formation of *P. aeruginosa* biofilm by close to 90%. This observation is in stark contrast to the pyocin-resistant control strain PAO1 and pyocin-sensitive clinical isolate In7 subjected to treatment with *E. coli* systems missing either the pyocin S5 or E7 lysis gene.

To visualize the extent of biofilm inhibition, biofilm cells with green fluorescence were grown in the presence of engineered *E. coli* on glass slide substrate and examined with confocal laser scanning microscopy (CLSM). Figure 3-7 shows that the morphology of *P. aeruginosa* biofilm developed from a culture of *P. aeruginosa* treated with engineered *E. coli* appears sparse and falls short of the dense, elaborated honey-combed structures apparent in control experiments. This observation may imply that our engineered *E. coli* have the capacity to inhibit biofilm formation during the initial attachment and prevent subsequent progression into mature micro-colonies. Collectively, our results suggest that our engineered *E. coli* carrying the final system, which contains the sensing and killing, and lysing devices, can effectively inhibit the growth of *P. aeruginosa* in both its planktonic and sessile state.

Figure 3-6. Biofilm inhibition assay with engineered *E. coli*, by measuring Percentage survival of *P. aeruginosa* biofilm carrying chloramphenicol-resistant plasmid

Pseudomonas biofilm was grown in a polystyrene 24-well plate in the presence of the engineered *E. coli* for 18 hours and quantified by viable cell count using chloramphenicol selection. The results imply that the formation of *Pseudomonas* biofilm was inhibited by close to 90% with the engineered *E. coli* carrying the final system (pTetR-LasR-pLuxR-S5-pLuxR-E7) as compared to biofilm grown with wild type *E. coli* or incomplete *E. coli* system missing either pyocin S5 or E7 lysis genes. *P. aeruginosa* PAO1, which pyocin S5 was derived from, was included as a negative control.

**In7 biofilm cultured with *E. coli*
pTetR-LasR-pLuxR-S5-pLuxR-E7**

**In7 biofilm cultured with *E. coli*
pTetR-LasR-pLuxR-S5**

In7 biofilm cultured with *E. coli*
pTetR-LasR-pLuxR-E7

In7 biofilm

Figure 3-7. Biofilm inhibition assay with engineered *E. coli* by CLSM microscopy

Biofilm inhibition observed under CLSM microscopy. *Pseudomonas* biofilm with green fluorescence was grown on glass slide in the presence of engineered *E. coli* and visualized under CLSM microscope after 18 hours. Images reconstructed from biofilm Z-stacks using Zeiss 2.5D software implied that the initialization and progression of biofilm cells into multi layers were strongly inhibited for *Pseudomonas* grown with *E. coli* carrying the final system as opposed to lush and elaborated biofilm formation observed in *Pseudomonas* grown alone or with incomplete *E. coli* system missing either pyocin S5 or E7 lysis genes. Scale bar: 50μm. Z stack: 40μm.

3-4- Conclusion

This report presents a novel method of treatment against infection by *P. aeruginosa* by adopting engineering concepts from synthetic biology. As mentioned earlier in previous chapter, all genetic devices involved in our system (e.g. sensing, killing and lysis device) were designed, assembled and characterized in terms of their working efficiency. Furthermore we went on to verify the effectiveness of lysis device in releasing pyocin S5 to the environment and antimicrobial efficiency of overall sensing-killing system to attack *P. aeruginosa* in planktonic and biofilm state .

Our study is one of the first examples of how synthetic biology can be used for fighting bacterial infections. The first phase of this project was for a proof of concept, and the results strongly advocate the feasibility of using engineered sensing-killing construct for treatment against *P. aeruginosa* infections. As compared to current methods being employed in the medical industry, the new proposed method is superior in terms of potency and strain specificity. Given the stalled development of new antibiotics and the increasing emergence of multi-drug-resistant pathogens, this study might provide the evidence that synthetic biology has great potential to overcome those bottlenecks in combating infectious diseases. For instance, by using standard biological parts, our pathogen sensing and killing scheme can readily be expanded to eradicating other infectious pathogens such as *Vibrio cholera* *Mycobacterium tuberculosis*, which will greatly benefit from the synthetic biology framework harnessed in this study, including standardization, abstraction, and decoupling. Additionally, system optimization can be conveniently carried out by integrating supplementary amplifying circuits to attain

performance of enhanced sensitivity, signal output or killing ratio (Nistala, Wu et al. 2010).

As an expansion of this work, we are currently developing characterization models that can be harnessed in other microbial chassis including residential microbes present in the upper respiratory tract. This presents the possibility of engineering potentially beneficial microbiota from the *Neisseria*, *Capnocytophaga* and *Veillonella* genera as therapeutic bioagents in arresting *Pseudomonas* infection (Brook 2005; Charlson, Chen et al. 2010).

The next step of this work is to further optimize our current system in terms of; (i) its sensitivity to detect inducer concentration, (ii) increasing the dynamic and steady state production of pyocin S5 and (iii) controlled production of lysis protein to release pyocin S5 to the extracellular environment. In the following chapter I have proposed and validate a mathematical model which can fully describe the dynamic and steady state behavior of our sensing device in term of downstream protein expression.

4- Characterization of quorum sensing device for synthetic biology design: experimental and modeling validation

4-1- Introduction

Synthetic biology involves engineering biological systems with novel functions by using standard, reusable, interchangeable biological parts. The use of these standard biological parts enables the utilization of engineering principles such as standardization, decoupling, and abstraction for more efficient engineering of biological systems (Endy 2005). Synthetic biology has shown that its framework can be implemented to develop novel biological systems to produce biofuels (Steen, Kang et al. 2010), to degrade pollutants in water (Sinha, Reyes et al. 2010), to produce drugs (Ro, Paradise et al. 2006), and to sense and kill a pathogen (Saeidi, Wong et al. 2011) .

There are now a number of registries of biological parts such as MIT registry of standard biological parts (<http://partsregistry.org/>) and BglBricks (Anderson, Dueber et al. 2010). These biological parts range from promoters to composite of parts which form devices with more complex functions. In order to provide important and useful information about these parts for the development of new complex biological systems, these parts need to be well characterized. To address the challenge of characterizing standard biological parts, Canton et al proposed a framework in which both static and dynamic performance of a device were characterized and presented in a form of datasheet for easy reference (Canton, Labno et al. 2008). Static and dynamic performances of devices are important characteristics. Static performance describes the steady state relationship between the

input and output of the device while dynamic performance describes the dynamic response of the device to a step increase at time zero. These two characteristics are useful in predicting the output amount and studying the response time of composed systems (Canton, Labno et al. 2008). To enable more efficient design of complex biological systems, it is important that this information about the characteristics of the parts is readily accessible by computer aided design (CAD) tools. Consequently, it has been proposed that each standard biological part should have a corresponding standard model that can provide necessary characteristics about the parts to perform modeling of biological systems *in silico* (Rouilly, Canton et al. 2007). Because it is important to have quantitative, reproducible models that can stimulate and reproduce the static and dynamic characteristics of standard biological parts/devices, in this study, we developed a mathematical model that is able to simulate both the dynamic and static performance of a standard biological device and embed information such as rate constants for reactions involved into the model to facilitate integration of different parts into a composed system.

This paper presents the development of the quantitative model that is able to simulate both static and dynamic performance of a biological device. In this work, we modeled a quorum sensing device fused with green fluorescent protein (GFP). This sensing device is able to sense *Pseudomonas aeruginosa* (a human pathogen) based on quorum sensing and was utilized in a larger biological system which is capable of sensing and killing *P. aeruginosa* (Ling, Saeidi et al. 2010; Saeidi, Wong et al. 2011). Experiments were performed for parameters estimation and model validation. As the model should be machine readable and searchable so that the model and its content can be readily accessed by CAD tools, our model is stored using system biology markup language (SBML) with

all the entities in the model fully annotated based on MIRAM, the Minimum Information Requested in the Annotation of Models (Novere, Finney et al. 2005). To ensure that our model is reproducible and reusable, we adhered to the recently introduced MIASE requirements, the Minimum Information About a Simulation Experiment (Waltemath, Adams et al. 2011).

This study presents the development of quantitative model that is able to simulate both static and dynamic behavior of a biological device. In this work, we modeled a quorum sensing device, which is capable of sensing *P. aeruginosa* (a human pathogen), fused with green fluorescent protein (GFP) producing device as a case study. This quorum sensing system was utilized in a larger biological system which is capable of detecting the quorum sensing molecules, (Acyl Homoserine Lactone-AHL) produced by *P. aeruginosa* and destroying the pathogen using a type of bacteriocin (i.e., pyocin S5) (Ling, Saeidi et al. 2010). Experiments were performed for parameters estimation and validation of the model.

4-2- Methodology

4-2-1- Strains and media

All cells involved in cloning and characterization experiments are *E. coli* TOP10 (Invitrogen). Supplemented M9 media (M9 salts, 1 mM thiamine hydrochloride, 0.4% glycerol, 0.2% casamino acids, 0.1 M MgSO₄, 0.5M CaCl₂) was used as the medium for the characterization. Ampicillin (100 µg/mL) was added to the culture media for antibiotic selection where appropriate. Homoserine lactone (3OC₁₂HSL; Sigma Aldrich) was used for characterization experiments. All enzymes were purchased from New

England Biolabs (NEB). Table 4-1 summarizes all plasmids, BioBrick parts and devices used in this study. Genetic maps are shown in Appendix Figure 1.

Table 4-1. List of all genetically encoded parts, devices and systems used in this work.

The part number, functional description and symbol used are listed for each component. Descriptions of all BBa parts can be found in the MIT Registry of Standard Biological Parts while the rest are explained in text.

Part Number	Description	Symbol
BBa-R0040	TetR repressible promoter
BBa-R0062	LuxR & AHL inducible promoter
BBa-B0032	Ribosome Binding Site (medium)
BBa-B0034	Ribosome Binding Site (strong)
BBa-C0179	LasR coding region
BBa-E0040	Green Fluorescence Protein
BBa-K117000	Lysis Protein
BBa-B0015	Terminator
pTetR-LasR-pluxR	Sensing device
pTetR-LasR-pluxR-GFP	Sensing device with reporter protein

4-2-2- System assembly

The genetic constructs developed in this study were assembled using standard synthetic biology protocols (Shetty R 2008). Details have been explained previously in section 2-2-2.

4-2-3- Design of the quorum sensing device

The mechanism behind the design of the sensing device has been previously described in section 2-3-1. As shown in Figure 4-1, the formation of the LasR-3OC₁₂HSL complex, which binds to the *luxR* promoter will lead to the production of GFP which serves as a reporter to characterize the steady state and dynamic performance of the quorum sensing device.

We have focused on sensitivity to AHL as the key specification of the sensing device. Characterization of the sensing device in our earlier study indicated that *P. aeruginosa* produced 1.0E-7 to 1.0E-6 M of 3OC₁₂HSL (Saeidi, Wong et al. 2011) and previous studies estimated extracellular concentration of 3OC₁₂HSL to be in the range of 1.0E-6 to 1.0E-4 M within proximity to the site of *P. aeruginosa* infection (Charlton et al., 2000; Pearson et al., 1995). Hence, the sensing device needs to be sensitive to the 1.0E-7 to 1.0E-6 M of AHL. The performance of the sensing device should be reproducible under same condition. It is worth noting that the current device response time is about 2-3 hours.

Figure 4-1. Schematic of the quorum sensing system modeled.

The system is based on the type I quorum sensing mechanism of *P. aeruginosa*. The TetR promoter (pTetR), which is constitutively on, produces a transcriptional factor (LasR) that binds to AHL (3OC₁₂HSL). The *LuxR* promoter (pLuxR), to which LasR-AHL activator complex binds, serves as the inducible promoter in our sensing system. The formation of the LasR-AHL complex, which binds to the *luxR* promoter, leads to the production of GFP as the reporter protein.

4-2-4- Characterization of sensing device (pTetR-LasR-pLuxR-GFP) with 3OC₁₂HSL

Single colonies of *E. coli* (Top10) with pTetR-LasR-pLuxR-GFP were each inoculated into 5 mL of pre-warmed supplemented M9 ampicillin for overnight culture in a shaking incubator at 37°C. After overnight growth, the cultures were diluted to OD₆₀₀ of 0.02 and allowed to incubate further to OD₆₀₀ of 0.5 under the same condition. Cultures were then transferred into a transparent, flat-bottom 96 well plate in triplicate aliquots of 200 µL for induction with 3OC₁₂HSL at varying molar concentrations (0, 5E-10, 1E-9, 1E-8, 5E-8, 1E-7, 5E-7, 1E-6, 5E-6, 1E-5, and 1E-4 M). The plate was incubated at 37°C with rapid shaking in a microplate reader (BioTek) and assayed for green fluorescence. Time-series fluorescence and OD₆₀₀ data were obtained at intervals of 10 minutes for a total run time

of 5 hours. The experiments were done with two biological replicates and three technical replicates. The experimental result was zeroed with supplemented M9 to remove background fluorescence and OD₆₀₀.

We chose these concentrations as our sensing device is designed to sense the presence of *P. aeruginosa* (Saeidi, Wong et al. 2011). Therefore, for our sensing device to be useful it should be able to detect the native 3OC₁₂HSL concentration produced by *P. aeruginosa* as described in Section 2.3.

4-2-5- Model of the sensing device

The model developed comprises of 6 first order ordinary differential equations (ODEs) which represent the reactions involved and the kinetics of the quorum sensing device. Figure 4-1 illustrates the steps involved in triggering the production of reporter protein GFP.

The constitutive production of LasR is described by Equations 4-1 and 4-2. We assume that LasR mRNA and LasR protein are produced according to the law of mass action at the rate of k_1 and k_2 and are degraded with the rate of y_1 and y_2 respectively.

$$\frac{d[mRNA_{LasR}]}{dt} = k_1[pTetR] - y_1[mRNA_{LasR}] \quad (4-1)$$

$$\frac{d[LasR]}{dt} = k_2[mRNA_{LasR}] - y_2[LasR] \quad (4-2)$$

The rate of complex formation between LasR protein and 3OC₁₂HSL is represented in Equation 4-3 where LasR-AHL denotes the complex. The complex formation rate is

directly proportional to the concentration of LasR and 3OC₁₂HSL present, where k_3 denotes the association rate constant (Fagerlind, Nilsson et al. 2005) and k_4 denotes the dissociation rate.

$$\frac{d[LasR - AHL]}{dt} = k_3[LasR][AHL] - k_4[LasR - AHL] \quad (4-3)$$

Equation 4-4 represents the change in concentration of 3OC₁₂HSL (AHL).

$$\frac{d[AHL]}{dt} = -k_3[LasR][AHL] + k_4[LasR - AHL] - \gamma_3[AHL] \quad (4-4)$$

Equation 4-5 represents the binding of unoccupied *pLuxR* promoter (pLuxR) to LasR-AHL complex, in which k_5 denotes the association rate, k_6 denotes the dissociation rate and A.pLuxR represents the occupied form of pLuxR (Hooshangi, Thiberge et al. 2005).

$$\frac{d[A.pLuxR]}{dt} = k_5[LasR - AHL][pLuxR] - k_6[A.pLuxR] \quad (4-5)$$

GFP concentration follows Equation 4-10. T in Equation 4-10 denotes the time constant which represents the time it takes for the system response to reach 63.2% of its maximum output. GFP_{max} is the maximum GFP (amount of GFP at steady state) produced at different AHL concentrations. Hence, Equation 4-6 describes the production rate of GFP by the active complex (A.pLuxR), T and GFP_{max} in equation 4-6 have the same meaning as in equation 4-10. We have assumed that AHL concentration is negligible when compared to pLuxR concentration. Furthermore, because we are using PSB1A2 which is a high copy number plasmid (100-300 per cell), considering pluxR in excess is a

reasonable assumption. Hence, we consider that A.pluxR concentration mainly depends on the AHL concentration. T and $GFP_{\max}$, both are function of AHL concentration. Because experimental results show that $1/T$ and $GFP_{\max}$ varied in a Hill equation like manner when AHL concentration increases, two Equations 4-6.1 and 4-6.2 were introduced to represent the change of $1/T$ and $GFP_{\max}$ with AHL, respectively. $1/T$ is introduced in equation 4-6.1 because it represents the speed of reaction (dynamic performance) while $GFP_{\max}$ in equation 4-6.2 denotes the final output (steady state performance). Equations 4-6.1 and 4-6.2 are functions of the A.pLuxR.

$$\frac{d[GFP]}{dt} = \frac{1}{T} (GFP_{\max} - [GFP]) \quad (4-6)$$

$$\frac{1}{T} = k_7 + \frac{k_8 [A.pLuxR]^{n_1}}{k_9^{n_1} + [A.pLuxR]^{n_1}} \quad (4-6.1)$$

$$GFP_{\max} = k_{10} + \frac{k_{11} [A.pLuxR]^{n_2}}{k_{12}^{n_2} + [A.pLuxR]^{n_2}} \quad (4-6.2)$$

4-2-6- Parameter derivation

Parameters for Equation 4-1 to Equation 4-5 were obtained derived from literature. k_1 and k_2 were derived based on information provided in BioNumbers, the database of useful biological numbers. BioNumber ID: 104902 (RNA polymerase transcription rate of mRNA in different media and doubling times) was used to calculate k_1 (Vogel and Jensen 1994) While BioNumber ID:100059 (Rate of translation by ribosome) and distance between ribosome on each mRNA (Bremer 1996) were used to derive k_2 . Table 4-2 summarizes the parameters related to equations 4-1 to 4-5. To derive the parameters

for Equations 4-6, experiments in which the quorum sensing device was characterized were performed. Two independent experiments were carried out. Each experiment had two biological replicate and three technical replicates. The data from the first experiment was used to estimate the parameters of Equations 4- 6.1 and 4-6.2 while the data from the second experiment was used to verify the model. Table 4-4 shows the parameters value of equations 6.1 and 6.2.

In this experiment, time-series fluorescence and OD₆₀₀ data were obtained at intervals of 10 minutes for a total run time of 5 hours. Relative Fluorescence Unit (RFU) which is an indirect way of GFP concentration measurement was then divided by OD₆₀₀ at each time point. The data was converted to absolute units (CFU/well and [GFP](M)) using Equations 4-7, 4-8 and 4-9. These equations were derived by calibrating the BioTek microplate reader (Canton, Labno et al. 2008). Details about the steps to calibrate the micropalte reader have been explained in the following section.

$$CFU / well = OD_{600} \times 3E + 8 \quad (4-7)$$

$$[GFP](M) = RFU \times 1.28E - 8 \quad (4-8)$$

$$\frac{[GFP](M)}{CFU / well} \approx \frac{RFU}{OD_{600}} \times 4E - 17 \quad (4-9)$$

To obtain parameters for Equations 4-6.1 and 4-6.2, GFP_{max} and T at different AHL concentrations need to be first derived. This was achieved by fitting Equation 4-10 to the curve representing the GFP production over time (dynamic characteristics) for each

different AHL concentration. The parameter t in equation 10 refers to the time as the variable

$$[GFP] = GFP_{\max} (1 - e^{-t/T}) \quad (4-10)$$

Parameters in Equations 4-6.1 and 4-6.2 were then estimated by fitting $1/T$ at different AHL concentrations and by fitting $GFP_{\max}$ at different AHL concentrations, respectively.

All fitting of curves were performed using cftool in Matlab (The Mathworks, Natwick, MA, USA) and CellDesigner 4.1 with SBML ODE solver and Copasi (Funahashi, Morohashi et al. 2003; Funahashi, Matsuoka et al. 2008) was used to construct the model run the simulation.

4-2-7- Data calibration

Data measured using the BioTek microplate reader were converted to absolute units for better repetition of the experiments and to allow comparison of data. Absorbance measurements were related to colony forming units (CFU) by growing a culture of *E. coli* top10 carrying pTetR-LasR-pLuxR-GFP to different cell densities and then plating an appropriate dilution on LB agar plates containing 100µg/ml ampicillin. For each culture density, three identical plates were made and the resulting colonies were counted. A straight line ($R^2 = 0.99$) was used to fit the data relating the number of colony forming units (CFU) per ml of culture to the absorbance measurements. The equation of the straight line was used to convert all absorbance measurements to CFU/well (Equation 4-7) (Sambrook and Russell 2001).

Fluorescence measurements were converted to an absolute number of GFP molecules by relating the fluorescence of cultures of *E. coli* top10 carrying pTetR-LasR-pLuxR-GFP to the fluorescence of purified GFP using a two-step calibration. In the first step, purified GFP (1.0 mg/ml) from GFP quantification kit (Cell Biolabs Cat # AKR-120) was serially diluted in assay diluent provided by the kit. Fluorescence of the GFP dilutions was measured in the microplate reader and the data plotted against the mass of GFP in each dilution. A straight line ($R^2 = 0.99$) was fitted to the data.

In the second step, cultures of *E. coli* top10 carrying pTetR-LasR-pLuxR-GFP were induced using (0,1E-10,1E-9,1E-8,1E-7,1E-6 M) of AHL at $OD_{600}=0.42$ leading to varying intracellular concentrations of GFP. Sample fluorescence was measured in the fluorimeter and the remainder of the cultures were lysed in the following way.

Samples were centrifuged at 13000rpm for 4 min at 4°C. After removing the supernatant, they were resuspended in 10mM Tris buffer containing 100mM NaCl adjusted to pH=8.0. Then, samples were centrifuged as before and cell pellets were resuspended in lysis buffer (provided by the kit + EDTA free complete protease inhibitor). After allowing about 30 min for complete lysis, the fluorescence was read. It should be noted that to avoid GFP degradation, samples were kept on ice before measuring the fluorescence. The fluorescence of the lysed samples was measured and plotted against the fluorescence prior to lysis. A straight line ($R^2=0.99$) was used to fit the data. The fit equations for the purified GFP dilutions and the lysed samples were combined to derive a straight-line relationship between the fluorescence of unlysed cultures and the number of

GFP M/well. This relationship was used to convert all fluorescence measurements to GFP molecules per well (Equation 4-8).

4-2-8-Sensitivity analysis assay

To analyze the sensitivity of the various parameters in the model, sensitivity analysis was performed. To carry out the sensitivity analysis, each parameter was varied in range of -80% to +100% (at an interval of 20%) from the original value which was obtained either from literature or from parameter fitting. To compare the sensitivity assay results obtained from simulation, two graphs were drawn: percentage difference in original output value at 300 min versus percentage change in parameter value at AHL concentration of $5\text{E-}7$ M as the medium input of the quorum sensing device, and percentage change in time to reach steady state versus percentage change in parameter value. We chose this concentration as our sensing device should be able to detect the AHL in the range of $1\text{E-}7$ to $1\text{E-}6$. Hence we picked $5\text{E-}7\text{M}$ as the average AHL concentration.

4-2-9-Model validation

To verify the reproducibility of our proposed model, an independent set of experimental results was obtained to compare with the simulation results derived using the proposed model. To validate the simulation results for steady state GFP concentration, simulations with different concentrations of AHL (0, $5\text{E-}10$, $1\text{E-}9$, $1\text{E-}8$, $5\text{E-}8$, $1\text{E-}7$, $5\text{E-}7$, $1\text{E-}6$, $5\text{E-}6$, $1\text{E-}5$, and $1\text{E-}4$ M) were performed for 100 time steps over 300 min. Furthermore, the ODEs were solved at steady-state to analytically derive the transfer function from AHL

to GFP. The steady state value of GFP obtained using the simulation results and analytical transfer function were compared to the experimental results obtained using different concentrations of AHL. To validate the simulation results for dynamic GFP production, simulation was run using the same conditions as before at 3 different AHL input concentrations: low, medium and high input (i.e., $5\text{E-}10$, $5\text{E-}7$ and $5\text{E-}6$ M of AHL, respectively) and simulation results were compared with experimental results under same conditions (e.g. same AHL concentrations and over 300 min). Results are shown in section 4-3-4.

4-2-10-Format and annotation of the model

To ensure that our model and the content in the model are searchable and readable by machines, standard SBML format was used and all parts and reactions were fully annotated according to MIRIAM. Further, to ensure that the simulation results are reproducible, we adhered to the MIASE requirements.

MIRIAM is a standard for reference correspondence dealing with syntax and semantics of the model and an annotation scheme that specifies the documentation of the model by external knowledge (Novere, Finney et al. 2005). Annotation is composed of two key components. Firstly, it gives an attribution to the entity, covering the absolute minimum information that is required to associate the model with both a reference description and an encoding process. Secondly, it is linked to external data resources. Although the MIRIAM promotes the communication and reusability of quantitative biochemical models, it is not enough for efficient reuse of models in a quantitative setting. Consequently, MIASE which describes the minimal information that must be provided

with a simulation experiment for others to use was recently introduced. It is essential to include the core information required to perform the simulation of quantitative models (Waltemath, Adams et al. 2011).

4-3-Results and discussion

4-3-1-Characterization of sensing device (pTetR-LasR-pLuxR-GFP) with 3OC₁₂HSL

To characterize and evaluate our proposed model of the quorum sensing device, GFP was ligated downstream to the sensing device (i.e. pTetR-LasR-pLuxR-GFP). To obtain the characteristics of the sensing device, GFP concentration over the number of cells in one well was monitored at a range of concentrations of inducer 3OC₁₂HSL. The results from two independent experiments were obtained (Figure 4-2). In both experiments, the results show that there was a sharp increase in GFP production as the 3OC₁₂HSL concentration increased. This transition occurred at inducer concentration from 0 to 1E-6 M, after which it started to decline. We observed a decline in GFP response at AHL concentration greater than 1E-6. We observed a decline in GFP response at AHL concentration greater than 1E-6. This decline could be due to the over production of GFP and its toxicity to the cells, causing *E. coli* to die. The switch point of the sensing device is at 2.4E-7 M. This shows that the optimum detection range of 3OC₁₂HSL for this quorum sensing device is between 1E-7 M and 1E-6 M.

4-3-2-Parameters derivation

Table 4-2 summarizes the parameters that were derived from literature. These parameters were used in equations 1 to 5 of our proposed model. Most of the parameters were extracted directly from the corresponding literature (Elowitz and Leibler 2000; Basu, Gerchman et al. 2005; Fagerlind, Nilsson et al. 2005; Hooshangi, Thiberge et al. 2005; Kaufmann, Sartorio et al. 2005), except for k_1 and k_2 . These two parameters represent LasR mRNA and LasR protein synthesis rate respectively. k_1 was calculated to be 0.0622 s^{-1} or 3.734 min^{-1} by considering that the length of LasR mRNA is 723 nucleotides and mRNA chain elongation rate to be $45 \text{ residues s}^{-1}$ (according to BioNumber ID:104902). For k_2 derivation, by taking into account the length of LasR which is 241 amino acids and translation rate of $16 \text{ amino acids s}^{-1}$ which is obtained from (BioNumber ID: 100059), rate of a LasR synthesis was calculated to be $16/241 = 0.07 \text{ s}^{-1}$. To calculate the number of active translation sites on a LasR mRNA we also considered the distance between each ribosome on the mRNA and the length of LasR mRNA. Based on our doubling time which is 60 min, the required distance between each ribosome is 85 nucleotides. As the length of $\text{mRNA}_{\text{LasR}}$ is 723 nucleotides, the number of active translation site on a $\text{mRNA}_{\text{LasR}}$ is 8.5 (Bremer 1996). Therefore, k_2 was calculated to be 0.595 s^{-1} or 35.7 min^{-1} .

To estimate the parameters in Equations 4- 6.1 and 4-6.2, $1/T$ and GFP_{max} at different concentrations of AHL were first derived by fitting equation 10 to the first set of experimental data (summarized in Table 3). Overall, the R^2 of the fittings related to AHL

concentration from 1E-8 to 1E-6 M were more than 0.9, showing high correlation between experimental data and fitted graphs.

Using the $1/T$ and GFP_{max} derived, equations 6.1 and 6.2 were fitted to the curve $1/T$ versus AHL concentrations and GFP_{max} versus AHL concentrations, respectively. Table 4 shows the parameters that were derived for Equations 4- 6.1 and 4-6.2. The experimental results show that both GFP_{max} and $1/T$ increases in a Hill equation like manner as the concentration of AHL increases. GFP_{max} and $1/T$ correspond to the steady state and dynamic performance of the quorum sensing device to different AHL concentrations, respectively. This implies that the steady state amount of GFP and the rate of GFP production increase when AHL concentration increases from 1E-8 to 1E-6 M and reaches a plateau after 1E-6 M.

Figure 4-2. Characterization results of sensing device coupled with GFP reporter.

GFP concentration per cell over time at different 3OC₁₂HSL inducer concentrations, high GFP production per cell was observed when the input 3OC₁₂HSL concentration was in the range of $1\text{E}-6$. (A) First experiment set was used to derive parameters of the model. (B) Second set was used for modeling validation.

Table 4-2. List of all the parameters for equations 4-1 to 4-5, which are taken from literatures.

Parameters	Definition	Value	Reference
k_1	Synthesis rate of mRNA (LasR), min^{-1}	3.734	http://Bionumbers.harvard.edu Derived based on BioNumber ID: 104902
y_1	mRNA (LasR) decay rate, min^{-1}	0.348	(Elowitz and Leibler 2000)
k_2	Synthesis rate of LasR, min^{-1}	35.7	http://Bionumbers.harvard.edu Derived based on BioNumber ID: 100059 and 102364
y_2	Decay rate of LasR, min^{-1}	0.0696	(Basu, Gerchman et al. 2005)
y_3	Decay rate of 3OC ₁₂ HSL, min^{-1}	2.83E-4	(Kaufmann, Sartorio et al. 2005)
k_3	Association rate of LasR and 3OC ₁₂ HSL, $\text{M}^{-1}\text{min}^{-1}$	9.6E+6	(Fagerlind, Nilsson et al. 2005)
k_4	Dissociation rate of LasR and 3OC ₁₂ HSL, min^{-1}	0	Assumption for long chain AHLs
k_5	Association rate of LasR-3OC ₁₂ HSL and pLuxR, $\text{M}^{-1}\text{min}^{-1}$	1.98E+6	(Hooshangi, Thiberge et al. 2005) (tuned)
k_6	dissociation rate of LasR-3OC ₁₂ HSL and pLuxR, min^{-1}	10.2	(Hooshangi, Thiberge et al. 2005) (tuned)

Table 4-3. Derived parameters using 1st set of experiment, to find out GFP_{max} and 1/T

AHL Concentrations	GFP _{max} (M)	T(min)	1/T(min ⁻¹)	R ²
5.00E-10	7.5E-16	210	0.0048	0.826
1.00E-09	8.5E-16	205	0.0049	0.866
1.00E-08	8.90E-16	190	0.0053	0.904
5.00E-08	1.20E-15	135	0.0074	0.960
1.00E-07	1.90E-15	115	0.0087	0.937
5.00E-07	6.20E-15	90	0.0111	0.953
1.00E-06	9.3E-15	89	0.0112	0.936
5.00E-06	1.03E-14	85	0.0118	0.922

Table 4-4. Parameters after estimated empirically for Equations 4- 6.1 and 4-6.2

Parameter	Equation	Value
K ₇ (1/min)	6.1	0.0041
K ₈ (1/(min))	6.1	0.0096
K ₉ ([M])	6.1	9.74E-08
n ₁	6.1	2
K ₁₀ ([M]/cell)	6.2	6.50E-16
K ₁₁ ([M]/cell)	6.2	1.00E-14
K ₁₂ ([M])	6.2	2.40E-07
n ₂	6.2	2

4-3-3-Sensitivity analysis

There are a number of parameters in the model developed. To determine the parameters which have large effect on the model in terms of steady state GFP produced and time for GFP production to reach steady state, sensitivity analysis was performed. Figure 4-3A and Figure 4-3B show the sensitivity analysis results based on changes in GFP_{max} and time for GFP production to reach steady state, respectively.

For sensitivity analysis of changes in $\text{GFP}_{\max}$, the simulation results (Figure 4-3A) show that k_{11} (maximum output) and k_{12} (switch point) of Equation 4-6.2 were the two most sensitive parameters that greatly affect the final output of the quorum sensing device.

Figure 4-3. Sensitivity analysis.

(A) Sensitivity analysis on all the parameters for Equations 4-1 to 4-6, ranging from -80% to +100% of original value, changes in output taken at 300 min and with inducer concentration of $5E-7$ M. It was observed that k_{11} (switch point) and k_{12} (maximum output) of Equation 4-6.2, are the most sensitive parameters. (B) Sensitivity analysis on all the parameters for Equations 4-1 to 4-6, ranging from -80% to +100% of original value, changes in time to reach steady state. It was observed that that k_8 ($1/k_8$ represents the minimum time to reach 63.2% of the final output) and k_7 ($1/k_7$ represents the time to reach 63.2% of the final output when there is no induction) of Equation 4-6.1 are the most sensitive parameters to define the time which our system needs to reach steady state condition.

For sensitivity analysis of changes in time to reach steady state, Figure 4-3B shows that k_8 ($1/k_8$ represents the minimum time to reach 63.2% of the final output) and k_7 ($1/k_7$ represents the time to reach 63.2% of the final output when there is no induction) of Equation 4-6.1 were the two most sensitive parameters. These two parameters greatly affect the time at which the system reaches steady state. The other parameters are relatively insensitive mainly because reactions 1 to 5 are very fast reactions as compared

to reaction 6. The sensitivity of the parameters is independent of AHL concentrations, except k_{11} and k_{12} . An observation made is that k_{11} (maximum output) becomes more sensitive while k_{12} (switch point) becomes less sensitive at higher AHL concentration.

4-3-4-Model validation

To verify the accuracy of our proposed model, an independent second set of experimental data was obtained and compared with the simulation results computed by our model. Figure 4-4A and Figure 4-4B show the dynamic and steady state comparison between the experimental and simulated data. For the dynamic comparison (Figure 4-4A), at all three different inducer concentrations (low, medium and high), the simulated result matched well with the experimental data. The close matching of simulated and the experimental data shows that our proposed model is able to simulate the dynamic performance of quorum sensing system using the derived parameters. Figure 4-4B shows that for the steady state performance, both simulation results and analytical transfer function match well with the experimental result with $R^2 = 0.98$ and $R^2 = 0.93$ respectively. Hence, it is verified that the maximum GFP production can be reproduced using the proposed model. Equation 4-11 shows the analytical transfer function.

$$GFP = 6.5E-16 + \frac{1E-14[AHL]^2}{2.4E-7^2 + [AHL]^2} \quad (4-11)$$

(A)

(B)

Figure 4-4. Modeling validation.

(A) Validation of dynamic performance, for three different inducer concentration, (low, medium and high input). It was observed from the data that the dynamic performance of the quorum sensing device simulated by the proposed model matched well with the experimental data. (B) Steady state modeling validation, based on the output taken at 300 min, it is verified that the maximum GFP production for different input concentration can be predicted by the proposed model as well.

4-3-5-Model format and annotation

The quorum sensing system mathematical model is stored using SBML format and it follows both MIRIAM and MIASE requirements (Novere, Finney et al. 2005; Waltemath, Adams et al. 2011). One of the essential elements to create a standard biological model is to encode it in a machine-readable and searchable format such as SBML (Hucka, Finney et al. 2003; Endler, Rodriguez et al. 2009) and CellML (Cuellar, Lloyd et al. 2003). SBML was chosen in our study mainly because it has been more widely used, although saving in other formats is possible. These bioparts description languages enable the description of qualitative and quantitative characteristics of biochemical networks as well as definition of modules and hierarchies between them (Rouilly, Canton et al. 2007). In these languages, all elements can be annotated and commented in both human-readable and machine-interpretable forms, allowing extensive documentation and external references to be directly included in the model file. It allows for easier sharing of quantitative models among the community which is making them available. That is why a set of rules in a standardized approach (MIRIAM) has been proposed to the curation of model collection and encoding. Table 4-5 shows all the species and biological reactions in our developed model which are fully annotated following MIRIAM. This enables the species and biological reactions to be searchable as they will have unique identity.

Although MIRIAM promotes the communication and reusability of quantitative biochemical models, it is not enough for efficient reuse in a quantitative setting. Nowadays, it is essential to include the core information required to perform the

simulation of quantitative models. MIASE describes the minimal information that must be provided with a simulation experiment for others to use (Waltemath, Adams et al. 2011). To improve the reusability of the model, in this study we have adhered to the MIASE, which is important to extend the usefulness of the model for synthetic biology design. One of the key points in presenting a simulation experiment is the initial/boundary conditions of different components in the system. Table 4-6 shows all the species in our model with their corresponding initial concentration.

Table 4-5. Annotation of species and reactions following MIRIAM

Species	Database	ID
pTet-LasR	Pubmed	9092630
mRNA _{LasR}	CHEBI	CHEBI:33699
LasR	UniProt	P25084
3OC ₁₂ HSL	CHEBI	CHEBI:56080
LasR-AHL	Gene Ontology	GO:0005667
pLuxR	PubMed	1987152
A.pluxR	Gene Ontology	GO:0000428
GFP	UnitProt	P42212
Eq. 1	Gene Ontology	GO:0009299
Eq. 2	Gene Ontology	GO:0006412 and GO:0030163
Eq. 3	Gene Ontology	GO:0008134
Eq. 4	Gene Ontology and Pubmed	GO: 0008134 and PMID: 15623555
Eq. 5	Gene Ontology	GO:0010843
Eq. 6	Gene Ontology	GO: 0006412

Table 4-6. Initial concentration of species

Species	Initial concentration
pTet-LasR	1E-5 M
mRNA _{LasR}	1E-6 M
LasR	1E-6 M
3OC ₁₂ HSL	Range from 0 to 5E-6 M
LasR-AHL	0 M
pLuxR	1E-5 M
A.pluxR	0 M
GFP	0 M/cell

4-4-Conclusion

To facilitate the design of complex systems using the bottom-up approach, we need the help of computational tools. These tools can help us to understand the system behavior *in silico* first before proceeding to the labs to carry out the actual construction but there are challenges in using CAD tools. One of the challenges is that these tools need information about the parts. We developed a model which can describe the kinetics of a quorum sensing device as well as its dynamic and steady state performance. The quorum sensing device has been used in an earlier study as part of a larger biological system which is able to sense and kill *P. aeruginosa* (Saeidi, Wong et al. 2011). Our model is in standard SBML format and it complies to both MIRIAM and MIASE requirements. These requirement are crucial for the models, which are submitted to BioModels.net a database of annotated, published models. This resource allows biologists to store, search and retrieve mathematical models. Additionally, those models can be used to generate sub-models, can be simulated online, and can be converted between different representational

formats. It should be noted that, Our model has been recently submitted to these data base with submission ID of MODEL1301110000. Our model provides detailed annotated reactions involved during the gene expression of the quorum sensing device under certain conditions. One could imagine having a datasheet for the model similar to that proposed by Canton et al for biological parts. The model datasheet, together with the model will complement the datasheet of biological parts, facilitating future use of the biological parts (Temme, Zhao et al. 2012).

All the parameters related to equations 1 to 5 were derived from literature. Parameters of equation 6 with sub-equations 6.1 and 6.2 which were derived based on experimental data fitting related to $1/T$ and GFP_{max} , respectively. Our experimental findings show that $1/T$ which represents the time for the system to reach 63.2% of the final output follows a Hill equation manner. The experimental results show that the rate at which the device reaches to 63.2% of the final output increases sharply at AHL concentration of $9.74E-8$ M (switch point) and reaches a max rate at AHL concentration of $5.0E-6$ M. The GFP_{max} produced also follows similar trend, the switch point for equation 6.2 occurs at AHL concentration of $2.4E-7$ M and maximum amount of GFP_{max} is produced at AHL concentration of $5.0E-6$ M. In our study, data fittings of equations 6.1 and 6.2 were done based on three assumptions: (i) Both Hill coefficients (e.g. n_1 and n_2) are assumed to be 2 because quorum sensing transcription factors generally dimerise prior to binding to the promoter region as reported by earlier studies (Fuqua, Parsek et al. 2001; Zhu and Winans 2001; Nasser and Reverchon 2007). By considering this assumption, the curve was able to fit well with the experimental results. (ii) We considered that the

concentration of active complex A.pLuxR is equal to the input AHL concentration. Because in our construct LasR is constitutively being expressed under the control of pTetR promoter, we assumed that LasR amount is an excess when we introduced AHL. Furthermore, we are using PSB1A2 which is a high copy number plasmid (100-300 per cell). Taken together, considering pluxR in excess is a reasonable assumption. Because LasR and pLuxR are in excess, LasR-AHL and A.pLuxR amount will mainly depend on the input AHL concentration. (iii) The model is simulated deterministically which does not include the randomness that exists in natural systems, while stochastic model do. Nonetheless, the simulation results show that the model is able to reproduce the steady state and dynamic performance of the quorum sensing device under these assumptions.

Sensitivity analysis was performed to study the effect of individual parameter on the final output of the device and the time for the output to reach steady state. The simulation results from sensitivity assay shows that the most significant parameters are in equation 6. k_{11} and k_{12} of equation 6.2 were the most sensitive parameters in term of output amount while k_8 and k_7 of equation 6.1 had the most significant effect on the time to reach steady state condition. In equation 6.2, k_{11} is the most sensitive parameter in which a change in k_{11} will cause a similar percentage change in the final output, whereas a 100% change in k_{12} will cause about 30% change in the final output. On the other hand, in equation 6.1, a change in k_8 will cause a similar change in the time to reach steady state, while a 100% change in k_7 will cause about 20% change in the time to reach steady state point. The other parameters in equations 1 to 5 which were derived from literature have shown negligible sensitivity on the output amount and the time to reach steady state. Therefore our approach to use literature for these data derivation is reasonable. Although

the sensitivity analysis shows that only these 4 parameters are sensitive to the overall device performance, we embedded the reactions into the model in order to facilitate future merging of the models of different parts/devices to create the model of the desired composed system, in a similar manner described by semantic SBML (Krause, Uhlenendorf et al. 2010) and OREMPdb (Umeton, Nicosia et al. 2012). When there is a large number of well-validated models of biological parts in databases like BioModels.net, researchers working on new systems could search and import relevant models to be used in their own system modeling. This will enable more efficient building of models.

Although our proposed model was able to characterize the steady state and dynamic performance of the sensing device under similar conditions, the model can be further improved to take account different conditions such as different growing medium, different host strains, different plasmids etc. One possible approach is to study and establish the relationship of the parameters in the model with respect to a change in the conditions, for example changing the medium. This relationship can then be incorporated into the model to enable the model to capture a change in the medium.

The simulation results show that our proposed model agrees well with the experimental results. The accuracy of our proposed model was validated by carrying out dynamic and static modeling verification. Consequently, the model developed could be used to predict the GFP production under that current set of experimental conditions (e.g. temperature is fixed at 37°C). The model could be extended in the future to capture characteristics when these experimental conditions vary so that the model can be more versatile. The model

could provide useful information about the biological parts for future CAD applications for synthetic biology design.

5- Conclusion and future direction

In this thesis we adopted a synthetic biology framework for describing how to design and construct engineered devices/systems that have the potential to solve healthcare problems. Due to the emergence of multi-drug-resistant bacteria we aimed to use a synthetic biology approach to tackle infectious disease. By using a synthetic biology approach we engineered bacteria that can detect and kill pathogenic strains. In this work we chose *P. aeruginosa* because of its pathogenicity, which is mainly due to its ability to form biofilms. Biofilm formation makes *P. aeruginosa* resistant to antibiotic treatment. We took a bottom-up approach to assemble basic parts to form devices and used these devices to form the final system. In Chapter 2 and 3 we examined the efficiency of the individual devices as well as the final system.

To facilitate the design of complex systems using the bottom-up approach, we also needed the help of computational tools. These tools helped us to understand the system behavior in silico first before proceeding to the labs to carry out the actual construction. Although we attempted to optimize the circuits, there are challenges in using CAD tools. One of the challenges is that these tools need information about the parts and models of the parts. In order to optimize our existing genetic construct, we modeled individual devices, starting with our sensing device. The mathematical model of the sensing device was explained in Chapter 4.

5-1- The novel engineered *E. coli* to sense and kill *P. aeruginosa* a human pathogen

In Chapters 2 and 3 we used the tools of synthetic biology to create an *E. coli* cell that can break into pathogenic bacteria and blow up, killing other pathogens as well as itself. In this work we have shown the future possibilities of microorganism manipulation to tackle infectious diseases. Based on synthetic biology techniques, *E. coli* was engineered to perform functions that it is naturally incapable of carrying out, i.e. killing another microorganism. *P. aeruginosa* was selected as the target pathogenic strain because it can cause a wide range of infections, especially in patients with compromised defense mechanisms. At present, the development of new antimicrobial strategies is a serious concern. This is mainly due to a lack of progress among new generation antibiotics together with the emergence of multi-drug-resistant, extensively drug-resistant, and pandrug-resistant pathogens (Magiorakos, Srinivasan et al. 2012).

We have proposed a novel method to tackle *P. aeruginosa*, which is inspired by the natural quorum sensing mechanism. When pathogenic strains of *P. aeruginosa* sense other species, they can communicate with members of their own species through chemical signals and release pyocin. These communication and defense mechanisms allow *P. aeruginosa* to form biofilms in an infected area. In our novel engineered construct, we have used the *P. aeruginosa* defense system against itself.

We called our novel design a “sensing-killing device.” Our system functions as follows: First our engineered host cell, *E. coli*, detects the presence of *P. aeruginosa* by means of

the 3OC₁₂HSL (the signaling molecule produced by the quorum sensing system in *P. aeruginosa*). Second, the production of pyocin S5 as the toxic protein is activated, together with the E7 lysis protein. This causes the host cell to lyse and release the accumulated pyocin S5 to the external environment to target *P. aeruginosa*. Our engineered construct releases the pyocin only when *P. aeruginosa* is present and this toxic protein is not killing other types of bacteria which might be beneficial to the human body. We have also demonstrated that our engineered system worked not only in the planktonic state of *P. aeruginosa*, but also when the bacteria forms biofilm.

Although we have demonstrated the merits of this concept, our proposed system has not been tested on animals yet. Our current system would have to undergo a lot of work before it could be delivered to humans. If we could prove that it worked *in vivo*, this system could potentially be used in probiotic drinks for immunocompromised patients to prevent infections caused by *P. aeruginosa*. This novel synthetic system may provide a novel synthetic biology-driven antimicrobial strategy that could eradicate *P. aeruginosa* as well as other infectious pathogens such as *Vibrio cholera* or *Helicobacter pylori*.

In order to improve the efficiency of our current system and increase the concentration of pyocin S5, we have modeled the sensing device of the pathogen killing system. This mathematical modeling helps to understand the sensitivity of various parameters of the sensing device and their effect on the steady state response and dynamic response of the system.

5-2- Modeling the quorum sensing system

Mathematical modeling of synthetic genetic devices is a key component of designing novel biological circuits and predicting their expected behavior. In Chapter 4 we modeled our quorum sensing system to further optimize our current sensing-killing device. This work represents an example of how to develop mathematical equations that can describe the kinetics of standard biological parts as well as their dynamic and steady state behavior. It is essential to have a database with quantitative descriptions of parts. All parts should have their corresponding mathematical models in a machine-readable format that complies with MIRIAM and MIASE (Novere, Finney et al. 2005; Waltemath, Adams et al. 2011).

We modeled our quorum sensing device, which is capable of *P. aeruginosa* detection in the external environment (Saeidi, Wong et al. 2011). The proposed mathematical model is in standard SBML format and follows MIRIAM and MIASE rules. These rules are crucial for the models, which are submitted to BioModels Database—a database of annotated, published models. Our model has been recently submitted to this database with submission ID of MODEL1301110000. Most of the parameters in the equations are derived from the literature (Equations 4-1 to 4-5), while some of them are derived experimentally and based on data fitting (Equation 4-6). After data fitting, the results show that parameters in Equations 4-6.1 and 4-6.2, which represent $1/T$ and $GFP_{\max}$ respectively, are following Hill-like equation manner. $1/T$ represents the dynamic response while $GFP_{\max}$ represents the steady state response. In order to check the importance of individual parameters on the overall system, we did sensitivity analysis. A

sensitivity assay was done from both dynamic and steady state points of view. The results show that the parameters derived from experimental data fitting play the most important rule, while other parameters have less effect on the system. Finally, to validate the proposed model, we compared an independent set of experimental data to the simulation results. Our findings confirmed that our proposed model agreed well with the experimental results, and we showed that our proposed model can predict GFP production without running wet-lab experiments. This piece of work is an example of how we think the whole field of synthetic biology will migrate to documented quantitative descriptions.

Another challenge of using CAD tools is to have a database that can predict the strength of synthetic/genomic components like promoters. This database will improve the *in silico* design of biological systems. Recent research has calculated the strength of ribosome binding sites (Salis, Mirsky et al. 2009; Salis 2011). It is important to choose the right promoter to get the desired output before proceeding with wet-lab experiments. The following section discusses the proposed future direction of this promoter work and explains preliminary tasks.

5-3- Future direction

5-3-1- A model to predict the Synthetic promoter strength in *E. coli*

5-3-1-1- Introduction

The correlation between the promoter sequence and its strength was extensively studied in the 1980s (Stefano and Gralla 1982; Mulligan, Hawley et al. 1984; Mulligan, Brosius et al. 1985; Mulligan and McClure 1986; Berg and von Hippel 1987; Ayers, Auble et al. 1989; Gardella, Moyle et al. 1989; O'Neill 1989; Kobayashi, Nagata et al. 1990; Strohl 1992; Kumar, Malloch et al. 1993; Straney, Krah et al. 1994; Burr, Mitchell et al. 2000). Several studies used *E. coli* promoters that correspond to the sigma 70 subunit of RNA polymerase. A promoter consists of two conserved regions known as -35 box (TTGACA) and -10 box (TATAAT), with approximately 17 bp between them (Siebenlist, Simpson et al. 1980; Mulligan, Brosius et al. 1985; Harley and Reynolds 1987; Strohl 1992; Lissner and Margalit 1993). These studies have shown that promoters that are closer to the consensus sequence have stronger activities. Promoters with Spacer length of 17 have the highest activity. However, there is a lack of quantitative models to predict the strength of constitutive sigma 70 promoters in *E. coli* genome. Modeling and predicting the strength of the genomic and synthetic *E. coli* promoter from its sequence is without doubt a promising research area in the emerging field of synthetic biology.

5-3-1-2- Approach and results

In transcription regulation, DNA binding of sigma 70 and open complex formation are the rate limiting steps (McClure 1980). Therefore, considering the binding energy between the sigma 70 factor and the promoter can be promising to model the strength of *E. coli* promoters. Using the approach used by Kiryu *et al.* (Kiryu, Oshima et al. 2005) and Weindl *et al.* (Weindl, Hanus et al. 2007), we have found the energy landscape for 897 sigma 70 promoters from the Regulon DB database. The equations listed below were used to extract the binding energy.

$$E(s) = \sum_{k=1}^6 e(n_k, k) + \sum_{k=7}^{k=12} e(n_{k+s}, k) + \varepsilon(s)E \quad (5-1)$$

$$E(i) = \min_{s \in [15;19]} [E(s, i)] = \min_{s \in [15;19]} \left[\sum_{k=1}^6 e(n_{i+k-1}, k) + \sum_{k=7}^{k=12} e(n_{i+k+s-1}, k) + \varepsilon(s) \right] \quad (5-2)$$

$$\bar{E}(i) = \frac{1}{N} \sum_{I=1}^N E_I(i) - \bar{E}_{ran} \quad (5-3)$$

$E(i)$ is calculated at each position of DNA, using the weight matrix. The matrix represents the sigma factor, which slides along the DNA and does not change. The nucleotide sequence at position i determines which values of the matrix should be used in Equation 5-2. The weight matrix shown in Figure 5-1 is an important tool for the detection and analysis of protein binding sites; it assigns a score to each position of a sequence. Berg and Von Hippel have shown that the logarithm of the nucleotide frequencies is proportional to their binding energy contribution (Berg and von Hippel 1987).

Figure 5-1. Binding energy contribution of sigma 70 factor

(A) Weight matrix contribution of 12 nt to the binding energy. (B) Spacer length contribution to the binding energy. (C) -35 and -10 box contribution to the binding energy.

Figure 5-2 illustrates the energy landscape found by using Equations 5-1 to 5-3 and Figure 5-1.

Figure 5-2. Average binding energy of known sigma 70 promoters,

Average binding energy is calculated according to Equation 5-2 of 897 known sigma70 promoters aligned to the transcription start site (TSS). $E(i)$ zero corresponds to the energy E_{ran} (-0.4423) of random sequences. $E(i)$ shows three significant minimas at -35 and -10 box as well as UP element.

Based on the work of Weindl J *et al.* (Weindl, Hanus et al. 2007), we hypothesized that we can find the correlation between the minimum binding energy of genomic promoters at position (-38:-32) and the gene expression level of the corresponding gene. From this model we might be able to predict the strength of synthetic promoters of *E. coli*. After extracting the gene expression data of the *E. coli* K12 MG1655 from the GEO database, we could not find any meaningful trend between the minimum binding energy related to -35 box and the gene expression level (data not shown). According to our findings, we have assumed that considering the minimum binding energy is not enough to predict the strength of sigma 70 promoters in *E. coli*.

We proceeded to change our approach and to use our synthetic promoter library to find the model; this library is constructed by using the De Mey M *et al.* approach (De Mey, Maertens et al. 2007). Figure 5-3 illustrates the promoters' activity by doing a GFP assay. For specific values, refer to Table 5-1 through 5-3.

Figure 5-3. Promoter library 1-70 arranged in ascending order of activities

GFP-R primer 5' to 3' (TGGGTATCTCGCAAAGCATTGAAGAC) was designed to sequence all 70 promoters for further studies. The 5' to 3' consensus sequence of *E. coli* promoter library is:

WTWNWNNNAWWNNTTCTTGACATNTTNNNNNNNWTRTGRTATAATDNN
NNNRTWW.

Table 5-1. Weak promoters (Activities <200 RFU/OD₆₀₀)

	Average RFU/OD	Stdev
Ctrl	0.00	0.00
Plib2	0.00	0.00
Plib26	7.84	0.47
Plib9	46.02	1.74
Plib20	64.42	0.74
Plib15	73.84	4.21
Plib1	74.57	0.92
Plib10	89.07	0.96
Plib14	94.88	1.42
Plib6	95.68	1.56
Plib7	96.54	8.27
Plib11	103.74	5.16
Plib4	115.16	1.62
Plib12	122.54	1.10
Plib41	131.00	0.38
Plib24	150.05	1.04
Plib18	153.70	0.80
Plib56	155.32	2.49
Plib33	170.96	0.75
Plib13	189.14	0.91
Plib19	199.17	0.44
Plib37	200.29	0.12

Table 5-2. Moderate promoters (200 RFU/OD₆₀₀<Activities <500 RFU/OD₆₀₀)

	Average RFU/OD	Stdev
Plib3	218.79	0.92
Plib17	229.20	1.80
Plib16	234.91	0.51
Plib55	236.18	4.54
Plib69	241.24	1.78
Plib8	246.81	3.46
Plib38	253.06	2.51
Plib5	253.32	3.57
Plib25	284.76	3.30
Plib39	284.89	2.18
Plib28	287.96	2.47
Plib30	291.07	1.23
Plib35	295.03	0.47
Plib21	296.15	0.36
Plib42	302.68	1.68
Plib23	350.74	2.38
Plib40	351.77	5.64
Plib22	355.25	0.69
Plib27	356.97	1.97
Plib36	361.78	6.10
Plib45	372.50	4.57
Plib47	379.86	10.88
Plib51	407.53	5.44
Plib61	416.78	4.90
Plib31	422.72	5.94
Plib49	443.85	9.78
Plib52	451.76	4.18
Plib54	490.22	6.64
Plib32	491.82	13.64
Plib29	497.50	5.83

Table 5-3. Strong promoters (Activities >500 RFU/OD₆₀₀)

	Average RFU/OD	Stdev
Plib53	507.77	2.61
Plib62	512.03	4.29
Plib65	522.32	4.69
Plib34	553.32	1.97
Plib57	566.24	1.09
Plib58	577.77	4.42
Plib50	583.89	4.38
Plib70	586.06	2.58
Plib46	595.54	5.35
Plib59	596.40	11.31
Plib43	612.23	5.44
Plib68	618.39	13.77
Plib60	735.60	14.51
Plib63	766.94	2.10
Plib66	817.59	6.77
Plib48	829.83	9.91
Plib64	837.74	11.27
Plib44	855.85	19.77
Plib67	1157.92	7.18

5-3-1-3- New proposed approach

A recent study by Rhodius *et al.* (Rhodius and Mutalik 2010) used a Positioned Weight Matrix (PWM) to predict transcription factor binding in *E. coli* sigma E promoters. We might be able to use this approach to assign scores to the different sites of the sigma 70 promoter (e.g. -35 and -10 box, UP element, spacer, etc.) and then find the correlation between promoter scores and promoter activity. Seventy percent of our sample (n=70) can be used to train the data, while the other 30% can be used to validate our obtained correlation. PWM can be constructed using the method proposed by Stormo *et al.*

(Stormo 1990), and sequences can be scored as described in (Rhodius, Suh et al. 2006). The approach mentioned above is very close to what we did in the first place, but it includes more parameters than just -35 box. By taking additional elements in to account such as UP element, spacer length, -10 box, etc., we hope to achieve significant correlation between promoter scores and their activity. Based on a quantitative model that can predict the strength of synthetic promoters without running any wet-lab experiment, synthetic biologists can choose a promoter that is well-matched to their desired output more efficiently.

A-Appendix Figures

Appendix Figure 1. Genetic maps of engineered system/devices in pSB1A2 vector.

(A) Our final engineered system, pTetR-LasR-pLuxR-S5-pLuxR-E7. The system recognizes input chemical signals from *P. aeruginosa* and produces S5 pyocin and E7 lysis proteins. (B) Sensing device coupled to GFP, pTetR-LasR-pLuxR-GFP. This construct was used as a measurement tool for characterization of the sensor (pTetR-LasR-pLuxR-). (C) Sensing device with E7 lysis, pTetR-LasR-pLuxR-E7. This construct was used for the characterization of E7 lysis protein whose function is to disrupt cell membrane for the release of pyocin. (D) Sensing device with pyocin S5, pTetR-LasR-pLuxR-S5. This construct was used as a control to compare the efficiency of lysis device in mediating protein release.

Appendix Figure 2. Inhibition of *P. aeruginosa* by the engineered *E. coli* induced with 3OC₁₂HSL.

(A) *P. aeruginosa* cells stained using the LIVE/DEAD cell viability assay. The results show that more PI-stained *P. aeruginosa* cells were present when *P. aeruginosa* was exposed to supernatant of the *E. coli* carrying the final system that was induced by 3OC₁₂HSL, whereas all *P. aeruginosa* cells exposed to supernatant of wild-type *E. coli* (control) were stained with SYTO 9 (green). Scale bar: 5 μ m. (B) CFU count of *P. aeruginosa* (carrying chloramphenicol-resistant plasmid pAWG1-1) in a mixed culture with engineered *E. coli*. To study whether the engineered *E. coli* carrying the final system can inhibit growth of *P. aeruginosa* in mixed culture, clinical isolate In7 and pyocin resistant control strain PAO1 was co-cultured with engineered *E. coli* in the ratio 1:4 and quantified by viable cell count of *Pseudomonas*. Additionally, In7 was also co-cultured with control *E. coli* missing either the pyocin S5 or E7 lysis devices.

The results show that only the final system (i.e. pTetR-LasR-pLuxR-S5-pLuxR-E7), complete with sensing, killing and lysis devices are capable of inhibiting the growth of *P. aeruginosa* for 15 hours.

Appendix Figure 3. Fluorescent Microscopy of *In7* expressing GFP

B- List of Publications

Publications:

- Saeidi N, Arshath M, Chang MW, Poh CL* (2012) **Characterization of a quorum sensing device for synthetic biology desing : both experimental and mathematical modeling** . *Chemical Engineering Science*. In press.
- Saeidi N, Wong CK, Lo T, Nguyen HX, Ling H, Leong SSJ, Poh CL, and Chang MW* (2011) **Engineering microbes to sense and eradicate *Pseudomonas aeruginosa*, a human pathogen.** *Molecular Systems Biology*. 7:521. "Featured article" [PubMed: 21847113]
- Ling H, Saeidi N, Rasouliha BH, and Chang MW* (2010) **A predicted S-type pyocin shows a bactericidal activity against clinical *Pseudomonas aeruginosa* isolates through membrane damage.** *FEBS Letters*. 584:3354-3358. [PubMed: 20580355]

Patents:

- Chang, Matthew Wook, Poh, Chueh Loo , Wong, Choon Kit and **Saeidi, Nazanin** (2013) **Isolated nucleotide molecule and method of sensing and killing of pathogenic microorganism** , United States Patent Application 20130052164.

Conference presentations:

- **Saeidi N**, Arshath M, Chang MW, Poh CL* (2013) **Characterization of a quorum sensing device for synthetic biology desing : both experimental and mathematical modeling**, SB6.0 the Sixth International Meeting on synthetic Biology, 9th-11th July 2013.
- **Nazanin Saeidi**, Choon Kit Wong, Tat-Ming Lo, Hung Xuan Nguyen, Chueh Loo Poh, and Matthew Wook Chang (2011) **Engineered cells that detect and kill a pathogen: a novel synthetic biology-based antimicrobial strategy**, AICHE annual meeting, 16th-21st Oct 2011.
- **Nazanin Saeidi**, Choon Kit Wong, Tat-Ming Lo, Hung Xuan Nguyen, Chueh Loo Poh, and Matthew Wook Chang (2011) **Engineered cells that detect and kill a pathogen: a novel synthetic biology-based antimicrobial strategy**, SB5.0 the Fifth International Meeting on synthetic Biology, 15th-17th June 2011.
- **Nazanin Saeidi**, Mohamed Arshath, Matthew Wook Chang, Chuel Loo Poh (2011) **Models for Synthetic Biology Design**, 15th-17th June 2011.
- Tat-Ming Lo, Choon Kit Wong, **Nazanin Saeidi**, Chueh Loo Poh, and Matthew Wook Chang (2011) **Characterization of Colicin E7 lysis protein for controlled release of macromolecules**, SB5.0 the Fifth International Meeting on synthetic Biology, 15th-17th June 2011.

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