Development of quorum-sensing-based genetic circuits that enable programmable functionalities in escherichia coli

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**DEVELOPMENT OF QUORUM-SENSING-BASED
GENETIC CIRCUITS THAT ENABLE PROGRAMMABLE
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**SCHOOL OF CHEMICAL AND BIOMEDICAL
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2014

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Summary

Pseudomonas aeruginosa is a prevalent nosocomial pathogen which is a major cause of urinary tract disease and hospital secondary infections. With the emergence of antibiotic resistant bacteria, the number of methods to eradicate *P. aeruginosa* has become more limited. This is especially true when *P. aeruginosa* is in the biofilm state. The biofilm is the form whereby the cells are encompassed by a matrix comprising of exopolysaccharides, DNA and proteins. Because of the antibiotic-resistance property conferred by the matrix, the biofilm usually leads to chronic infections and is very difficult to treat. *P. aeruginosa* biofilm formation is regulated by quorum sensing.

Quorum sensing regulates gene expression in response to changes in population density of bacteria. Bacteria communicate by releasing and detecting the intercellular signalling molecules called autoinducers. As the population density increases, the concentration of autoinducers increases correspondingly. Once the threshold concentration of autoinducers is reached, a density-dependent change in gene expression is triggered, which eventually regulates physiological activities such as virulence, symbiosis, motility and biofilm formation. *P. aeruginosa* produces the autoinducer N-acyl homoserine lactone (AHL) as their primary specific signalling molecule for quorum-sensing. With its high specificity, the quorum sensing mechanism of *P. aeruginosa* can be exploited for the targeted and inducible regulation of desirable proteins to perform specific useful functions.

The work described in this thesis aimed to develop *P. aeruginosa* quorum-sensing-based genetic circuits that would enable clinically relevant programmable functionalities in *Escherichia coli*: protein release, directed motility, and pathogen killing. Toward this aim, I have developed three systems with a quorum sensing device

as a control system: (1) a *P. aeruginosa* quorum sensing-based genetic circuit that enabled cell density-dependent autoregulatory lysis for the release of macromolecules, (2) a probiotic strain with integrated *P. aeruginosa* quorum sensing device for specific sensing of *P. aeruginosa* and eventually killing of clinical isolates of *P. aeruginosa*, and (3) a genetic circuit that enables engineered *E. coli* to move distinctly towards *P. aeruginosa* and kill the human pathogen.

CHAPTER 1

Introduction

1.1 BACKGROUND

Pseudomonas aeruginosa is a prevalent nosocomial pathogen which is a major cause of urinary tract disease and hospital secondary infections. With the emergence of antibiotic resistant bacteria, the number of methods to eradicate *P. aeruginosa* has become more limited. This is especially true when *P. aeruginosa* is in the biofilm state. The biofilm is the form whereby the cells are encompassed by a matrix comprising of exopolysaccharides, DNA and proteins. Because of the antibiotic-resistance property conferred by the matrix, the biofilm usually leads to chronic infections and is very difficult to treat. *P. aeruginosa* biofilm formation is regulated by quorum sensing.

Quorum sensing regulates gene expression in response to changes in population density of bacteria. Bacteria communicate by releasing and detecting the intercellular signalling molecules called autoinducers. As the population density increases, the concentration of autoinducers increases correspondingly. Once the threshold concentration of autoinducers is reached, a density-dependent change in gene expression is triggered, which eventually regulates physiological activities such as virulence, symbiosis, motility and biofilm formation. *P. aeruginosa* produces the autoinducer N-acyl homoserine lactone (AHL) as their primary specific signalling molecule for quorum-sensing. With its high specificity, the quorum sensing mechanism of *P. aeruginosa* can be exploited for the targeted and inducible regulation of desirable proteins to perform specific useful functions.

There are many scientific discoveries which could lead to great applications in our daily lives. Synthetic biology is one option that can bridge the gap between science and application for the benefits of the society. A synthetic biologist utilises the discovery of science and constructs novel biological devices which when combine, leads to complex systems. Like engineering, it is modular, and governed by frameworks like abstraction and combinatory rules of simple biological parts. With these frameworks and rules to specify methods of assembly with a more predictable outcome, it allows much easier manoeuvring and designing of complex systems from a combination of simple devices. Geneticists identify the correct DNA frame and clone these DNA sequences into DNA parts (PCR products) before ligating into a well-characterised plasmid. In this manner, the desired proteins' properties could be investigated. This is similar to the synthetic biologist. What differentiates between them is that a synthetic biologist joins the parts from the scratch to form a complicated system before evaluating the combinatory proteins effect whether targeted performance is achieved. These newly engineered cells would then be able to perform desired functions. On the other hand, a genetic engineer tends to amend existing DNA strands instead of creating novel DNA strands. Because of the standards of each DNA part (from synthetic biology), the same enzymatic sites can be used repeatedly which could join multiple DNA parts without having restriction of limited enzymatic sites that could be used for each DNA part. Hence, synthetic biology has unleashed unlimited possibilities of creating complex systems to allow cells to perform desired functions. Thus, synthetic biology platform could be implemented to build a novel system with the knowledge of regulation of quorum sensing from science, to be able to specifically induce only when in the presence of AHL or its source *P. aeruginosa*.

1.2 OBJECTIVES AND SIGNIFICANCES

This thesis aims to develop quorum sensing derived genetic circuits in *Escherichia coli* for biotechnology applications, in particular therapeutic purposes. Toward this aim, I have developed three *P. aeruginosa* quorum sensing device-coupled systems integrated in three different *Escherichia coli* strains according to their desired functions. With the benefits of using quorum sensing device as the inducible system, in chapter 3, I described the work focused on engineering a bacterial cell that autolyses and releases the cellular contents, especially desired expressed proteins and DNA. *E. coli* has been widely used as a molecular cloning tool for biotechnology applications. However, it is often necessary to lyse cells before extraction of desired proteins or DNA, and these reagents or equipment are often costly and/or not environmentally friendly. This is because of the inability of *E. coli* to secrete macromolecules. Hence, I showed that by a novel quorum sensing genetic circuit could provide a more economical and viable alternative for extraction of macromolecules, like DNA and proteins.

Chapters 4 and 5 focus on describing novel quorum sensing derived genetic circuits that enabled clinically relevant functionalities in *Escherichia coli* for therapeutic applications. With the difficulty of eradicating *P. aeruginosa*, which is one of the main sources of infectious diseases, and the emergence of antibiotic-resistant bacteria, other options besides antibiotics should be made available. Furthermore, quorum sensing has a primary role in disease and the quorum sensing molecules have been identified in clinical samples of patients. The synthetic biology approach with the ease of design with predicted outcome and unlimited possibilities could create a possible alternative to specifically sense *P. aeruginosa* with eventual targeting against *P. aeruginosa* in biofilm state that is especially resistant to antibiotics.

In chapter 4, the host strain used is a probiotic *E. coli* as this is a step closer toward implementation in humans. To sense *P. aeruginosa*, a quorum-sensing device from *P. aeruginosa* was integrated into the design so that engineered *E. coli* would not produce unnecessary proteins in the absence of *P. aeruginosa*. Furthermore, this could enhance specificity in our system of responding only in the presence of *P. aeruginosa*. Our novel engineered probiotic would (1) only be activated in the presence of *P. aeruginosa* and (2) effectively kill *P. aeruginosa* in *in vivo* settings with a *Caenorhabditis elegans* model. On the other hand, I aimed to utilize a different method to release *P. aeruginosa*-targeting proteins instead of lysing. To ensure a continuous supply of therapeutic agents, a secretory tag was chosen to secrete *P. aeruginosa*-targeting proteins (chapter 5). I also aimed to enhance the *P. aeruginosa*-targeting protein activity by engineering *E. coli* to move toward *P. aeruginosa* so that the proteins would be closer to the source. With a known motility gene that can direct toward the source, *cheZ* was the ideal candidate. Furthermore, different proteins from chapter 4 were characterized to identify more *P. aeruginosa*-targeting proteins. Hence, I developed another system that seeks and kills in chapter 5 to provide another option to eradicate *P. aeruginosa*.

1.3 THESIS OUTLINE

This thesis consists of six chapters. Chapter 1 focuses on the specific objectives, significance, and motivations of the works presented in the thesis. Chapter 2 is based on the comprehensive literature that is related to the works presented in chapters 3 to 5. Chapter 3 shows that the incorporation of *P. aeruginosa* quorum sensing and AHL-production devices allows autolysis of the host cells without external reagents or

mechanisms to release the cellular content of the popular cloning tool, *E. coli* Top10. In both chapters 4 and 5, the synthetic biology approach is implemented in the biomedical field, specifically targeting the human pathogen *P. aeruginosa*, In7 and PAO1, respectively. In chapter 4, a comprehensive evaluation of the synthetic biology design integrated into a well-known probiotic *Nissle 1917 E. coli* strain was optimized for its *P. aeruginosa*-sensing device and tested against both states of clinical *P. aeruginosa*, planktonic and biofilm, states. Besides *in vitro* settings, *in vivo* settings with infected *C. elegans* were also evaluated. In chapter 5, engineered *E. coli* was designed using the synthetic biology approach to seek and kill *P. aeruginosa*. The strain used was Δ cheZ *E. coli* strain to reduce basal native motility for a better control of the *P. aeruginosa*-seeking property. A recently discovered microcin protein, MccS, and a well-known degradation enzyme, bpDNaseI, are evaluated for their effects on *P. aeruginosa* for the first time. Similar to the *P. aeruginosa*-sensing device, cheZ, MccS, and bpDNaseI are made into devices and characterized individually before being combined into a system to perform a seek-and-kill task against *P. aeruginosa*. Finally, in chapter 6, the conclusions and significance of this thesis as well as the considerations for future research are discussed.

CHAPTER 2

Literature Review

2.1 CHRONIC AND ACUTE INFECTIONS CAUSED BY READILY-ACQUIRED RESISTANCE BACTERIA

Bacteria could cause an infection that may be acute or chronic, depending on the characteristics the infection has. The infection is said to be acute when the bacteria spread and grow rapidly. On the other hand, chronic infection, although slower in multiplication, persists for longer periods (Bjarnsholt, 2013). Usually, planktonic or free-moving cells are associated with acute infection, whereas biofilm or sessile cells are more commonly linked to chronic infection (Bjarnsholt, 2013; Donlan & Costerton, 2002). The commonly known infections caused by microbes are tuberculosis and malaria (Kuo et al., 2003), which could cause substantial morbidity and mortality in humans, leaving the immunocompromised patients especially vulnerable. The first serendipitous discovery of a drug in 1940 that could counter these deadly pathogens was of penicillin (Ban, 2006). This brought about the study of how the drug works against the pathogens.

Fig 2.1 Bacteria choose which strategy to employ. Either acute or chronic infection will occur. Acute infection is usually linked with free-moving cells while chronic infection with sessile cells.

Four main mechanisms on how the drug could work against the pathogens were investigated (Levy, 2002). Penicillin works by interfering with enzymes involved in peptidoglycan layer synthesis, hence inhibiting cell wall synthesis. The second type of mechanism is through interference of the subunits of the ribosome, which results in bacterial cell growth inhibition. The third and fourth mechanisms disrupt the DNA synthesis and structure of bacterial membranes (Mason et al., 2006), respectively.

Fig 2.2 Schematic of the four main mechanisms of how most drugs work.

Initially, penicillin could successfully tide over the bacteria infection, but as the usage of the antimicrobial drug was raised, resistance against the drug was observed (Donkor, Newman, & Yeboah-Manu, 2012; Tenover, 2006). The prolonged therapy of using antibiotics could result in the development of low-level resistance, rendering therapy not as effective as in the initial stage (Leekha, Terrell, & Edson, 2011). The development of resistance may sometimes be undetectable. Reports and cases have shown that these pathogens easily acquire antibiotic resistance. For instance, methicillin-resistant *Staphylococcus aureus* (MRSA) is a good example of

Staphylococcus aureus (*S. aureus*) gaining antibiotic resistance (Gordon & Lowy, 2008). The resistant bacteria are prevalent in health care institutes, hence rendering antibiotic therapy less effective, as mentioned earlier. The rise in resistant *Pseudomonas aeruginosa* (*P. aeruginosa*) and *S. aureus* raised another concern as this trend was associated with increasing mortality rates in immunocompromised patients observed (C.-I. Kang et al., 2003; Naber, 2009). Hence, researchers investigated how the bacteria gained antibiotic resistance (Dever & Dermody, 1991). Some bacteria are innately resistant to one or more antibiotics (Obritsch, Fish, MacLaren, & Jung, 2005). However, others gained antibiotic resistance through *de novo* mutation (Gullberg et al., 2011). On the other hand, horizontal gene transfer occurred, whereby genes were transferred through conjugation, transformation, or transduction with transposons. Resistance genes that the pathogen could acquire could be of a variety (Dzidic & Bedekovic, 2003). For example, an efflux system could be obtained, which allowed the drugs to be pumped out (Louw et al., 2009). Alternatively, the modification of a drug's target sites or obtaining the metabolic pathway that bypasses the drug's action could be detrimental, which led to the pathogens being a step closer to becoming more resistant (Huttunen, Raunio, & Rautio, 2011; Tenover, 2006). With the emergence of antibiotic-resistant bacteria coupled with a slow trend of discovery of new antibiotics, this could pose a threat to our health (Spellberg et al., 2008).

2.1.1 *P. AERUGINOSA* AS OPPORTUNISTIC PATHOGENS

P. aeruginosa is a Gram-negative bacteria and one of the prevalent nosocomial pathogens worldwide. Its metabolic flexibility in adaption to its environment caused it to be a serious threat to the public health (Livermore, 2002). Thus, tremendous efforts were made in investigating the virulence determinants of *P. aeruginosa* (Ben Haj Khalifa, Moissenet, Vu Thien, & Khedher, 2011; Veessenmeyer, Hauser, Lisboa, & Rello, 2009). It is also one of the most common causes of severe sepsis, with its primary colonization site being intestinal tract reservoir, leading to subsequent infection (Ben Haj Khalifa et al., 2011; Matsumoto et al., 1999; Romanowski et al., 2011). This is especially prevalent in gut of immunocompromised patients (Boukadida et al., 1991). These multidrug-resistant *P. aeruginosa* have the ability to disrupt the intestinal epithelial barrier and therefore sepsis. Hence, this leads to the study of *P. aeruginosa* in animal models like *C. elegans* (Kirienko et al., 2013; Tan, Mahajan-Miklos, & Ausubel, 1999) and mice to take a step closer to tackling these pathogens (Fink et al., 2011).

Current treatments of *P. aeruginosa*, like phage therapy (Keen, 2012) and chemotherapy (Poole, 2005), were proposed. Chemotherapy involved using a combination of different but effective antibiotics (Igbinosa et al., 2012), while phage therapy involved utilization of strain-specific bacteriophages that penetrate the targeted pathogen and hijacks the cellular machinery of the pathogen (Debarbieux et al., 2010). Although these therapies are successful, these methods may not be as effective for subsequent usage. The main problem would be the rapid mutation by *P. aeruginosa* that leads to antibiotics tolerance. On the other hand, after employment of phage-dependent therapy, an infected host would develop immunity to the introduced virus

(Loc-Carrillo & Abedon, 2011). Hence, *P. aeruginosa* still remained as a threat to the human.

2.1.2 BIOFILM FORMATION

The biofilm state of *P. aeruginosa* played a prominent role in enhancing its antibiotic resistance (Billings et al., 2013; Lewis, 2001). The biofilm is the form whereby the cells are encompassed by the exopolysaccharide matrix (Donlan & Costerton, 2002). Because of the antibiotic-resistance property, they usually lead to chronic infections and hence are more difficult to treat. In fact, the bacteria residing in biofilm is 1,000 times more resilient than their planktonic counterpart (Liao & Sauer, 2012). The chronic infections like urinary and pulmonary tract infections occurred commonly for *P. aeruginosa* (Kalra & Raizada, 2009). One hypothesis of why biofilm are more resistant is the delayed or restricted penetration to the biofilm by the antimicrobials (Mah & O'Toole, 2001). There are also reports mentioning that the susceptibility to antimicrobials was proportional to the bacterial cell growth (M. R. Brown, Collier, & Gilbert, 1990). The second hypothesis is the availability of oxygen that could affect the antimicrobial effect (Eini et al., 2013). Some antibiotics' activities are oxygen dependent (Shima, Szaszák, Solbach, Gieffers, & Rupp, 2011). Lack of oxygen would render the antibiotics ineffective. Bacteria undergo physiological, metabolic, and phenotypic changes from planktonic to biofilm states (Rotem et al., 2010; Sauer & Camper, 2001). Oxidative stress-related genes and genes involved in anaerobic processes are up-regulated, while flagellar-related genes are down-regulated (A. Kang, Tan, Ling, & Chang, 2013). There is a possibility that phenotypic changes and biofilm resistance is closely related from the discovery of a regulatory protein that regulates

the conversion between antibiotic-resistant and antibiotic-susceptible form (Drenkard & Ausubel, 2002).

Fig 2.3 Antibiotic resistance mechanism mediated by resistant variants. After antibiotic treatments, most of the susceptible biofilm fraction was eradicated. Remaining resistant variants survived from treatment and biofilm developed once treatment was stopped.

There have been studies on how biofilm is formed using confocal scanning laser microscopy on the green fluorescent protein-tagged bacteria (Stapper et al., 2004; Stretton, Techkarnjanaruk, McLennan, & Goodman, 1998). The biofilm development is initiated by free-moving bacteria, which then attach to the surface and form a layer. After multiplication, microcolonies are formed, and polymer matrix production occurs surrounding the microcolonies. Mushroomlike or towerlike structures would appear. When the biofilm is fully matured, the biofilm is optimally resistant to antibiotics. Infection spreads when biofilm disperses and free-moving cells attach to a new surface (Sauer, Camper, Ehrlich, Costerton, & Davies, 2002). Figure 2.4 illustrates the biofilm development.

Fig 2.4 Schematic of the biofilm development from planktonic cells to mature biofilm. There are three main stages of biofilm development; Attachment, maturation and dispersion. When enough cells accumulate, quorum sensing molecules start to accumulate and trigger relevant protein expression. When external condition is suitable for dispersion, cells from biofilm disperse and infection spreads.

2.1.3 BACTERIOCINS SECRETED BY BACTERIA

The emergence of the multi-antibiotic-resistant bacteria and the limited type of antibiotic available that may eventually be resistant call for attention to derive new alternatives of antibiotics. Furthermore, antibiotics action is usually a broad spectrum, which may lead to unselective killing of beneficial bacteria in human (Chopra & Roberts, 2001). There the development of new antimicrobial agent is necessary. An alternative to antibiotics is bacteriocin. They are natively produced by bacteria, are usually peptides, and have bactericidal action against other bacteria as a component of their innate defense mechanism (Munoz, Jaramillo, Melendez Adel, C, & Sanchez, 2011). They are classified into two broad classes: peptides that undergo posttranslational modification (class I) and peptides that do not (class II) (De Kwaadsteniet, Fraser, Van Reenen, & Dicks, 2006). There are numerous advantages of utilizing bacteriocins compared with antibiotics (Cotter, Ross, & Hill, 2013). The most

important property of bacteriocin is its narrow spectrum of targets, which makes it a highly specific drug unlike antibiotics. The narrow spectrum bacteriocins can continue to target the desired pathogens without negatively influencing the beneficial bacteria. However, there are also some bacteriocins that have a broad spectrum of action like antibiotics. The second advantage is its composition of amino acids; hence, modification of the bacteriocin to cater for specific activity or targets would not be difficult. Bacteriocin is composed of three components: translocation, receptor-binding, and cytotoxic domain. Hence, by changing the receptor binding of the desired bacteriocin to a known receptor-binding domain that has shown to target desired species, the desired bacteriocin target would be changed easily to target the desired species. Reports have shown that the switching of receptor-binding domain to another bacteriocin's receptor-binding domain is functional and leads to a desirable outcome (Kageyama, Kobayashi, Sano, & Masaki, 1996). The third important advantage is its low toxicity to the treated host, and its high potency makes it an ideal candidate for antibacterial therapy (Cotter et al., 2013). Moreover, the bactericidal activity of the bacteriocin could be further enhanced when used in synergy with other antimicrobials (Cotter et al., 2013). Hence, bacteriocins could be a viable alternative to antibiotics.

P. aeruginosa like other bacteria secretes bacteriocins. Most of the *P. aeruginosa* strains (~90%) produce a class of bacteriocins called pyocins (Elfarash, Wei, & Cornelis, 2012). Pyocins are categorized into three categories: R, F, and S types (Michel-Briand & Baysse, 2002; Waite & Curtis, 2009). The activity of the pyocins occurs through the binding of the pyocins to the selective receptors from the outer membrane of the target cells. As bacteriocins, they are produced to protect the host; hence, most strains are resistant to their own pyocins. Besides pyocins, the host will produce the immunity protein of the pyocins that help to protect themselves against

their own pyocins. R-type pyocin is the first identified pyocins (Nakayama et al., 2000). It consists of four major components: a hollow cylinder comprising (i) an inner core and (ii) outer sheath, and both are attached to (iii) the base plate that extends (iv) six tail fibers. All R-type pyocins are nuclease resistant and protease resistant (Bakkal, Robinson, Ordonez, Waltz, & Riley, 2010). Takeya discovered a second type of pyocin, F-type pyocin (Kuroda & Kageyama, 1981). Like the R-type pyocin, it is nuclease resistant and protease resistant too. Unlike the R-type, F-type pyocins comprised three major components: (i) the core, (ii) the base plate, and (iii) the tail fibers comprising several short and long filaments. Afterward, S-type pyocins were discovered. It is soluble and protease sensitive, unlike both R and F types. S-type pyocins comprise two main protein components (Michel-Briand & Baysse, 2002). The larger component contains the killing activity, whereas the smaller component contains the immunity protein that protects the host. It is usual for a single strain to produce more than one type of pyocins. The more common pyocins are the R and F types, which comprise 90% of the *P. aeruginosa* strains, while the S type comprises 70% (Saeidi et al., 2011a).

The S-type pyocins like S1, S2, S3, and S5 have similar modes of action as colicins, a bacteriocin produced by *E. coli*. S-type pyocins are composed of two parts: the killing and the immunity component. The killing component, in general, consists of receptor-binding, translocation, and killing domains that span from 498 to 776 amino acids. On the other hand, the smaller immunity component span from 87 to 153 amino acids to provide immunity for their host (Elfarash et al., 2012; Ling, Saeidi, Rasouliha, & Chang, 2010).

Fig 2.5 (A-C) Schematic depicts the structures of the three types of pyocins, (A) R, (B) F, (C) and S type. Both (A) and (B) contain (1) core, (2) tail fibres, (3) and base plates. However, (A) has additional component, the inner core and has less tail fibres than (B). (C) contain two main components; immunity component, with killing component comprising of 3 parts, translocation domain (T), receptor-binding domain (R), and cytotoxic domain (C).

The pyocin S5, which was recently characterised, contains translocation, receptor-binding and killing domains with 46% homology of colicin Ia which has a pore-forming mechanism. Ling and his group verified that S5 pyocin efficiently eradicate seven clinical *P. aeruginosa* strains (Ling et al., 2010). Among the seven strains was a clinical isolate from cystic fibrosis patients, DWW3. They demonstrated that pyocin S5 mode of action is membrane damage which was observed from leakage of cellular materials and distortion of the cell shape and surface of *P. aeruginosa* upon treatment with pyocin S5. Another group from Ron Weiss lab engineered a novel colicin that targets *P. aeruginosa* by exchanging the binding receptor and translocation domain of colicin E3 with that of pyocin S3 (Gupta, Bram, & Weiss, 2013).

2.2 SYNTHETIC BIOLOGY

Synthetic biology implements an engineering approach to design and construct genetic circuits (Figure 2.6). There are differences between synthetic biology and the traditional genetic engineering. The major advantages of utilizing the synthetic biology approach is the possible reduced time and cost to complete the project because of greater predictability. Synthetic biology is a framework that incorporates biological function from genetic circuit. It is based on engineering the three main principles: abstraction, standardization, and decoupling. Synthetic biology has been using the concept of abstraction for simplifying the complex biological processes from the lessons drawn from engineering fields. Biological components can be easily seen as simple modules (Figure 2.6). Besides abstraction, the other element, standardization, enables the creation of standardized sets of biological modules with predicted outcome. This resulted in the ease of reusing in combination, coupled with composition rules that define the assembling method of such modules (Andrianantoandro et al., 2006). Not only does standardization of biological parts aid in reusability, the outcome of interoperability of parts in different devices or systems could also be predicted. This is very crucial as standardization practically allows modular units to be built upon to form a complex system while known characterized data allow the ease of optimization, the desired outcome safely, and better control of the synthetic biological systems (Endy, 2005). The reusability and combination of the parts were being verified and proofed to be a potential approach to unify all the characterization experiments of the biological parts (Canton, Labno, & Endy, 2008). At present, inducible promoters like the quorum sensor that receives N-(3-oxohexanoyl)-L-homoserine lactone and constitutive promoters from σ^{70} promoters were characterized for other scientist usage

(BioBrick Registry). This provides transcriptional regulation adjustment according to the design needs.

Fig 2.6 Schematic representing the linkage between synthetic biology and engineering (Andrianantoandro, Basu, Karig, & Weiss, 2006)

One of the most popular synthetic biology standards that are adopted by scientists is the BioBrick standard. Dr. Tom Knight from Massachusetts Institute of Technology, USA, proposed the idea of the BioBrick standard, which was later extended by Dr. Drew Endy, also from MIT (Shetty, Endy, & Knight, 2008). The BioBrick standard is composed of biological deoxyribonucleic acid (DNA) parts (Figure 2.7). As long as the biological genetic component conforms to the BioBrick standard, it would be known as a BioBrick part. A BioBrick part is a strand of DNA that codes for a

particular biological function that could be a promoter or ribosomal binding site or gene or terminator. Promoter initiates transcription upon RNA polymerase binding. Ribosomal binding site enables translation while protein coding sequences code for functional proteins. Terminator terminates transcription via formation of hairpin loop, which is a secondary structure that deters the formation of the DNA-mRNA-RNA polymerase ternary complex. From basic level of DNA, it can be assembled to form parts, then devices and finally systems to perform desired functions like oscillators or logic gates.

Fig 2.7 Schematic depicts the hierarchy from synthetic biology standard parts to systems.

These devices could be introduced in the form of plasmid DNA into the host cells, where they perform new activities or initiate new metabolic processes. In Nazanin and Choon Kit's work, the three devices were required for the *E. coli* host cell to perform the tasks of sense *P. aeruginosa*, autolysed and therefore released bacteriocin S5, which then killed the pathogen *P. aeruginosa* (Saeidi et al., 2011a). The three devices are the quorum-sensing device, the lysis device, and the killing device. Both the quorum-sensing and killing devices are derived from another species of bacteria, *P. aeruginosa*.

The BioBrick assembly required a universal connector to join two BioBrick parts together. The connectors present in a BioBrick part are *EcoRI*, *XbaI*, *SpeI*, and *PstI*. *EcoRI* and *XbaI* form the prefix, while *SpeI* and *PstI* form the suffix (Figure 2.8). Isocaudomeric is defined as two enzymes possessing different restriction enzyme recognition sites but generate similar termini upon cleavage. *XbaI* and *SpeI* are isocaudomeric, which is present in a BioBrick part in the prefix and suffix, respectively, enabling two BioBrick parts to anneal and form devices by forming scar (Shetty et al., 2008). In this manner, iterative restriction, enzymatic digestion, and annealing enabled the assembly of individual BioBrick parts to form a large composite. This not only reduces the hassle of finding new restriction enzymes to digest and ligate to existing plasmid but also enables unlimited BioBrick parts' iterative add-ons to preexisting plasmid.

Fig 2.8 Schematic depicts the BioBrick assembly method. E: EcoRI, X: XbaI, S:SpeI, P:PstI

However, there is an issue that could be detrimental when applying the BioBrick assembly method when composing protein-fusion parts, especially peptide tags. As protein engineering becomes more prevalent, using the BioBrick assembly method would not be a favorable approach. Interestingly, multimodular domains can be combined to form a range of larger polypeptide sequences (Dueber et al., 2009; Dueber, Yeh, Chak, & Lim, 2003). But the eight-nucleotide-scar sequence formed by the digestion and relegation of XbaI and SpeI sites deters the construction of functional chimeric proteins. There are two reasons why chimeric proteins cannot be functional when the BioBrick assembly method is used. First, the scar sequence (TACTAGAG) encodes a stop codon. Second, a frame shift would occur between the two coding

sequences because of the eight nucleotide scars. To overcome these issues, a new standard called BglBrick parts is composed in UC Berkeley (J Christopher Anderson et al., 2010) (Figure 2.9). Instead of using *XbaI* and *SpeI* restriction enzyme sites, *BglII* and *BamHI* restriction enzyme sites are flanked at the 5' and 3' ends of BglBrick parts, respectively (Figure 2.9A). The BglBrick standards offer more benefits than the BioBrick standards. First, the reliability is assured because the restriction enzymes used are the common enzymes used in the laboratory. Second, the efficiency of restriction enzymatic digestion is high in the same buffer used, across all the four restriction enzymatic digestions. Third, they are not influenced by overlapping dam/dcm methylation. Lastly, the resultant six-nucleotide-scar sequence (GGATCT) encoding glycine-serine does not affect the formation of functional protein-fusion complex in a range of host cells used (Evers, van Dongen, Faesen, Meijer, & Merkx, 2006; Suda et al., 2008).

Fig 2.9 A) An overview of the main difference between BioBrick and BglBrick standard. BioBrick part generates 8 nucleotide scar while BglBrick part generates 6 nucleotide scar. Unlike BioBrick part, BglBrick part allows successful protein fusion. B) Schematic of joining BglBrick parts has similar experimental procedures as joining BioBrick parts.

There is a wide range of applications of synthetic biology ranging from biofuels to biomedical applications. The first application of synthetic biology is biosensor. Natively, bacteria produce and receive signal molecules as a form of communication before deciding which function for the energy to channel to. They are also sensitive to other environmental signals like pH, temperature, and chemical substances that trigger specific proteins synthesis for specific functions. Reporter gene such as green fluorescence protein (GFP) could be coupled downstream of the promoter sensitive to the external signal. The intensity of the florescence obtained would be directly related to the quantity of input signals. Hence, by genetic circuit design, the input signals become quantifiable. Levaskaya and his team used red light to switch the states of the engineered cells with measureable lacZ activity, while Aleksic and his team engineered bacteria to detect harmful arsenic in water, which resulted in a change in pH that is measureable.

Besides application in biosensor, an important application of synthetic biology is the metabolic engineering. Modification on the native metabolic pathways from microbes can be implemented for a more cost-effective chemical and drug synthesis. Complex drugs or new drugs can be synthesized by microbes and bypassed the need to include multiple additional steps for protection and deprotection of the pharmaceutical molecules. (Fenical & Jensen, 2006; Gantet & Memelink, 2002; Pollard & Woodley, 2007). An important application of synthetic biology in medical field is the control of malaria. However, a protozoan of the genus *Plasmodium* impedes the control of malaria. It is a detrimental disease as it caused an estimation of one to three million deaths annually, with a worldwide infection of 300 to 500 million (Al-Jabri, 2004; Jacobs-Lorena, 2003). There are drugs like artemisinin, which has been proven to be effective in malaria treatment, but it requires high production cost. Hence, it would not

be practical to solve the malaria situation in developing countries like Africa. The production cost of artemisinin has been the limiting factor until Jay Keasling successfully implemented the synthetic biology approach to engineer *E. coli* to produce artemisinin, which could lower the cost significantly from US\$2.40 per dose to US\$0.25 per dose (Ro et al., 2006).

Besides depending on only metabolic engineering to produce pharmaceuticals, others extend the knowledge to combine biosensor with metabolic engineering to produce pharmaceuticals. Anderson and his group successfully rewired a cell that can sense and kill tumor (J. C. Anderson, Clarke, Arkin, & Voigt, 2006).(J. C. Anderson et al., 2007). Only when both conditions, anaerobic and dense population, were fulfilled, engineered bacteria would be activated whereby they will invade the cancer cells selectively and subsequently produce toxins to kill them (J. C. Anderson et al., 2006). This idea was further extended by integrating multiple signals in the form of AND gate in the biosensor section for specificity enhancement (J. C. Anderson et al., 2007) (Figure 2.10).

Fig 2.10 Schematic representing AND gate genetic circuit. Two input signals were required to trigger GFP expression. First promoter transcribes amber suppressor tRNA *supD* while second promoter transcribes T7 RNA polymerase (*T7ptag*). *T7ptag* is a modified T7 RNA polymerase with two amber stop codons. When *supD* is transcribed, the two stop codons are translated as serine. Hence, functional polymerase is expressed when both *supD* and *T7ptag* are transcribed. Only a functional polymerase could trigger T7 promoter for GFP expression (J. C. Anderson, C. A. Voigt, & A. P. Arkin, 2007).

Fuel is a renewable resource and is running out. Several groups are working on engineering microbes to produce biofuels as an alternative to fuels (Atsumi et al., 2008; Collas et al., 2012; T. Liu & Khosla, 2010; Soma et al., 2012). There is even a greater stride made in synthetic biology application: the construction of minimal life (Luisi & Stano, 2011) (Figure 2.11). With minimal life formed, only minimum proteins are required to express to support cell growth, which could save unnecessary energy for functions that are not required like motility. Other energy can therefore channel to desired functions like production of useful biofuels and pharmaceuticals.

Fig 2.11 Schematic representing the steps leading to the construction of synthetic cell-like systems(Luisi & Stano, 2011).

2.3 QUORUM SENSING

Quorum sensing regulates gene expression in response to the changes in population density of bacteria (M. B. Miller & Bassler, 2001). Bacteria release and detect the signaling molecule called autoinducers as a form of communication. As the bacteria population density increases, the concentration of autoinducers will also increase correspondingly. A change in gene expression is triggered upon reaching a threshold concentration of autoinducers. In this manner, both Gram-negative and Gram-positive bacteria communicate and regulate an array of physiological activities such as virulence, symbiosis, motility, and biofilm formation (M. B. Miller & Bassler, 2001). One of the most prevalent autoinducers is N-acyl homoserine lactone (AHL), which is produced by Gram-negative bacteria (Cha, Gao, Chen, Shaw, & Farrand, 1998; Gray, Passador, Iglewski, & Greenberg, 1994; M. B. Miller & Bassler, 2001); oligo-peptides produced by Gram-positive bacteria (Reading & Sperandio, 2006); and autoinducer-2 (AI-2), which is sensed by both Gram-negative and Gram-positive bacteria (Bassler, 1999; Federle & Bassler, 2003). *P. aeruginosa*, like the other Gram-negative bacteria, AHLs is the primary specific signaling molecules involved in the quorum-sensing mechanism (J. J. Huang, Han, Zhang, & Leadbetter, 2003; Wu et al., 2000). Substrates such as S-adenosyl methionine (SAM) and acylated acyl carrier protein (Acyl-ACP) are both metabolites, and they produce AHL (M. B. Miller & Bassler, 2001; Parsek & Greenberg, 2000) (Figure 2.12). In the *Las* quorum-sensing system of *P. aeruginosa*, *LasI* autoinducer synthase is expressed, which then converts SAM and Acyl-ACP into diffusible N-3-oxododecanoyl-L-homoserine lactone (3OC-12-HSL) (Hoang, Sullivan, Cusick, & Schweizer, 2002; Parsek & Greenberg, 2000; Watson et al., 2002).

Fig 2.12 AHL synthesis pathway using S-Adenosyl Methionine (SAM) and acylated acyl carrier protein (Acyl-ACP) as the substrates (Watson, Minogue, Val, von Bodman, & Churchill, 2002).

The *Las* quorum-sensing system includes *LasI*, the autoinducer synthase that produces 3OC-12-HSL, and the *LasR*, the R-type transcriptional activator protein (J. P. Pearson, Pesci, & Iglewski, 1997). Since both *LasI* and *LasR* are involved in the quorum-sensing system, their expressions are greatly affected by the change in cell density and therefore the change in concentration of 3OC-12-HSL in the extracellular medium (Fig 2.13).

Fig 2.13 *P. aeruginosa* produces and secretes 3OC-12-HSL by enzymatic conversion of SAM and Acyl-ACP.

Studies have shown that the range of concentration of PAI (first type of quorum-sensing system in *P. aeruginosa*) in the extracellular medium is between 10^{-6} M and 10^{-4} M (J. P. Pearson et al., 1997; Pesci, Pearson, Seed, & Iglewski, 1997), which is at close proximity to the infection site (Charlton et al., 2000b; J. P. Pearson, Passador, Iglewski, & Greenberg, 1995; Seed, Passador, & Iglewski, 1995). 3OC-12-HSL forms an activated complex with LasR protein. A positive feedback loop is triggered whereby the threshold activated complex is reached, which then initiates the *lasI* gene expression. This results in a drastic rise in 3OC-12-HSL and therefore more activated complex formed. This brings about a rapid rise in expression of virulence genes such as *lasB* elastase, exotoxin A, and alkaline protease (Gambello & Iglewski, 1991). The other quorum-sensing systems, such as Rhl system, are also positively regulated. However, the Rhl system is more dependent on a different signaling molecule, N-butanoyl-L-homoserine lactone (C4-HSL) (Kiratisin, Tucker, & Passador, 2002; Pesci et al., 1997; Rampioni et al., 2006). Rhl system is the second quorum-sensing system of *P. aeruginosa* (J. P. Pearson et al., 1997). The Rhl system produces C4-HSL with smaller concentration of C6-HSL. The Rhl system is composed of 11 *rhlR* transcriptional regulators and *rhlI* synthases. It is activated only after Las system is initiated. Expression of *rhlAB* operon is regulated. The operon encodes for rhamnosyl-transferase that synthesize rhamnolipid (Ochsner, Fiechter, & Reiser, 1994; Ochsner & Reiser, 1995). Rhamnolipid is a biosurfactant that emulsifies hydrocarbon substrate for utilization by the cells. Being a virulence secondary metabolite (VSM), like the Las quorum-sensing system, the Rhl quorum-sensing system, also controls the expression of virulence genes like *lasAB* and pyocyanin (Brint & Ohman, 1995; Pesci et al., 1997; Wagner & Iglewski, 2008).

With a disparity in length and hydrophobicity of the side groups, the uptake and secretion of C4-HSL and 3OC-12-HSL would differ. C4-HSL, being smaller, would be able to diffuse through the cells more easily than 3OC-12-HSL; and so 3OC-12-HSL requires a more efficient system, an active efflux system to be able to be transported out of cells (James P. Pearson, Van Delden, & Iglewski, 1999). The different side chains coupled with the specific binding R-proteins made the activation highly specific in action (Pesci et al., 1997). Hence, lasR protein would only be activated by 3OC-12-HSL. On top of the highly specific binding of signaling molecule with the R-proteins, the activated complex also binds only to specific promoters. However, the Las quorum-sensing system and the Rhl quorum-sensing system are dependent on each other (Figure 2.14). The Las system regulates the Rhl system through competitive binding upstream to RhlR operon (Pesci et al., 1997). Therefore, 3OC-12-HSL could be considered as the primary source of *P. aeruginosa*'s virulence (Van Delden & Iglewski, 1998). Down-regulation of the Las system could also occur upon expression of RsaL, which is an inhibitor for lasI transcription (de Kievit, Seed, Nezezon, Passador, & Iglewski, 1999; Rampioni et al., 2006).

Fig 2.14 Schematic of quorum sensing system in *P. aeruginosa*. Positive feedback loop for 3OC-12-HSL production is triggered when activated complex reached threshold. Activated complex also positively regulates expression of other quorum sensing mechanisms and virulence factors. RsaL inhibitor counters the positive feedback effect of activated complex.

2.4 MOTILITY

Bacterial chemotaxis is a phenomenon whereby bacteria actively respond via movement according to the changes in the environment such that they will approach chemoattractants (advantageous chemicals) and avoid chemorepellents (unwanted chemicals) (Bren & Eisenbach, 2000). Because of limited options for simple bacteria to adjust to the environment from survival, they adopt strategic movement from one location to another. Hence, in this manner, bacteria can move to a more favorable environment for its survival. Chemotaxis is also important in extreme conditions, in particular stress conditions, as it provides communication between the cells, which allows cell recruitment to enhance its chances of survival (Baker, Wolanin, & Stock, 2006). Most bacteria are motile, especially the rod-shaped bacteria (Stephen & Mark, 2006). However, the modes of motility and stimuli vary for each bacteria species. The type of motility is also highly dependent on the environment and the number of

flagellar that the species has. Out of all the modes of motility, swimming is the most common approach that the bacteria adopted. Swimming is driven in the form of flagellar rotation. Among swimming mode, *P. aeruginosa* and *E. coli* differ slightly, with *P. aeruginosa* possessing a less directional movement and a more passive reorientation. Another mode of motility that requires flagellar is swarming. Unlike swimming, swarming requires hard surface condition and is often associated with quorum sensing (Daniels, Vanderleyden, & Michiels, 2004; Kearns, 2010). Swarming often associates with colony movement rather than individual. Other modes of motility do not involve flagellar, gliding, and twitching (Kearns, 2010; R. M. Miller et al., 2008).

Different species of bacteria respond to different stimuli (Soutourina, Semenova, Parfenova, Danchin, & Bertin, 2001). It is highly dependent on the environment that the bacteria live in (Table 2.1).

Table 2.1 Table showing different stimuli has different effect on different bacteria species (Eisenbach, 2001).

Reagent	Chemoattractant for	Chemorepellent for
Phenol	<i>Escherichia coli</i>	<i>Salmonella typhimurium</i>
Leucine	<i>Bacillus subtilis</i>	<i>Escherichia coli</i> , <i>Salmonella typhimurium</i>
Valine	<i>Bacillus subtilis</i>	<i>Escherichia coli</i> , <i>Salmonella typhimurium</i>
Tryptophan	<i>Bacillus subtilis</i> , <i>Chromatium vinosum</i>	<i>Escherichia coli</i> , <i>Salmonella typhimurium</i> , <i>Rhodobacter sphaeroides</i>
Acetate	<i>Chromatium vinosum</i>	<i>Escherichia coli</i> , <i>Salmonella typhimurium</i> , <i>Rhodobacter sphaeroides</i>
Benzoate	<i>Pseudomonas putida</i>	<i>Escherichia coli</i>

Bacteria flagellar is composed of three main components: a basal body, a hook, and a filament (Erhardt, Namba, & Hughes, 2010; K. Zhang, Tong, Liu, & Li, 2012). The basal body component of *E. coli* comprises four rings on a rod: M ring (membrane), S ring (supramembrane), P ring (peptidoglycan), and L ring (lipopolysaccharide) (Figure 2.15). The hook is made up of one protein, FlgE, which links the filament with the basal body (Eisenbach, 2001). The filament is also built from mono-protein FliC. It is

connected to the hook via hook-associated proteins. Rotation of the filament relies on the flagellar motor (Berg, 2003).

Fig 2.15 Schematic of structure of *E. coli* flagellar. CM, OM and PL represent cytoplasmic membrane, outer membrane and peptidoglycan layer respectively (Eisenbach, 2001).

The cell cycle, cell division, and growth phase are factors that could affect the initiation of assembly of flagellar. Once the flagellar is assembled, they will respond according to the stimuli present. In the presence of chemoattractants, the flagellar will rotate counterclockwise and will enable a smooth swim. However, in the presence of chemorepellant or absence of chemoattractant, the flagellar will rotate clockwise and hence tumble about the axis and reorientate itself toward the chemoattractant or away from the chemorepellant. Through the rotation and reorientation of the flagellar, the

bacteria cell would be able to move toward an environment of higher concentration of chemoattractant (Topp & Gallivan, 2007).

CHAPTER 3

Designing a synthetic genetic circuit that enables cell density-dependent auto-regulatory lysis for macromolecule release

3.1 INTRODUCTION

Escherichia coli has been widely used in manipulating genes and expression of proteins because it is well characterized with easy manipulation. With the existence of a wide range of molecular tools available it is an ideal candidate to be used as a host for biotechnology applications. Although there are numerous benefits of using *E. coli* as a host, *E. coli* lack the ability to secrete macromolecules (Goeddel et al., 1979; Jung & Lee, 2011). Therefore, external intervention is required to extract the macromolecules after expression through mechanical and/or chemical means (R. B. Brown & Audet, 2008) which required costly reagents and equipments and sometimes may be time consuming, inefficient and/or cause protein denaturation.

To address these concerns, Morita and his team (Morita, Asami, Tanji, & Unno, 2001) implemented programmed cell lysis using T4 bacteriophage lytic proteins. It is a holin that perturbs the membrane of the cells (Park, Struck, Deaton, & Young, 2006). Hence in this manner, contents of the cells which include the proteins of interest could be released spontaneously without additional steps. However, the draw back in the method used was the expression of this holin protein was regulated by an inducible promoter that requires supplementation of chemical inducers which could amount to high cost especially when the proteins of interest produced in large scale (J. M. Lee, Lee, Kim, & Lee, 2013). To reduce the cost, Yun and his team (Yun, Park, Park, Kang, & Ryu, 2007) utilized an inducible promoter that spontaneously express the protein of interest without the requirement of external chemical inducers. The promoter used was

the promoter of the *ptsG(ptsGPL)* derived from *E. coli*, with the expression level dependable on depletion of glucose. *ptsG* is a gene that regulates a major glucose transporter. The group placed the lysis gene under the regulation of *ptsGPL*. The advantage of their approach is that auto cell lysis would only occur after the glucose in the medium is depleted which allows the cell to grow to sufficient quantity. However, although the issue of additional cell disruption steps and the usage of external chemical inducers were resolved, inefficient lysis may pose a problem (Yun et al., 2007). The inefficient lysis system was greatly attributed by the low promoter activity of *ptsGPL*. On top of this drawback, the promoter used could be regulated by other factors like oxidative stress (Rungrasamee, Liu, & Pomposiello, 2008) and oxygen concentration (J. Y. Jeong et al., 2004), besides glucose. Therefore, in industrial context, *ptsGPL* 's unspecific activation with low activity, may not be desirable. On the other hand, *csiDp*, the sigma-38 factor regulated promoter of *csiD*, only activates when carbon is depleted and/or stationary phase is reached (Battesti, Majdalani, & Gottesman, 2011; Marschall et al., 1998; M. Metzner, J. Germer, & R. Hengge, 2004).

Hence, to address the limitations of the previous works of the engineered systems for macromolecule release, this study aimed to design a system that allows auto-regulatory lysis in *E. coli* via its cell density. Towards this goal, we have developed a transducer-switch genetic circuit with the integration of (i) the transducer, using the glucose depletion or stationary phase inducible promoter *csiDp*, (ii) the switch, *las* quorum sensing (*lasQS*) device, and lastly (ii) the lytic protein, colicin E7 (Fig 3.1 A & B). We exploited the usage of *lasQS* device derived from *Pseudomonas aeruginosa* (Gray et al., 1994; Pesci et al., 1997; Smith & Iglewski, 2003; Venturi, 2006) in consideration of the foreign proteins expressed that could directly affect the host innate regulatory pathway. In this chapter, we demonstrated that our reprogrammed cell was able to

display cell density-dependent auto-regulatory lytic behaviour. Maximum cell growth was allowed to reach before lysis occur at stationary phase and/or carbon depletion. Last but not least, we validated the practicability of our design circuit in biotechnological application by quantifying plasmid DNA and fluorescence proteins released as the macromolecule of interest to be extracted.

Fig 3.1 (A) General structure of a colicin. It is composed of a translocation domain (T domain), a receptor-binding domain (R domain) and a cytotoxic domain (C domain). (B) Schematic outlining the mechanism of *csiDp*. Briefly, the promoter *csiDp* may be activated by stationary phase sigma-38 transcription factor and/or carbon starvation signal cAMP-CRP complex.

3.2 MATERIALS AND METHODS

3.2.1 Strains and plasmids

E. coli K-2 MG1655 strain was used as a template for amplifying *csiDp*. *P. aeruginosa* PAO1 was used as the template for amplification of *lasQS* device. All cloning and expression of the constructed plasmids were done in *E. coli* TOP10 strain (Invitrogen). The vectors used in this study to construct the plasmids were pBbS8a and pBbE8k which were BglBrick standard plasmid obtained from the Joint BioEnergy institute (JBEI), USA. pBbS8a is a low copy number plasmid (pSC101; ~4-6 molecules per cell), carrying an ampicillin marker. pBbE8k is a high copy number plasmid (ColE1; ~50-70 molecules per cell), carrying a kanamycin resistance marker. The test plasmid used was pSB1K3, a high copy (pMB1; ~100-300 molecules per cell BioBrick standard plasmid with a kanamycin resistance marker, containing constitutively expressed RFP (www.parts.igem.org/wiki/index.php?title=Part:BBa_J04450).

3.2.2 Promoter and quorum sensing system characterization

For promoter and quorum sensing system characterization, reporter proteins used were red fluorescence protein (RFP) and green fluorescence protein (GFP). RFP was integrated downstream of *csiDp* whereas GFP was integrated downstream of *lasQS* device. Biotek Synergy HT microplate reader was used to detect the activities of the promoter (RFP; excitation at 535 nm and emission at 600 nm) and *lasQS* device (GFP; excitation at 485 nm and emission at 540 nm). Cells containing promoter *csiDp* (in pBbE8k), were grown to exponential phase at $A_{600}=1.5$. The supernatant of the cells were substituted by the M9 minimal media supplemented with 0.2 % (w/v) casamino acids and a varying range of 0 to 0.5 % (w/v) of glucose concentration. RFP fluorescence from the cell culture was detected for a period of 8 hours. For *lasQS*

device (in pBbE8k) characterization, cells were grown to stationary phase in Luria broth medium (LB) before dilution of 100X in a fresh LB medium. Again, the cells were grown to exponential phase at $A_{600}=0.5$ before the addition of 3-O-C12-HSL (Sigma Aldrich) to the final concentration of 10^{-10} M to 10^{-5} M. The fluorescence measurement of the cell culture was detected in microplate reader for a period of 10 hours. To examine the transducer-switch (*csiDp* coupled to *lasQS* in pBbS8a) behaviour, cells were grown to stationary phase in LB medium before dilution to $A_{600}=0.3$ in fresh LB medium supplemented with 0 to 0.3 % (w/v) of glucose concentration. . The green fluorescence measurement of the cell culture was detected in microplate reader for a period of 6 hours.

3.2.3 Cell growth measurement

Population density of *E. coli* were quantified by using 300 μ L of the sample and detected by microplate reader at absorbance of 600nm.

3.2.4 Membrane damage assay

To observe the membrane damage caused by the auto-regulatory lysis construct (*csiDp-las-Lysis*), cells were stained with propidium iodide (PI from Sigma Aldrich). Briefly, cells were cultured in 100 mL fresh LB for 14 hours until stationary phase was reached. 1ml of the cell culture was aliquot and rinsed before resuspension in 0.85 % NaCl solution. After the addition of 0.3 μ L of PI dissolved in DMSO to the 100ul cell suspension, the mixture was incubated at room temperature in the dark for 15 minutes. 1 μ L of the mixture was used for visualization under a fluorescence microscope (Zeiss Axio Scope A1) equipped with appropriate filters. Cell containing the auto-regulatory GFP construct (*csiDp-las-GFP*) was used as a control.

3.2.5 Macromolecular product release assay

To quantify the release of proteins from the engineered cells, the green fluorescence released into the extracellular medium was detected using the microplate reader. Cells containing auto-regulatory Lysis with GFP construct (*csiDp-las-Lysis-GFP*) was tested against the control cells containing only auto-regulatory GFP construct (*csiDp-las-GFP*). Both are similarly grown in fresh 5ml LB for 14 hours until stationary phase was reached. Additional 6 hours of incubation at 37 °C for the cells were done to ensure maximal lysis had already obtained. Cells were then pelleted via centrifugation at 5,000 x g for 5 minutes. Supernatant was collected and filter sterilized with 0.2 µm filter (Sartorius Stedim Biotech). GFP fluorescence was detected using microplate reader.

To quantify the release of DNA plasmids from the engineered cells, contrasformation of auto-regulatory Lysis with GFP construct (*csiDp-las-Lysis-GFP* in pBbS8a) and test plasmid with constitutively expressed (RFP in pSB1K3) was done. Cells were cultured in 5ml LB supplemented with 0.3 % (w/v) glucose for 24 hours until stationary phase was reached. Cells were then pelleted via centrifugation at 5,000 x g for 5 minutes and rinsed with LB, followed by addition of fresh LB medium supplemented with 0.3 % (w/v) glucose concentrations or without. Cells were incubated further for additional 8 hours. 5 mL of cell culture was aliquot and filter sterilized with 0.2 µm filter. 0.7 volume of isopropanol was added to the collected filtrate. The mixture was cleaned up by allowing it to pass through the spin column with 2 washings of PE buffer using Qiagen's QIAprep kit and following its protocol. Only the spin column, elution buffer and washing buffer were used. The eluted plasmids were further analysed for its concentrations and purity using NanoDrop 1000 spectrophotometer.

3.3 RESULTS AND DISCUSSION

3.3.1 Characterization of the exclusive carbon depletion and/or stationary phase inducible promoter *csiDp*

To demonstrate that the promoter *csiDp* fitted the feature of a transducer, *E. coli* containing *csiDp* was ligated to a reporter protein RFP in pBbE8k was exposed to varying glucose concentrations. An inverse dependence of glucose concentration on the activity of *csiDp* was observed (Fig 3.2). Hence, maximal activity of 184.9 ± 3.3 a.u. per A_{600} was observed in the medium without glucose at the 8th hour steady state time point. Additional 0.5% (w/v) glucose led to the lowest *csiDp*'s activity of 24.3 ± 0.3 a.u. per A_{600} with a difference of ~ 7.6 -fold from the activity without glucose. Regular interval of decline of 0.1% glucose from 0.5% led to an accelerated decline in activity of *csiDp*: 27.7 ± 0.8 , 44.2 ± 0.6 , 79.1 ± 2.1 , 145.6 ± 2.5 a.u. per A_{600} respectively.

Fig 3.2 Characterization of the cells containing red fluorescent protein (RFP) regulated by *csiDp* at different glucose concentrations ranging from 0 to 0.5% (w/v). (A & B) RFP was expressed when the cells reached stationary phase. Addition of glucose was observed to reduce the fluorescence values.

Besides the control of *csiDp*'s activity, activating cell density was also manipulated with varying glucose concentration. An increase in glucose concentration led to higher rate of RFP expression due to a higher activating cell density (Fig 3.3). Interestingly, we found out that the operating range of glucose concentration was narrow; between 0 and 0.3% (w/v) with corresponding range of A_{600} 3.05 ± 0.13 to 4.65 ± 0.21 . In addition, the results also suggested that the activation of the *csiDp* occurred at the stationary phase which corresponded well with previous studies (Battesti et al., 2011; Marschall et al., 1998; Martin Metzner, Jens Germer, & Regine Hengge, 2004).

Fig 3.3 The activating cell density was determined when RFP expression per A_{600} started increasing. We observed that the adjustable glucose range for *csiDp* is between 0% and 0.3% (w/v) in M9 minimal medium.

However, even when the system above worked well as a transducer, it is often not desirable to have varied promoter activity (red fluorescence) which heavily relied on the signal input intensity (glucose). This may lead to varying efficiency of lysis that is dependable on the glucose concentration used. Hence, we proposed the implementation of a switch that could have a more control on the lytic device. We aimed for our reprogrammed cell to be responsive to a range of input but at the same time, be able to produce a consistent output. To achieve this, we coupled *las* quorum sensing (*las*QS) device to serve as a switch to produce a consistent lysis efficiency.

3.3.2 Characterization of *las*QS device

In this study, the *las*QS device was composed of three parts: the *lasI* promoter to which activated complex binds to, transcription factor LasR that couples with signalling molecule 3-O-C₁₂-HSL (AHL) to form the activated complex, and AHL synthetase LasI that catalyzed the synthesis of AHL. The activation complex involved 2 substrates, LasR and AHL. To design the sensor such that transcription of downstream genes would be triggered only and immediately when AHL is present, LasR has to be constitutively expressed. A common class of constitutive promoters that was used was the sigma-70 promoter. J23108 was the chosen promoter derived from the BioBrick parts registry (http://parts.igem.org/wiki/index.php?title=Part:BBa_J23108).

Fig 3.4 Schematic of characterization of the *las*QS device. Exogenous 3-O-C12-HSL (AHL) molecules were added to the cells bearing the *las*QS-regulated green fluorescent protein (GFP). Once the concentration exceeded the nano-molar range, GFP fluorescence became consistent.

To assess the activity of the *las*QS-GFP device (Fig 3.4), the cells containing such device was subjected to varying AHL concentration of 10^{-10} M to 10^{-5} M. The *las*QS device that was used to assess its activity was composed of GFP that was coupled downstream of the pLasI promoter with constitutive expression of LasR. *las*QS-GFP device was able to produce a consistent output, green fluorescence in spite of the different input signal (AHL concentration) with a sensitivity limit of 10^{-9} M (Fig 3.5). An increment of AHL concentrations more than 10^{-8} M did not significantly increase the expression of GFP. Hence, we have proved that *las*QS device would ideally serve as the switch due to the constant output expression once 10^{-8} M AHL was exceeded.

Fig 3.5 Characterization graph of *las*QS device. Green fluorescent protein (GFP) regulated by *las*QS was triggered on addition of exogenous 3-O-C12-HSL (AHL) molecules. When the added AHL concentration exceeded the nano-molar range, GFP fluorescence became consistent.

3.3.3 Performance by the combination of *csiDp*-regulated LasI transducer and *las*QS switch

To evaluate whether after the individual device, transducer *csiDp* and switch *las*QS were combined, they still performed as the designed function; a constant output regardless of varied signal input and activating cell density. To address this, *lasI* gene was coupled downstream of *csiDp*, making *lasI* regulation dependent on *csiDp*, and *las*QS-GFP device was integrated (Fig 3.6).

Fig 3.6 Schematic of the coupling of the *csiDp-lasI* (transducer) and *lasQS*-GFP (switch) and using GFP to determine the behaviour of the coupling effect.

When the *csiDp-lasI* and *lasQS*-GFP device were coupled, in the presence of 0 to 0.1% (w/v) glucose, constant GFP was expressed at an average steady state of 1638.1 ± 35.2 a.u. per A_{600} . When higher concentration of 0.2 to 0.3% (w/v) of glucose concentration was added, the steady state GFP expression was reduced by ~5 fold to 344.2 ± 1.8 a.u. per A_{600} for 0.3 % (w/v) glucose (Fig 3.7 B). The kinetics of the constant steady state GFP expression was varied from 0 to 0.3% (w/v) glucose (Fig 3.7 B) with the fastest and earliest onset at 0% (w/v) of glucose. GFP expression at 0% (w/v) glucose was triggered at mid-log phase while cells grown at 0.1 and 0.2% (w/v) expressed GFP at early stationary phase and at stationary phase respectively (Fig 3.7 A & B). Only at 0.3% (w/v) glucose, the GFP expression was entirely repressed in the period of 6 hours. The results obtained correlated well with the Boolean device that generates single logical output (0 or 1), regardless of the varying glucose concentration (signal inputs) of 0 – 0.3% (w/v). Hence, the coupling of the *csiDp-lasI* and *lasQS* device (transducer-switch system) could generate a constant output signal regardless of the varying input signal.

Fig 3.7 Characterized behaviour of the *csiDp*-regulated 3-O-C12-HSL (AHL) synthetase LasI coupled with the *las* quorum sensing switch, with cell growth indicated in (A) and GFP expression in (B). The combined *csiDp-las* system enabled users to control GFP output expression by varying glucose input concentrations. 0–0.1% (w/v) glucose led to a consistent elevated GFP output, while 0.2–0.3% (w/v) glucose repressed the system. The high initial GFP expression was due to *E. coli* cells being diluted from the stationary phase where the cells were first grown un-repressed in LB medium only.

3.3.4 Evaluation of the synthetic lysis system

To enable our reprogrammed *E. coli* to display cell density-dependent auto-regulatory lytic behaviour, we integrated the transducer-switch system (*csiDplasI-lasQS*) that we had created and validated in the lysis genetic circuit. Hence, a lytic gene, colicin E7 (Saeidi et al., 2011a) was placed downstream of the transducer-switch system. *E. coli* that produces colicin E7 natively, export bacteriocin in stress conditions (Chak, Kuo, Lu, & James, 1991). E7 lysis protein damages the inner membrane and may also be involved in the outer membrane disruption via activation of the outer membrane phospholipase A (Burow, Mabbett, Borrás, & Blackall, 2009). The ability of colicin E7 to lyse *E. coli* with a known lytic mechanism made it an ideal choice to be served as the lytic gene in our lysis genetic circuit (*csiDp-lasQS-Lysis*).

To demonstrate the behaviour of our lysis genetic circuit, we quantify the macromolecules, protein (green fluorescence) and DNA released from our reprogrammed cells. To demonstrate the practicability of our lysis genetic circuit on the protein released, lysis genetic circuit with GFP (*csiDp-lasQS-Lysis-GFP*) was used. Transducer-switch system with GFP only, without the lysis gene (*csiDp-lasQS-GFP*) served as our control. After 3.5 hours, upon reaching stationary phase, a prominent decrease in cell growth in the lysis genetic circuit but not the control that did not bear the lysis gene (Fig 3.8).

Fig 3.8 Characterization of the lytic effect by GFP-encoding synthetic lysis circuit (*csiDp-lasQS-Lysis-GFP*) was compared against the control (*csiDp-las-GFP*) upon reaching stationary phase. Detection at absorbance 600nm was made for a duration of 6 hours. Only synthetic lysis system reduced the absorbance measurement but not the control after 3.5 hours.

To validate that the observation was due to lysis effect, the amount of GFP released into extracellular medium was quantified (Fig 3.9). The GFP green fluorescence released into the extracellular medium from the auto lysis genetic circuit showed approximately 3 fold higher GFP than the control without the lysis gene. This confirmed that when the cells reached the stationary phase, lysis occurred and the green fluorescent proteins were released into the extracellular medium.

Fig 3.9 Characterization of the extracellular GFP released into the extracellular medium between the GFP-encoding synthetic lysis circuit (*csiDp-las*QS-Lysis-GFP) and the control (*csiDp-las*-GFP). Cells carrying the synthetic lysis system released nearly 3-folds more GFP than the control.

To visualise the lysis effect and further validate that the membrane was damaged with the expression of lysis protein at stationary phase, *E. coli* cells containing aforementioned lysis genetic circuit was stained with propidium iodide (PI). PI is a large molecule that will enter only cells with damaged membrane to emit red fluorescence (excitation: 490nm, emission: 635 nm). Significantly more red cells were observed for the cells with the lysis genetic circuit than the control with only the transducer-switch system (Fig 3.10).

Fig 3.10 Visualization of propidium iodide-stained cells for membrane damage assessment. Control cells (*csiDp-las*-GFP). and the cells carrying *csiDp-las*QS-Lysis-GFP were compared. More red cells carrying *csiDp-las*QS-Lysis-GFP than control indicated more cells carrying synthetic lysis system were membrane-damaged than the control cells.

To demonstrate the capability of our lysis genetic circuit on the macromolecule plasmid DNA released could be manipulated easily by varying the glucose concentration, our lysis genetic circuit (*csiDp-las*QS-Lysis) was used. Usually, in molecular cloning, successful recombinant plasmid was extracted using costly reagents to lyse the cells. However, our lysis genetic circuit was present in low copy number (~4 – 6 molecules per cells) which may pose difficulty for both visualization in a electrophoresis gel and accurate quantification from nanodrop. Hence, co-transformation of the high copy pSB1K3-RFP (~100 – 300 molecules per cells) with our lysis genetic circuit was done. Cells were initially treated with 0.3 % (w/v) glucose to repress lysis activity (GFP was repressed in Fig 3.7 when exposed to 0.3 % (w/v) glucose) until stationary phase was reached. Medium was then replaced with fresh LB medium supplement with 0.3 % (w/v) glucose or without. When glucose that was used to repress the promoter *csiDp* activity was removed, LasI which was regulated by the

promoter *csiDp* would be transcribed and expressed and thus, downstream genes like lysis genes would in turn be transcribed (*csiDp-lasQS-Lysis*). However for the control which was grown in fresh LB with continual supplementation of 0.3 % (w/v) glucose, promoter *csiDp* would continue to be repressed and no lysis would occur. To ensure that the lysis was complete and maximal amount of the DNA plasmid was released into the extracellular medium occurred, an addition of 8 hours incubation was done. Approximately 3 fold of plasmid could be extracted when the cells was removed of glucose compared to the cells with continual supplementation of 0.3 % (w/v) glucose (Fig 3.11).

Fig 3.11 Effects of glucose on the amount of macromolecule released. DNA plasmids extracted from the extracellular medium of the cells growing with and without glucose were compared. The *csiDp-lasQS-Lysis* system was activated when glucose was removed with a ~3 fold of higher amount of extracted plasmid released.

To ensure that the extracted plasmid using our lysis genetic circuit was intact and of good quality, nanodrop spectroscopy was used. Measurement values at absorbance of 260 nm and 280 nm was detected. A A_{260}/A_{280} ratio of 1.8 – 2.0 represents a pure nucleic acid sample. Our sample showed a A_{260}/A_{280} ratio of 1.78 ± 0.12 which indicated that the plasmid extracted was of good quality and integrity of the plasmid was not compromised. Further, to validate that the extracted plasmid was of correct identity (pSB1K3-RFP), the extracted samples was run in 0.8 % (w/v) agarose electrophoresis gel (Fig 3.12) and PCR was conducted using the standard primers from the BioBrick parts registry (VF2 and VR) to confirm the extracted plasmid was pSB1K3-RFP. All in all, we have achieved the aim of controlling lysis of cells through varying glucose concentration and created a more economical option of macromolecules extraction without addition of reagents (chemical, enzymatic lysis: eg. Lysozyme, buffers) and usage of external costly equipment (mechanical lysis: fast prep, sonication).

Fig 3.12 Extracted DNA samples were run in 0.8% (w/v) agarose gel at 120 V for 20 min using Thermo Scientific GeneRuler 1 kb DNA ladder as a marker. No band was observed for the sample repressed with 0.3% (w/v) glucose, highlighting the effect of 0.3% (w/v) glucose on the synthetic lysis system. The expected band size of pSB1k3-RFP was ~3 kb.

3.4 CONCLUSION AND FUTURE WORK

In this study, we had created a novel genetic circuit which allowed cell density-dependent auto-regulatory lysis for a more economically viable alternative in macromolecules like plasmid DNA and proteins extraction. Our lysis genetic circuit was composed of a transducer-switch which utilised a carbon-depletion dependent promoter *csiDp* as the transducer, the *lasQS* device as the switch, and the lytic protein colicin E7 to release the macromolecule of interest. Our circuit design was able to (i) activate only when stationary phase was reached which enabled more cell growth and more products produced before extraction; (ii) control activating cell density through varying a range of glucose concentration (0 to 0.3 % (w/v)); and (iii) most importantly a reliable and constant output (lysis) that was independent of the input signal. The reason that colicin E7 was chosen was because of its known mechanism on membrane disruption on host cell and was applicable in *E. coli*. However, it could be substituted in our genetic circuit by any lytic protein that worked on the host cell. Finally, the practicability of our circuit design was demonstrated through plasmid DNA and green fluorescent protein extraction. This not only reduced the number steps of extraction of macromolecules but also gave a more economical option of utilizing costly reagents and equipment. The reduced usage of reagents in industrial scale could make extraction of macromolecules more environmental friendly because some reagents are toxic. We envision that this novel lysis genetic circuit design in our study could provide a simplified, more economical and environmental friendly option of extraction of macromolecules expressed in *E. coli*.

Future work which could be done would be to integrate the lysis genetic circuit into the genomic DNA. As there could be possible extraction of recombinant plasmid along with other the plasmid containing the lysis genetic circuit, genomic DNA integration

would serve a better option. Further, genomic DNA is stable and horizontal gene transfer would be minimized. Hence, Integration into the genomic DNA allows more degree of freedom and more feasibility in the recombinant plasmid DNA extraction in cloning. Since our design was tested in low copy vector, the expected expression of the lysis genetic circuit should not be drastically affected in genomic DNA.

CHAPTER 4

Engineering Probiotic Strain (EcN) with Optimized Sensing and Eradication against *Pseudomonas aeruginosa*

4.1 INTRODUCTION

P. aeruginosa is an opportunistic pathogen that causes life-threatening infection in individuals. It is commonly hospital-acquired and is often associated with higher mortality in immunocompromised patients than other Gram-negative bacterial infections (ALIAGA, MEDIAVILLA, & COBO, 2002). Although the airways is a major site of infection for *P. aeruginosa*, there are reports tracing back to the gut as a prominent reservoir that leads to its spread from intestine to the lungs (Alverdy et al., 2000; Laughlin et al., 2000; Okuda et al., 2010; Zaborina et al., 2006). As the *P. aeruginosa* can bind and disrupt the intestinal epithelial barriers, the gastrointestinal tract may be an important site to modulate the pathogen reservoir from developing into infections. Furthermore, it is reported to be capable of spontaneously acquire resistance to antibiotics, limiting the efficacy of current antibiotic treatments in use.

The biofilm formation conveys resistance toward antibiotics and plays a role in pathogenesis of many chronic infections (Bjarnsholt, 2013; Sanchez et al., 2013). Those remediable by antibiotics are associated with acute infections, which usually involved planktonic bacteria (Furukawa, Kuchma, & O'Toole, 2006; Wang et al., 2012). On the other hand, the more persistent chronic infection was derived from the other life-form, biofilm (Bjarnsholt, 2013; Byrd et al., 2011). Biofilm consists of an extracellular polymeric substance (EPS), which provides the matrix that holds the cells together and microcolonies within the matrix. Because the antibiotic has been proven to be ineffective against biofilms, biofilm degrading enzyme had been used to disrupt

the biofilm structure. Once disrupted, the sessile bacteria cells become susceptible to the antibacterial treatment. There are many reports that have already done so. Esterase EstA and DNase can affect the morphology of *P. aeruginosa* biofilms (J. B. Kaplan, 2009; Tielen et al., 2010). DspB (dispersin B) is another well-characterized biofilm degradation enzyme. It catalyzes the hydrolysis of polysaccharide found in the biofilm matrix. DspB cleaves poly- β -(1,6)-linked N-acetylglucosamine bonds (J. B. Kaplan, C. Ragunath, N. Ramasubbu, & D. H. Fine, 2003). Although the biofilm degradation has been shown on *E. coli* and *Staphylococcus epidermis*, its activity on *P. aeruginosa* (PAO1) biofilm has shown to be inconclusive (Itoh, Wang, Hinnebusch, Preston, & Romeo, 2005). However, in this study, we show that DspB is effective in degrading mature biofilm of *P. aeruginosa* (clinical isolate, In7).

After biofilm disruption, *P. aeruginosa* would become more susceptible to antibiotics (Bayles, 2007). Hence, it was not surprising when Lamppa and his team demonstrated a positive synergy of biofilm-disruptive enzyme with antibiotics against biofilm cells (Lamppa & Griswold, 2013). In fact, after biofilm dispersal, the free-moving cells would be released and spread out in biofilm development (Bayles, 2007; Rollet, Gal, & Guzzo, 2009). Therefore, current treatment regimen against *P. aeruginosa* infection mostly involves multiple unspecific-targeting antimicrobial agents due to the rapidly acquired resistance *P. aeruginosa*. However, these broad-spectrum-targeting antibiotics exacerbate a healthy human microbiome in the process. Hence, we not only will develop a single agent that can target both life-forms of *P. aeruginosa* but also the incorporation of selectively *P. aeruginosa*-targeting pyocin S5 (Michel-Briand & Baysse, 2002). This would deter targeting beneficial bacteria present in the gut.

Before the biofilm degradation enzyme DspB and the pyocin S5 could fulfill their role, they should be present extracellularly. Hence, like in our previous work (Saeidi et al.,

2011a), we would use E7 lysis gene that could efficiently release the *P. aeruginosa*-targeting proteins. Moreover, we would also be exploiting the quorum sensing of *P. aeruginosa* for specific detection and release of DspB and the pyocin S5. Only upon detection of AHL specifically produced by *P. aeruginosa* would the expression of these growth-unrelated proteins be produced to conserve resources from the host cell.

Probiotics are defined as live microorganisms that confer health benefits to the host when administered in adequate amounts (Nagpal et al., 2012). Several probiotic bacteria are already released into the market, and the range of the products with the addition of these bacteria to the food is on the rise (Farnworth, 2008; Khan & Ansari, 2007). One good example of the probiotic-containing food product is yoghurt. These probiotics are capable of superior GI tract colonization compared with pathogenic microbes, resulting in health benefit. Using antibiotics to treat pathogenic microbes may sometimes cause disruption in human microbiome, which may lead to diseases (Bjarnsholt, 2013). However, patient ingesting beneficial bacteria would be able to restore the balance (Rosenfeldt et al., 2002; Zoetendal et al., 2002). Therefore, taking advantage of this innate beneficial aspect, the notion of using genetically modified designer probiotics as novel therapeutic agents for GI diseases is gaining great interest (Baat et al., 2006; Steidler, 2003). Although there are various types of probiotics available, *E. coli* Nissle 1917 (EcN) is one of the well-characterized with its well-established therapeutic effects and commercialization as MUTAFLOR. It has also been used to alleviate various diseases of the digestive tract such as inflammatory bowel disease (IBD) and ulcerative colitis (Hernando-Harder, Büнау, Nadarajah, Singer, & Harder, 2008; Kruis, Chrubasik, Boehm, Stange, & Schulze, 2012). Thus, in the realm of synthetic biology, EcN will be a perfect candidate to be the chassis of carefully designed genetic circuit to be the new generation antimicrobial agent.

In this study, we have extended the idea of engineering a clinically relevant probiotic strain that can achieve more complete eradication scheme, by having highly efficient cell killing capability combined with the ability to prevent and actively degrade the biofilm of a clinical isolate *P. aeruginosa*, In7. The efficacy was extensively demonstrated in various *in vitro* conditions and growth states, and *in vivo* *C. elegans* model to simulate the mammalian gut environment.

4.2 MATERIALS AND METHODS

4.2.1 Strains and media

All cloning and characterization experiments are done in *E. coli* TOP10 (Invitrogen), unless otherwise stated. Commercial Luria-Bertani (LB) was used as the medium for cloning, unless otherwise stated. Kanamycin (30 µg/mL) was added to the culture media for antibiotic selection where appropriate. Homoserine lactone, 3OC-12-HSL (AHL; Sigma-Aldrich) was used for characterization experiments. All restriction and ligation enzymes were purchased from New England Biolabs (NEB). pBbE8k, which was a BglBrick standard plasmid obtained from the Joint BioEnergy institute (JBEI), USA, was used as the vector for our inserts. pBbE8k is a high-copy-number plasmid with ColE1 replication origin (~50–70 molecules per cell) and carries kanamycin cassette. *P. aeruginosa* ln7, a clinical isolate that is sensitive to S5 pyocin, was used throughout this study (Ling et al., 2010). The probiotic *E. coli* Nissle 1917 (EcN) was from Mutaflor (Canada).

4.2.2 System assembly

The genetic constructs developed in this study were assembled using standard synthetic biology protocols (Canton et al., 2008). Briefly, for front insertion of Bglbrick parts, purified insert and vector plasmids were digested with *EcoRI*/*BamHI* and *EcoRI*/*BglII*, respectively. For back insertion, the insert and vector plasmids were digested with *BglII*/*XhoI* and *BamHI*/*XhoI*, in that order. Digested fragments were separated by DNA gel electrophoresis and ligated with NEB T4 ligase in accordance with the manufacturer's instructions. Recombinant plasmids were verified by DNA sequencing.

4.2.3 Characterization of GFP containing constructs with AHL

Single colonies of the constructs in *E. coli* were inoculated into LB overnight. After overnight growth, the cultures were rediluted into fresh LB to low OD and allowed to incubate further to OD₆₀₀ of 0.5. Cultures were then transferred into a clear polystyrene 96-well plate in triplicate aliquots of 200 µL for induction with AHL at varying molar concentrations (0, 1.0E⁻⁹, 1.0E⁻⁸, 1.0E⁻⁷, 1.0E⁻⁶, 1.0E⁻⁵ M). The plate was incubated at 37°C with medium shaking in a microplate reader (Biotek) and assayed for green fluorescence (GFP; excitation at 485 nm and emission at 540 nm). Time-series fluorescence and OD₆₀₀ data were obtained for a total duration of 5 hours. A relative GFP production rate was derived as a ratio of background subtracted green fluorescence to OD₆₀₀ value. The experimental results were plotted in MATLAB. The results from the constructs, pTet LasR-pLux GFP expressed in Top10 and EcN, and J23108 LasR-pLas GFP in EcN were used for modeling.

For comparison of the percentage (%) of nutrients allocation for constitutive expression of LasR and inducible expression, GFP was coupled downstream of pTet and quorum sensor device, resulting in pTet-GFP and *lasQS*-GFP (pTet LasR-pLux GFP, J23108 LasR-pLas GFP) constructs. Characterization was done using 1.0E⁻⁶ M AHL for inducible expression. The percentage of nutrients allocation between the constitutive and inducible expression was based on the assumption that all nutrients were allocated for the expression of foreign proteins. The equation was as follows:

$$\begin{aligned} & \% \text{ resources allocation for expression of protein} \\ & = 100\% \times \frac{\text{RFU per OD from constitutive or inducible expression at time} = t}{\text{Total RFU per OD from both constitutive and inducible expression at time} = t} \end{aligned}$$

4.2.4 Characterisation of the enhanced quorum sensor in EcN

For the enhancement of quorum sensor, systematic changes were made to the translation and transcriptional levels. In that order, progressive modification to the *lasQS*; the vector, ribosomal binding site (RBS); and both constitutive and inducible promoters were made. GFP was regulated by the *lasQS* (*lasQS*-GFP). The OD₆₀₀ and relative GFP were measured and compared with each modification made to reach the final construct, J23108 LasR-pLas GFP. Characterization was done with the induction of 1.0E⁻⁶M AHL.

4.2.5 Enzyme assay of DspB protein expressed in EcN

A synthetic substrate, 4-nitrophenyl-N-acetyl-β-D-glucosaminide, was used to test the enzymatic activity of DspB. The supernatant was prepared by inducing the expression of DspB and E7 in Nissle for 4 hours by adding 1E⁻⁷ M AHL when the cells reached OD₆₀₀ of 0.6. The cells would have lysed and released DspB into the supernatant because of E7 expression. Enzyme reactions were carried out in 100 μL mixtures containing 4 mM substrate in PBS buffer and 10 μg/mL of cellular supernatant, and its absorption changes were immediately measured from the release of *p-nitrophenolate* with Biotek Synergy HT Multi-Mode Microplate Reader set at 405 nm. The protein concentration of the supernatant was determined using standard Bradford assay.

4.2.6 Cell viability testing of co-cultured engineered *E. coli* and *P. aeruginosa*

Overnight cultures of *P. aeruginosa* (In7 with plasmid containing constitutive

expression of GFP; pMRP9-1), *P. aeruginosa* (In7), and various sense-annihilation-lyse constructs (Figure 4C and D) were diluted and harvested at exponential phase.

EcN containing the constructs were added to In7 culture in an appropriate ratio and was subsequently transferred into a clear polystyrene 96-well plate in triplicate aliquots of 200 μ L. As In7 is constitutively expressing GFP from the plasmid, its death was determined via GFP fluorescence and was detected by using microplate reader after 5 hours. The same procedures were repeated for EcN containing the E7 construct (Nissle-E) as negative control. For cell viability assays, aliquots of In7 in the mixed culture were quantified by CFU count on chloramphenicol-selective agar plates at regular intervals of 12 hours up to 48 hours. The *P. aeruginosa*-carrying chloramphenicol-resistant plasmid was used for selection. The same procedures were repeated for EcN containing the E7 construct (Nissle-E) as negative control. Percentage survival of In7 was tabulated relative to Nissle-E.

4.2.7 Biofilm detachment of *P. aeruginosa* biofilm assay treated with engineered EcN

The wells of a 96-well microtiter plate were filled with 150 μ L of medium containing a single cell suspension of *P. aeruginosa* at OD₆₀₀ of 0.05 (CFU) and incubated at 37°C for 24 hours. The wells were rinsed with PBS, and 200 μ L of engineered EcN (Nissle-SE, Nissle-3SED) supernatant was added to each well, and the plates were incubated for an additional 4 hours. The wells were washed thrice with PBS; and the bacteria remaining attached to the surface were stained with crystal violet (0.1% w/v), rewashed under running tap water, and dried. The amount of biofilm mass was quantified by destaining the biofilms for 20 minutes with 200 μ L of 95% ethanol and

then measuring the absorbance of the crystal violet solution at 595 nm. The same procedures were repeated for EcN as negative control.

4.2.8 Biofilm formation & CLSM preparation

Mixed bacteria cultures of *P. aeruginosa* (In7 with pMRP9-1) and engineered EcN (Nissle-SE, Nissle-3SED) were grown in LB in 50 mL tubes containing sterile glass slide. Biofilm developed on the glass slides after 18 to 20 hours of growth was rinsed in PBS, dried, and visualized by LSM 510 confocal laser scanning microscope (Zeiss, Jena, Germany). Collected Z-stack biofilm images were reconstructed using Image J software. Likewise, biofilm detachment images were prepared after treating the mature biofilms with engineered EcN for 4 hours. Three-day-old (72 hours) *P. aeruginosa* biofilms were prepared using LB growth medium that was replaced with fresh medium every day. The same procedures were repeated for EcN as negative control.

4.2.9 *Caenorhabditis elegans* killing assay

The *C. elegans* strain, AU37, *glp-4 (bn2) sek-1 (km4)*, used in this study was obtained from the Caenorhabditis Genetic Center (<http://www.cbs.umn.edu/cgc>). AU37 is a pathogen-sensitive strain that exhibits temperature-sensitive sterilization. All nematode-related maintenance protocols were performed according to Powell and Ausubel (2008). Worms were allowed to grow in 15°C for maintenance or at room temperature for sterility or assay. Pathogenicity killing assay was performed with slight modifications. Adult nematodes were infected on slow-killing plate (SKP) with In7 (transformed with plasmid pMRP9-1) bacterial lawn for 24 hours. They were then washed off the plates with M9 buffer, spun down, and twice again in M9 buffer

containing 200 µg/mL kanamycin, and resuspended in M9 buffer. Nematodes were then transferred to 96-well microtiter plate with *E. coli* bacterial lawn for treatment. Nematodes were observed for infection with fluorescence microscope. Worms were counted before and after 16 hours of treatment, and the percentage (%) survival of *C. elegans* was tabulated. At least three replicates of 10 to 30 worms were carried out. The same procedures were repeated for EcN as negative control.

4.3 Results and Discussion

An overall schematic of the components of our biocide in this study is outlined in Figure 4.1. *C. elegans* infected with a clinical isolate *P. aeruginosa* In7 was used as the model for studying the biocide. *P. aeruginosa* residing in *C. elegans* produced the quorum-sensing molecule 3O-C12-HSL (AHL) that would be detected by our engineered probiotic strain, Nissle (EcN). The detection of AHL was by *lasQS* (quorum sensor, *sensing* module), which we reconstructed and boosted its expression in EcN. Both *annihilation* and *lysis* modules were produced simultaneously until lysis occurred and released the annihilation module that consisted of specific planktonic *P. aeruginosa* killing protein, pyocin and biofilm disruption protein, Dispersin B (DspB).

Fig 4.1 Schematic representation of engineered probiotic Nissle against *P. aeruginosa* in *C. elegans*. *C. elegans* infected with a clinical isolate *P. aeruginosa* In7 was used as the model to study therapeutic effect of engineered Nissle (EcN) against In7 in *in vivo* settings. Production of quorum-sensing molecule (AHL) from In7 residing in *C. elegans* would be detected by *sensing* module in engineered EcN. *Sensing* module was the crucial component affecting downstream expression, which we reconstructed for boosted downstream expression in EcN. Both *annihilation* and *lysis* modules were produced upon AHL detection, releasing the annihilation proteins that were consisted of specific planktonic *P. aeruginosa* killing protein (S5) and biofilm degradation protein (DspB).

4.3.1 Quorum-sensing device expression level characterization in EcN

The part that should be first finalized would be the quorum sensor. It is the backbone of our circuit design because we desired our functionality modules to be only expressed when necessary, which in this case would be during the presence of *P. aeruginosa*. To establish the expression rate of quorum sensor in the EcN, GFP coupled with the quorum sensor device from our previous work (Saeidi et al., 2011a) was transformed into EcN for expression characterization. Different expression levels of recombinant proteins had been shown when different strain was used (Casali, 2003; Humphrey et al., 2012; Yin, Lin, Li, & Wang, 2003). This would greatly affect the downstream protein expression and hence, the success of application. GFP expression (P_{Tet} LasR- P_{Lux} GFP) was induced by a range of concentration of inducer, AHL (3O-C12-HSL) and the expression rate in EcN was compared with that of *E. coli* Top10. The heat map clearly demonstrated that the rate of GFP expression per cell density in EcN was significantly lower than Top10 (Figure 4.2). Up to fivefold difference in expression rate was observed between two different strains of *E. coli* (Figure 4.2).

Fig 4.2 Characterization of P_{Tet} LasR- P_{Lux} quorum sensing device in EcN. Protein expression level was quantified in (A) Top10 and (B) EcN strain.

Furthermore, it was observed that the cell viability of the host cell was greatly affected when 1.0E^{-7} M or higher concentration of AHL was present (Figure 4.3). It is reported that the concentration of 3-O-C12-HSL found in the planktonic and biofilm states of *P. aeruginosa* well exceeds 1E^{-7} M (Charlton et al., 2000a). This implies that a decline in cell viability of engineered EcN when exposed to higher concentration of AHL would further decrease the total amount of protein expressed.

Fig 4.3 Cell growth upon induction by AHL concentration greater than 10^{-7} M was greatly affected in EcN.

From the characterization data, we have discovered that there was lowered expression in EcN than Top10 and, hence, raised the need to rederive the genetic circuit for maximal expression of proteins under quorum sensors.

In bacteria, there is a correlation between cell proliferation and the level of protein expression, and recent mathematical modeling equations deduced that the unnecessary inducible protein expression results in retarded cell growth (Bailey et al., 1987; William E. Bentley, Mirjalili, Andersen, Davis, & Kompala, 1990; Boe, Gerdes, & Molin, 1987; Scott, Gunderson, Mateescu, Zhang, & Hwa, 2010). In the quorum-sensing device, the activator protein, LasR, is subjected to constitutive expression under a strong promoter, P_{Tet} . While the fraction of cellular resources devoted for foreign protein is fixed, the amount of activator protein, LasR, is consuming approximately 40% of allocated resources (Figure 4.4). This can be considered as a metabolic burden, which may restrict the expression of downstream proteins (Scott et al., 2010; Wessely et al., 2011).

Fig 4.4 Estimated level of protein expressed under constitutive promoter P_{Tet} , and inducible promoter P_{Lux} by green fluorescence, shows constitutively high expression under P_{Tet} in EcN, which may be the cause of low viability in EcN subjected to high concentration of AHL.

Therefore, in addressing an optimized quorum-sensing device that is functional in EcN to express downstream proteins for pathogen-killing effect, we aimed to design a construct that has minimal effect on cell growth, particularly in EcN, while obtaining maximal protein expression induced by a broader range of AHL concentration.

4.3.2 Reconstruction of the quorum-sensing device for optimal expression with minimal metabolic burden

From the characterization data in EcN, to retain, if not improve, its effectiveness in counteracting *P. aeruginosa* (Saeidi et al., 2011a), we reconstructed the quorum-sensing device with the aim of improving the inducible expression level of downstream genes in EcN and minimize the effect of expression of inducible proteins on EcN growth. The replication of origin in the plasmid determines the copy number of plasmid per cell. Bentley and his team discovered that the primary factor that influenced the metabolic stress was the proteins encoded in the plasmid (William E. Bentley et al., 1990). Decreasing growth rate was observed to correlate with greater plasmid copy number (William E. Bentley et al., 1990; Boe et al., 1987). Besides that the copy number of plasmids may influence the growth rate, another team reported that smaller plasmid copy number could increase inducible expression (Susanna Zucca, Pasotti, Mazzini, Cusella De Angelis, & Magni, 2012). The available copy number of the plasmid, in addition to the regulatory circuit component such as promoter, will determine the resulting amount of transcript available for expression. These two factors appear to interact with each other, where relative strength of promoter is altered by the different copy number (Susanna Zucca et al., 2012). Based on this finding, we placed the quorum-sensing device in a lower-copy-number Bglbrick plasmid with 15–

20 copies (T. Lee et al., 2011). As anticipated, the sensor was slightly more responsive upon AHL induction in lower-copy-number Bglbrick plasmid from 2412 to 3396 (RFU/OD), which is ~1.5-fold (Figure 4.5). Although the change in expression was noticeable, the cell growth was not greatly improved by the copy number alone (Figure 4.5). The unaffected cell growth observed may be because of the inducible expression being the main metabolic burden that triggered the decline in cell growth. This was observed when we compared the cell growth without and with AHL induction with OD_{600 nm} of 1.01 and 0.33, respectively. When the engineered EcN was induced with AHL, the cell growth was approximately threefold lower than the cell growth without induction. To maintain a high production of advantageous downstream proteins, decreasing the inducible production was not an option. Hence, subsequent reconstruction involved further systematically boosting the regulatory circuit components to maximize the inducible expression while maintaining the minimal activator protein (LasR) expression.

First, the ribosomal binding site was replaced with a stronger part from BioBrick registry (BBa_B0034; RBS5) to increase the rate of translation. Second, the inducible promoter for downstream expression was replaced with a native inducible promoter of LasR protein, P_{Las}. Previous use of P_{Lux} LasR heterologous pairing for inducible expression may have resulted in suboptimal expression. These two changes resulted in a twofold and threefold enhancement in cell growth and inducible expression upon induction, respectively (Figure 4.5). Thus, the result confirmed our aim of improving the cell growth rate without compromising the inducible expression was feasible.

Fig 4.5 Schematic diagram and the changes made to maximize inducible protein expression and cell growth in EcN. Schematic diagram depicts the general genetic design for systematic amendments to achieve optimal downstream protein expression. Systematic changes in the vector, promoters and ribosomal subunits led to progressive improvement in expression of inducible protein in EcN and cell growth of EcN.

Cabrera and his team reported that a saturation effect is observed in bacteria (Cabrera & Jin, 2006). This happened in bacteria and not in other species because bacteria had only one type of RNA polymerase (RNAP). RNAP would be easily overloaded by the endogenous transcriptional and translational machinery, which would in turn compromise the production of not only growth-related genes from the genome but also its own synthesis (Cabrera & Jin, 2006). Therefore, when the cellular machinery for expression and the fraction of total protein allocated for foreign protein expression is fixed, to give a maximal boost in the inducible expression while maintaining cell growth, the proportion of constitutive LasR production would be an ideal area to be fine-tuned. Four σ^{70} constitutive promoters from BioBrick registry (Anderson promoter collection) with different strengths—J23102, J23108, J23105, J23113 (Supplementary Figure) along with widely used P_{Tet} —were placed for LasR expression to determine the optimal balance between LasR and inducible expression with minimal metabolic stress. These progressive modifications resulted in a new quorum sensor (P_{J23108} -LasR- P_{Las} -GFP) with enhanced inducible expression rate and cell growth upon induction in EcN (Figure 4.6).

Fig 4.6 (A,B) Inducible expression is maximized and cell growth is no longer affected by the inducible system. (C) Total fraction of LasR protein when the host cell was subjected to AHL, has been greatly reduced to allow maximal expression under inducible promoter in a fixed nutrient content of host cell.

4.3.3 Characterization of the biofilm-degrading module that degrades mature *P. aeruginosa* biofilm

After the completion of the customized quorum sensor in EcN, we could then add function modules to the sensor for application, in our case are the important modules relevant to targeting *P. aeruginosa*. One of the added functionalities to the construct compared with our initial framework is the incorporation of biofilm degradation module. Here, we have chosen DspB to catalyze the hydrolysis of polysaccharide found in the biofilm matrix. DspB is a well-characterized enzyme that cleaves poly- β -(1,6)-linked N-acetylglucosamine bonds (J. B. Kaplan et al., 2003). In addition, DspB has been tested in *in vivo* settings in the rabbit model (Darouiche, Mansouri, Gawande, & Madhyastha, 2009) and has been shown to be a promising antibiofilm molecule to be administered for bacterial infection. Although the biofilm degradation has been shown on *E. coli* and *S. epidermis*, its activity on *P. aeruginosa* (PAO1) biofilm has shown to be inconclusive (Itoh et al., 2005). However, in this study, we showed that DspB was effective in degrading mature biofilm of *P. aeruginosa* (clinical isolate, In7). DspB from *Actinobacillus actinomycetemcomitans* was subcloned for expression in EcN under the regulation of quorum sensor device upon AHL induction. For DspB to display its activity, lysis was required as native DspB has been purified from the cell lysate (Jeffrey B. Kaplan, Chandran Ragunath, Narayanan Ramasubbu, & Daniel H. Fine, 2003). Thus, we included E7 gene in the circuit design to allow AHL-responsive cell lysis for characterization of DspB released in its native state. After AHL induction, the resulting supernatant was collected and assayed for its enzymatic activity using a synthetic substrate, 4-nitrophenyl-N-acetyl- β -D-glucosaminide. Enzyme activity was monitored by looking at the absorption change that resulted from the conversion of the substrate to 4-nitrophenol, which increased linearly with time (Figure 4.7).

Fig 4.7 The enzymatic activity of the DspB-containing supernatant was characterized upon AHL induction. Glycosyl hydrolase activity of cellular supernatant upon induction of DspB and E7 lysis was tested for enzymatic activity using 4-nitrophenyl-*N*-acetyl- β -D-Glucosaminide as a substrate. The optical density at 405nm is proportional to the amount of 4-nitrophynolate released in the reaction.

To validate its activity on the mature biofilm of *P. aeruginosa*, which was cultured for 24 hours, crystal violet staining was carried out after treatment with a range of concentration of supernatant from induced engineered EcN. As low as 1.25 $\mu\text{g/mL}$ of protein in crude supernatant containing DspB could effectively reduce *P. aeruginosa* biofilm biomass by approximately 30% (Figure 4.8).

Fig 4.8 The effect of the DspB-containing supernatant on biofilm matrix was characterized upon AHL induction. Biofilms were cultured for 24h in unsupplemented LB, rinsed with PBS and then treated with varying total concentration of proteins (0, 0.625, 1.25, 2.5, 5 $\mu\text{g/ml}$) from cell extract containing DspB for 1 hour before staining with crystal-violet. Absorbance at 595nm is proportional to biofilm biomass. A minimum of 1.25 $\mu\text{g/ml}$ of total concentration of

4.3.4 Characterization of the biocide with different configuration of the annihilation and lysis modules on planktonic *P. aeruginosa*

After validation of DspB's activity on the mature biofilm and confirmation of the customized quorum sensor in EcN, the other component of the annihilation module, pyocin S5, that targets planktonic (Ling et al., 2010) and lysis module (E7) was added to the design of the final construct to be validated on its overall effect on planktonic *P. aeruginosa*. However, we aimed for selective trigger of expression of these proteins, which may have a negative effect on EcN growth shown from Figure 4.5, when GFP was expressed under our quorum sensor. The other condition was that the application of our biocide was targeting In7, so it was necessary to express the annihilation and lysis proteins only when In7 was detected. Hence, with these conditions in mind, both annihilation and lysis modules were coupled downstream of quorum sensor such that the annihilation and lysis modules would only be triggered in the presence of AHL from In7. This is known as multicistronic because different genes share the same promoter (Chang, Mead, Dhodda, Brumm, & Fox, 2009). It was reported that different lengths of downstream genes (second gene) to the GFP gene of the same transcript affected the GFP expression (Chang et al., 2009). The observed phenomenon was because of the addition of the second gene that stabilized expression from the promoter by 3' mRNA sequence (Chang et al., 2009; Schoner, Belagaje, & Schoner, 1986). In addition, though translation of first cistron is efficient, the succeeding cistrons heavily relied on intercistronic sequences (US Patent no: 6,060,273). Because we have three genes—S5, E7, and DspB—and all genes have to be coupled to the promoter of quorum sensor, P_{Las} , we derived different configurations and arrived with three configurations to be tested: SE, SDE, 3SED (Figure 4.9 A and B, Supplementary Figure) for its effect against planktonic In7.

A	Constructed plasmid	Insert(s)	Vector
	pLE7	Pcon LasR-pLasI E7	pBbe8K
	pLS5LE7	Pcon LasR-pLasI S5-pLasI E7	pBbe8K
	pLS5LDE7	Pcon LasR-pLasI S5-pLasI DspB E7	pBbe8K
	pLS5LDLE73	Pcon LasR-pLasI S5-pLasI DspB pLasIE7	pBbe8K

B	Recombinant <i>E. coli</i> strains	Properties
	Nissle-E	<i>E. coli</i> Nissle 1917 with pLE7
	Nissle-SE	<i>E. coli</i> Nissle 1917 with pLS5LE7
	Nissle-SDE	<i>E. coli</i> Nissle 1917 with pLS5LDE7
	Nissle-3SED	<i>E. coli</i> Nissle 1917 with pLS5LDLE73

Fig 4.9 Tables describing the host cells carrying different types of constructs.

To demonstrate the effectiveness of our customized construct on the planktonic *P. aeruginosa*, coculturing of engineered EcN with In7 carrying constitutive GFP expression was conducted at three different ratios of EcN to In7 at 1:1, 3:1, and 4:1. Control used was Nissle-E; EcN, which contained plasmid with E7 lysis gene. RFU was measured after 5 hours, which correlated with the survival of In7. Out of the three ratios, 1:1 and 3:1 gave better gauge of the effectiveness of engineered EcN on In7 (Figure 4.10). Higher ratio may not reflect a better or true effect of the engineered *E. coli*. This coincided with the reports of probiotic EcN having inherent property of outcompetition with other bacteria (Hancock, Dahl, & Klemm, 2010; Leatham et al., 2009). Hence, with more EcN introduced, it would be examining the inherent EcN effect of outcompeting In7.

Fig 4.10 Characterization of killing effect of engineered *E. coli* on planktonic *P. aeruginosa*. 200 μ l of the co-cultured engineered EcN with In7 was aliquot and read after 5 hours. Different ratios of different engineered EcN to In7 (1:1, 3:1 and 4:1) were mixed. RFU released from the constitutive expression of GFP from plasmid in *P. aeruginosa* at time t (h) over the RFU from GFP plasmid in *P. aeruginosa* at time t=0h. Both ratios of 1:1 and 3:1 gave the best contrasting result of control with samples (Nissle-SE, Nissle-SDE, and Nissle-3SED).

To complement the RFU result, CFU of the In7 after coculture was examined to determine the killing efficiency of the engineered EcN for a period of 48 hours. The results obtained from CFU of the samples were normalized to the control Nissle-E. A general trend of sigmoidal curve was observed. Generally, the engineered EcN performed consistently for all the three constructs at ratio 3:1 but not at ratio 1:1. However, Nissle-3SED performed consistently efficiently, ranging from 50% to 90% killing efficiency for both ratios (Figure 4.11). This made Nissle-3SED performed as

good as, if not better than, Nissle-SE even with additional DspB being expressed throughout the 48 hours period of coculturing EcN with In7 (Figure 4.11).

Fig 4.11 Validation of engineered *E. coli* on planktonic In7. 20ul of the co-culture was aliquot at every 12 hours and quantified by CFU. *P. aeruginosa* contained chloramphenicol plasmid which allowed selection against all the engineered EcN strains to obtain % survival of In7 relative to surviving In7 from treatment with control.

Furthermore, from Figure 4.11 and the reported configuration of the genes that could affect protein expression level (Chang et al., 2009; Schoner et al., 1986) (US Patent no: 6,060,273), in our case, the lysis, S5, and DspB protein expression levels would be affected depending on the position we placed the genes. Hence, we suspected that the reason why Nissle-3SED possessed greater killing efficiency than Nissle-SDE could be because of the earlier onset of lysis by Nissle-SDE, hence less total annihilation protein accumulation in Nissle-SDE than Nissle-3SED. To validate this, we investigated the engineered EcN growth upon artificial induction with synthetic AHL. As expected, Nissle-3SED (2 hours) has delayed lysis of 1 hour compared with Nissle-SDE (1 hours) (Supplementary Figure). Hence, we have shown the importance of the configuration of the genes that could influence the protein expression level.

4.3.5 Biofilm disruption and viability assay

Besides targeting planktonic In7, our other aim was for our engineered EcN to also target the persistent biofilm In7. Biofilm is held together and protected by a matrix. Before biofilm was formed, planktonic bacteria would adhere to a surface, thereafter producing a polymer matrix (Dunne, 2002). Hence, to have a detailed evaluation of our best engineered EcN against biofilm In7, investigation of its effect on biofilm inhibition would be crucial besides validating its effect on mature biofilm detachment and also the cells within the biofilm. To further support the reduced *P. aeruginosa* cells present in coculture condition after prolonged incubation (Figure 4.11), confocal images of the resulting biofilm were taken (Figure 4.12). As expected from our previous study (Saeidi et al., 2011a), markedly reduced and sparse biofilm formation was observed in cocultured sample, which supports that our engineered EcN (Nissle-3SED) could inhibit biofilm formation besides planktonic kill. The difference was marginally observed with Nissle-SE, more biofilm spread out than Nissle-3SED (Figure 4.12). This is expected as the primary mechanism of Nissle-SE solely depends on the decreased availability of resultant viable *P. aeruginosa* cells to proceed into forming biofilm.

Fig 4.12 Co-cultured EcN and In7 were also used to examine the engineered EcN effect on In7 biofilm inhibition. Nissle-3SED with DspB showed a greater extent of biofilm inhibition than Nissle-SE without DspB.

On the other hand, when mature biofilm (24 hours) was treated with Nissle-3SED, the coupling effect of S5 pyocin with DspB caused more noticeably sparse and thinner biofilm as compared with S5 pyocin alone (Figure 4.13A). Evaluation of the mature biofilm detachment and its killing efficiency on the biofilm viable cells protected by the biofilm was carried out via confocal microscopy, crystal violet (CV) staining in 96-well microplate, and CFU. To evaluate our engineered EcN on mature In7 biofilm, instead of coculturing EcN cells with the preformed In7 biofilm, supernatant of the induced engineered EcN was used to treat In7 biofilm prior to CV staining. This was done because of reports suggesting EcN has high capacity to colonize and form biofilm, which may make it hard to differentiate *P. aeruginosa* from EcN biofilm (Hancock, Witsø, & Klemm, 2011). The highest rate of In7 biofilm detachment was observed for biofilm treated with Nissle-3SED that expressed both S5 and DspB (Figure 4.13B). Approximately 40% (less than half) of biofilm matrix remained after treatment with Nissle-3SED compared with control. However, it is interesting to observe that the Nissle-SE, which only expressed S5 pyocin alone without DspB, was able to cause considerable detachment of the biofilm (Figure 4.13A and B) compared with the control EcN.

Fig 4.13 (A, B) In7 mature biofilm disruption was examined with the engineered EcN treating the mature biofilm developed after 24 hours. (A) Nissle-3SED gave the best confocal microscopy image and (B) qualitative disruption of biofilm matrix which was quantified by crystal violet staining.

After validation of our engineered EcN on the biofilm matrix, we desired to confirm its effect on the biofilm viable cells residing within the biofilm. This is because biofilm viable cells would lead directly to the next stage of biofilm formation, which is the production of polymer matrix with the biofilm matrix formed once again (Dunne, 2002; Woodworth, Tamashiro, Bhargave, Cohen, & Palmer, 2008). Hence, it was essential and desirable to show the effectiveness of our engineered EcN from biofilm inhibition to disrupting biofilm matrix and lastly eliminating the biofilm viable cells residing within the biofilm to give a comprehensive coverage of the effect of Nissle-3SED on In7 biofilm. To evaluate its effect on biofilm cells, instead of staining the biofilm matrix after treatment with engineered EcN, the resultant biofilm matrix was subjected

to sonication for the release of residing cells within the biofilm. The released cells were counted (CFU) after being subjected to antibiotic selection to select for *P. aeruginosa* cells from possible residue EcN cells. Nissle-3SED provided the best killing efficiency with *P. aeruginosa* cells reduced to only ~20% (Figure 4.14).

Fig 4.14 Percentage (%) of surviving In7 biofilm cells residing within the biofilm matrix was characterized and compared with the control. Nissle-3SED with DspB allowed the less surviving biofilm viable cells than Nissle-SE without DspB.

4.3.6 Characterization of the effect of final annihilation-lytic-AHL responsive

EcN on In7-infected *C. elegans*

The *C. elegans* strain, AU37, was used as an *in vivo* model to provide a more relevant validation of the therapeutic performance of our engineered EcN against clinical In7 in the *in vivo* settings. There were reports suggesting that numerous physiological responses that were observed in *C. elegans* were also observed in higher-order organisms like humans because of the high homology of the genes from *C. elegans* to human genes (Darby, Cosma, Thomas, & Manoil, 1999; Leung et al., 2008; Tan et al.,

1999). Figure 4.15 showed the outline of the methodology from the culturing of *C. elegans* to infection with In7 and treatment with engineered EcN.

Fig 4.15 Schematic describing the methodology from culturing of *C. elegans* to infection with In7, followed by treatment with engineered Nissle-3SED before subjected to counting of survival *C. elegans*.

To study the therapeutic effect of engineered EcN on In7-infected *C. elegans*, worms were counted before and after 24 hours of treatment, and the microscopy images of *C. elegans* were taken. The counting of worms was used to gauge the percentage of *P. aeruginosa*-infected *C. elegans* that were able to survive after treatment (Figure 4.16A), while microscopy images enabled a view of *P. aeruginosa* colonized in the gut of *C. elegans* before and after treatment (Figure 4.16B). Our engineered EcN, Nissle-3SED, was able to effectively prevent ~30% of the total number of infected *C. elegans* from death (Figure 4.3.16B), which was the highest amount of the control OP50 and EcN. OP50 was the common food source for *C. elegans* (Brooks, Liang, & Watts, 2009; So, Miyahara, & Ohshima, 2011).

Fig 4.16 (A) Percentage (%) survival of *P. aeruginosa*-infected *C. elegans* with engineered EcN. Nissle-3SED gave the best treatment for infected *C. elegans* compared to the control EcN and *E. coli* OP50 which was the food source for *C. elegans*. (B) Fluorescence imaging of *P. aeruginosa* colonized *C. elegans* gut and subsequent clearance due to the engineered EcN.

4.4 CONCLUSION AND FUTURE WORK

In this study, we aimed to extend the idea of engineering a clinically relevant and safe probiotic strain that has the capability to target both life-forms of a clinical isolate *P. aeruginosa*, In7, in planktonic and biofilm states. Toward this aim, we designed the genetic circuit with the production and release of anti-In7 proteins, which were triggered upon detection of AHL, which is secreted by In7. The host strain used was *E. coli* Nissle 1917 (EcN), which has a beneficial property of easy genetic manipulation; and being Gram-negative bacteria like *P. aeruginosa*, the quorum sensor (*lasQS*) adopted from *P. aeruginosa* can be easily translated. However, we concurred with previous reports that the expression level between different host strains, regardless of the same species, would vary. In our study, *E. coli* EcN shows up to a fivefold reduction in expression level compared with *E. coli* Top10. Hence, we have reconstructed the backbone of the design, the *lasQS*, to cater for the optimal inducible expression in EcN, with an additional feature of minimal metabolic burden to EcN.

To enable engineered EcN to function as a single agent to target both life-forms of In7, DspB was incorporated into the design besides free-moving In7-targeting pyocin S5. We have proven, for the first time, that DspB in supernatant of EcN was able to cause 30% reduction in mature In7 biofilm with as low as 1.25 µg/mL of total protein containing DspB. Its activity was robust, which increased linearly with time. We have also demonstrated that our final annihilation-lytic-AHL responsive EcN (Nissle-3SED) effectively killed planktonic In7 using a ratio (EcN to In7) as low as 1:1, with killing efficiency of 50% and 90% for a 3:1 ratio. The additional DspB protein production does not hinder its effect on planktonic In7. As expected, from the confocal images of the effect of Nissle-3SED on biofilm inhibition and mature biofilm matrix disruption, significantly sparser and thinner biofilm is shown. Only ~40% of the mature biofilm

remained after the treatment with Nissle-3SED. Moreover, biofilm viable cells were significantly reduced to only ~20%. Finally, to validate the efficacy of Nissle-3SED against clinical isolate In7, an *in vivo* *C. elegans* model was used to simulate the mammalian gut environment. A total of 30% of In7-infected *C. elegans* were revived, which is two- and threefold of the survived *C. elegans* treated with OP50 and EcN, respectively.

Taken together, our engineered probiotic was able to target both life-forms of clinical isolate *P. aeruginosa* and has advanced this therapeutic application further in *C. elegans* model that simulated the mammalian gut environment, which was proven to be successful. We envisioned that a similar approach would be possible to eradicate other species besides *P. aeruginosa* by changing the quorum sensing and annihilation module specific to the targeted species in the gut. Alternatively, the platform of the customized AHL quorum sensor in EcN could be utilized and incorporated to other genes for other biotechnology applications.

CHAPTER 5

Reprogramming microbes to be a pathogen-seeking killer

5.1 INTRODUCTION

Pseudomonas aeruginosa is currently among the leading causes of nosocomial infections due to its inherent resistance towards many antibiotics and antimicrobials. This resistance is likely mediated by the presence of effective multi-drug efflux systems, the ability to rapidly acquire drug tolerance, and notably, the biofilm formation (Spoering & Lewis, 2001). The biofilm matrix is mostly composed of extracellular polymeric substances that can act as a barrier to limit the accessibility of the antimicrobials to the cells, while providing the reservoir of nutrients for the biofilm cells. Therefore targeting the biofilm structure is potentially the primary key towards fighting pathogenic *P. aeruginosa*.

To achieve both antimicrobial and antibiofilm activities, a combinatorial approach comprising of purified biofilm degrading enzyme and antibiotics for synergistic effect are proposed (Anderl, Franklin, & Stewart, 2000; Schirmböck et al., 1994; Skurnik & Strauch, 2006). However, a single agent that is capable of targeting 2 growth states (planktonic and biofilm states) of *P. aeruginosa* has yet been reported. This is an important characteristic of a comprehensive pathogen killing agent as it is uncommon to find *P. aeruginosa* existing as solely in planktonic state or biofilm state when *P. aeruginosa* is lethal. There exists an equilibrium of proportion of *P. aeruginosa* in planktonic or biofilm state, depending on the environmental condition (Barraud et al., 2006). Alternative therapeutic approaches emerged due to the main reason of the inability of antibiotics to penetrate the resistant biofilm state of pathogens. A synthetic biology driven engineering of bacterial cells to fight human pathogen is one such approach that is gaining wide interest as a novel therapeutic strategy to address the

concerns of antibiotic resistance of *P. aeruginosa*. Despite the advances demonstrated in specific targeting ability, one notable limitation that is inherent in the design is the method used to expose the *P. aeruginosa* cells to the antimicrobial compounds. Currently, most of the engineered cells employ either secretion or lysis-mediated release (Ebendal et al., 1994; Gidijala et al., 2009; Gupta et al., 2013; Le et al., 2011; Widmaier et al., 2009) , thereby being highly dependent on the molecular diffusion to reach the targeting cells. A potential solution is an anti-bacterial system that migrates towards the targeting cell upon detection to localize the release of both antimicrobial and antibiofilm compounds for an effective killing of the pathogen.

Therefore, we propose to engineer *E. coli* cells that specifically recognize and mediate targeted cellular migration to localize at a closer proximity to *P. aeruginosa* PAO1 for an efficient cell killing activity. The advance of this work over other pre-existing synthetic biology driven antimicrobial strategy is in 2 ways. First, this approach addresses both planktonic and biofilm of the PAO1 as engineered *E. coli* will be secreting antimicrobial peptide, Microcin S (MccS) and biofilm degrading enzyme, bpDNaseI for efficient and sustained killing activity. Second, this work undertakes a novel approach of reprogramming chemotaxis response of killer *E. coli* cells to selectively swim towards PAO1. This selective motility augments the activity of secreted molecules from the killer *E. coli* as the source of secretion will be moving closer towards the targeting cells. This will create a higher chance of PAO1 cells to be exposed to the secreted molecules by motile killer *E. coli* compared to non-motile *E. coli* that relies on radial diffusion of the molecules for cell killing effect.

Specificity in recognizing the presence of *P. aeruginosa* is achieved by detecting N-3-oxododecanoyl homoserine lactone (3OC₁₂HSL), a quorum-sensing (QS) molecule produced uniquely by *P. aeruginosa*. The detection is followed by an array of

downstream protein expression by implementing a characterized QS device from our previous study (Saeidi et al., 2011b). In this study, a novel antimicrobial peptide MccS (Zschüttig et al., 2012) and nuclease DNaseI were placed under the QS device for cell killing and biofilm degradation, respectively. Nuclease was chosen to destabilize the biofilm as several studies have shown that exogenously added DNases can inhibit biofilm formation, detach preformed biofilms and sensitize biofilm bacteria to biocide killing (Alipour, Suntres, & Omri, 2009; Arciola, Montanaro, & Costerton, 2011; J. B. Kaplan, 2009; J.B. Kaplan, 2010; Nijland, Hall, & Burgess, 2010). We showed that through addition of secretion-tag, YebF, the secretion of the killer proteins were enhanced, accompanied with a more robust PAO1 cell killing (G. Zhang, Brokx, & Weiner, 2006).

Furthermore, we reprogrammed the chemotaxis signaling pathway of *E. coli* to swim towards *P. aeruginosa* by expressing the major determinant of chemotaxis, CheZ (Barraud et al., 2006). Modulation of CheZ expression can be employed to modify chemotaxis behavior as chemotaxis is sensitive towards the ratio between CheZ and CheY (Scharf, Fahrner, & Berg, 1998), a response regulator responsible for inducing cell tumbling when being phosphorylated. CheZ promotes smooth swimming by acting as an antagonistic phosphatase for CheY. There are several studies that places CheZ under an inducible expression system to reprogram chemotaxis behavior in *cheZ*-deleted strain through a process known as pseudotaxis (C. Liu et al., 2011; Sinha, Reyes, & Gallivan, 2010; Topp & Gallivan, 2007). Weiss team rewired motility protein, MotB, to be responsive towards AHL (Weiss et al., 2008). However, MotB has been shown to be involved in controlling motor speed (Che et al., 2008; Nakamura et al., 2009) but not in the cascade of reactions controlling the directional movement like CheZ.

Herein, we demonstrate that by coupling CheZ expression to QS device, reprogramming of *E. coli* to localize towards *P. aeruginosa* is achieved. These cells migrate preferentially up 3OC₁₂HSL concentration gradient, thus acquiring the unique ability to selectively swim towards PAO1 cells. In addition, we demonstrate that localization of these motile *E. coli* at closer proximity to PAO1 cells exhibit refined antimicrobial and antibiofilm activities.

5.2 MATERIALS AND METHODS

5.2.1 Strains and growth conditions

All bacterial cells were maintained in LB broth (BD) with the appropriate antibiotic(s) supplemented in all experiments and incubated at 37°C in a shaker at 225 rpm for all growth experiments. Ampicillin (100 µg/ml) and/or kanamycin (35 µg/ml) were added to the culture media for antibiotic selection where appropriate. *E. coli* Top10 was used for cloning, while the characterisation and expression was done in UU2685 unless otherwise stated. UU2685 is a CheZ deletion mutant, (*cheZ* Δ 4-211), derived from a wild-type chemotaxis strain, RP437 (Parkinson, 1978). This was a kind gift from Prof Parkinson, University of Utah. GFP reporter plasmid pMRP9-1 and chloramphenicol-resistant plasmid pAWG1.1 were transformed into PAO1 using a method described before (K.-H. Choi, Kumar, & Schweizer, 2006).

5.2.2 Plasmid construction

YebF and CheZ was PCR amplified from *E. coli* MG1655 genome. The codon-optimized sequence for *mcsS*, *mcsI* (microcin S immunity) and Bovine pancreatic DNaseI were synthesized (GeneArt, Germany) and introduced into Bglbrick vector for expression. The genetic constructs developed in this study were assembled using standard synthetic biology protocols (J Christopher Anderson et al., 2010). Briefly, for front insertion of Bglbrick parts, purified insert and vector plasmids were digested with EcoRI/BamHI and EcoRI/BglII respectively. For back insertion to upstream vector, the insert and vector plasmids were digested with BglII/XhoI and BamHI/XhoI 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. All plasmids used with the relevant genes are summarized in Table 5.1

Table 5.1 With the exception of CheZ, all proteins were expressed under strong ribosome binding site (RBS) sequence (Biobrick registry; BBa_B0034). CheZ was placed under a weak RBS (BBa_B0033).

Plasmid Name	Description of gene expressed	Origin of replication; Antibiotic resistance
pE8k-RFP	Constitutive LasR and RFP	ColE1; Kanamycin
pS8a-pLasI-CheZ	pLas controlled CheZ	SC101; Ampicillin
pS8a-pLasI-CheZ-SsrA	pLas controlled CheZ-SsrA	SC101; Ampicillin
pS8a-pLasI-CheZ-YbaQ	pLas controlled CheZ-YbaQ	SC101; Ampicillin
pE8k-pLasI-McsSH ₆	Constitutive LasR, pLas controlled McsS-His	ColE1; Kanamycin
pE8k-pLasI-yDnaseI	Constitutive LasR, pLas controlled YebF-bpDnaseI	ColE1; Kanamycin
pE8k-McsI-pLasI-yMcsS	Constitutive LasR and McsI, pLas controlled YebF-McsS	ColE1; Kanamycin
pE8k-McsI-pLasI-yMcsS-yDnaseI	Constitutive LasR and McsI, pLas controlled YebF-McsS and YebF-bpDnaseI	ColE1; Kanamycin
pS8a-pBAD-GFP	pBAD controlled GFP	SC101; Ampicillin
pS8a-pBAD-GFP-SsrA	pBAD controlled GFP-SsrA	SC101; Ampicillin
pS8a-pBAD-GFP-YbaQ	pBAD controlled GFP-YbaQ	SC101; Ampicillin

5.2.3 Motility assays

Agar medium was prepared (tryptone broth with 0.25% agar) and poured into petri dishes (85 mm dia). Prior to pouring, various concentrations of 3OC₁₂HSL (AHL) from aqueous stock solution was mixed for even distribution to induce expression of CheZ. Diluted cell suspensions from mid-log-phase cultures (5 µL, OD 0.5) were applied at the centre of the plate and the diameter of distance travelled was measured (outer ring).

For spatial localization, the agar medium was prepared, and solutions of AHL (100 nM)

or supernatant of PAO1 cell cultures, or tryptone broth alone, were applied in the pattern shown in Fig 5.5 by spotting with a micropipet (2 μ L/5mm), and the plates were air-dried for 30 min. Again, the diluted cell suspensions (5 μ L, OD 0.5) were applied at the location shown in Fig 5.5, the plates were dried in air for 15 min, and incubated overnight at 30 °C.

5.2.4 Protein secretion, purification and concentration measurement

For protein purification, 1L of *E. coli* cells with recombinant plasmid (pE8k-pLasI-McsSH₆) were grown to an OD₆₀₀ 0.8 and their expression was induced fully by 1 μ M exogenous 3-oxo-dodecanoyl-homoserine lactone (3OC₁₂HSL, Sigma Aldrich) for 2h at 37°C. Subsequently the cells were pelleted by centrifugation at 6000g for 20 minutes and homogenized using Emulsiflex-C3 homogenizer (Avestin, Inc). Purification of McsSH₆ was achieved using nickel affinity column, washed with 50 mM imidazole and eluted with 500 mM imidazole in PBS with 10% glycerol. The eluate was concentrated by ultrafiltration using a molecular mass cut-off membrane (Amicon Ultra-15 Centrifugal Filter Unit, Millipore). As the estimated size of the protein is approximately 12 kDa, first concentration was done using 30 kDa cut-off membrane where the flow-through was collected and subsequently passed through 5 kDa cut-off membrane. The resulting concentrate was quantified using Bradford assay and for subsequent use. Extracellular medium containing the secreted YebF-MccS was collected after 3h post-induction with AHL (1 μ M) and also concentrated using Amicon Ultra centrifugal filter units.

5.2.5 Protein electrophoresis and western blotting.

Proteins were separated on 17% SDS-PAGE gel and then transferred to a nitrocellulose membrane for immunoblotting. Trans-Blot[®] SD Semi-Dry Transfer Cell

from BIO-RAD was used to transfer the gel, which was run for 60 min at 150V in Tris/Glycine electrophoresis buffer. To detect the histidine tag, HRP-linked anti-6x His tag antibody (ab1187, Abcam) was used. Chemiluminescence detection system (ECL; Pierce Biotechnology) was used according to manufacturer's instructions for developing the membrane.

5.2.6 Cell killing assay

Optical density (600 nm) readings of PAO1 cells were taken using a Biotek Synergy HT Multi-Mode plate reader set to maintain 37°C at regular intervals over the course of the experiment. Total culture volume of purified MccSH₆ or YebF-MccSH₆ in a well of a 96-clear-bottom microtiter plate (Falcon, Oxnard, CA) was 150µl. For long experiments, gas-permeable adhesive film (BREATHseal, Greiner bio-one) was applied to the plate to prevent evaporation of the culture. Cell viability was determined by quantifying colony-forming units (CFU) after designated treatment with antimicrobial peptide.

5.2.7 Co-culture Assay

P. aeruginosa PAO1 expressing GFP and corresponding *E. coli* cell cultures were diluted and mixed to a final OD₆₀₀ of 0.015 PAO1 and 0.015/0.075/0.150 *E. coli* to set-up cell ratio of 1, 5 and 10 respectively. Cell cultures were then incubated at 37°C with agitation. Triplicates of 200 µL cultures were aliquoted at 0h, 8h, and 12h time points into 96-well clear bottom microtiter plate (Falcon, Oxnard, CA), and GFP fluorescence (ex: 485nm, em: 540nm) were measured as relative fluorescence unit (RFU) using the Biotek Synergy HT Multi-Mode plate reader. In addition, colony-forming unit (CFU) was measured at 12h time point. Briefly, 10-times serial dilutions were performed on the cell cultures before spotting onto selection plates and were subsequently incubated

overnight at 37°C. Colonies were counted and the CFU derived.

5.2.8 Detachment of mature *P. aeruginosa* biofilm by the secreted biofilm enzyme

The wells of a 96-well microtiter plate were filled with 150 µl of medium containing a single cell suspension of PAO1 cells at OD₆₀₀ of 0.05 and incubated at 37°C for 24 hours with pegs of polystyrene microtiter lid (catalog no. 445497; Nunc TSP system) immersed for bacterial biofilm formation.

The peg lid was rinsed with PBS, followed by immersing into microtiter plate with *E. coli* expressing YebF-bpDNaseI with or without YebF-MccS (200 µl/well), and the plates were incubated for 4, 8, and 16 h. The peg lids were washed with PBS for 3 times and the biofilm remaining attached to the surface were stained with crystal violet (0.1% w/v), rewashed under running tap water and dried. The amount of biofilm mass was quantified by destaining the biofilms for 20min with 200 µl of 95% ethanol and then measuring the absorbance of the crystal violet solution at 595nm.

Percentage biofilm survival was assayed after 16 h of incubation with *E. coli* cells. The peg lid was rinsed and recovered in fresh LB by sonication and centrifugation (810g, 20 min)(Moskowitz, Foster, Emerson, & Burns, 2004) and quantified by CFU count on chloramphenicol-selection plate (100µg/ml). The final CFU values were normalized to the control (PAO1 biofilm) and percentage survival after the *E. coli* treatment with respect to the control was graphed.

5.2.9 Transwell assay to assess the final *P. aeruginosa*-specific 'Seek and Kill' system

1ml of supernatant of PAO1 cell cultures was added into the bottom compartment of the transwells (3 µm porosity; Corning) in 6-well plates. Agar medium was prepared (tryptone broth with 0.25% agar) and poured into the top compartment of transwells

with a depth of 5mm. Diluted cell suspensions from mid-log-phase cultures (200 μ L, OD 0.5) was applied at the centre of the media agar. The plates were then incubated statically at 30°C for 16 h. The relative fluorescence unit (excitation 540nm, emission 600nm) of the reprogrammed *E. coli* in the bottom compartment was assessed as a marker of cell motility.

For killing assay, 300 μ L of PAO1 cell cultures (OD 0.002) was added into the bottom compartment of the transwells in 24 well plates. Agar medium was prepared and poured into the top compartment of transwells with a depth of 5mm. Diluted cell suspensions from mid-log-phase cultures (150 μ L, OD 1.5) was applied at the centre of the media agar. The plates were then incubated statically at 30°C for 16 h. The colony forming unit (CFU) of the PAO1 in the bottom compartment was assessed to reflect killing efficiency by the reprogrammed *E. coli* cells. A complementary killing assay which reflected the percentage of dead cells was tested. The treated PAO1 in the bottom compartment was stained with LIVE/DEAD BacLight kit (Invitrogen) according to manufacturer's instructions. Measurements of the fluorescence was done using microplate reader with detection of green fluorescence (excitation 485nm, emission 540nm) and red fluorescence (excitation 485nm, emission 645nm) that indicate live and dead cells, respectively. To assess the efficacy of the reprogrammed *E. coli* on the mature PAO1 biofilm and the viable biofilm cells, 1ml of PAO1 cells at OD₆₀₀ of 0.05 was added into each well and incubated at 37°C for 24 h before replacing the cell suspension with 300 μ L of PAO1 cell cultures (OD 0.002) and treated as the setup above at 30°C for 16 h. The total OD_{600nm} of the *E. coli* introduced was 0.05, 0.1, 0.225 respectively. CV staining was done on the treated PAO1 at the bottom of the transwell to assess the biofilm disruption of our

reprogrammed *E. coli*, while CFU was done to deduce the number of viable PAO1 biofilm cells after treatment. Control used was the untreated PAO1.

5.3 Results and Discussion

Overall scheme of the strategic approach for this study is divided into 2 modules, which are outlined in Figure 5.1. In the presence of QS molecule, AHL, the motility and killing modules will be expressed. The CheZ-mediated chemotaxis is initiated to allow *E. coli* to swim up the concentration gradient of AHL, thereby localizing the cells closer to *P. aeruginosa* PAO1 (*motility* module). Furthermore, the secretion of antimicrobial peptide (MccS) with antibiofilm enzyme (DNaseI) mediates ‘2-hit killing’ by targeting both planktonic and biofilm states of PAO1 (*killing* module).

Fig 5.1 Directed chemotaxis-guided motility of *E. coli* upon induction by AHL - reprogrammed ‘Seek and kill’ system in *E. coli*. In the presence of quorum sensing molecule (AHL) from *P. aeruginosa*, reprogrammed *E. coli* cells express CheZ to swim to towards the pathogen. Further gene expression is initiated in *E. coli* to secrete antibiofilm (DNaseI) and antimicrobial peptide (MccS) to degrade biofilm and kill planktonic or biofilm-residing cells that are released by the biofilm degradation.

5.3.1 Directed chemotactic motility of *E. coli* towards *Pseudomonas aeruginosa* PAO1

When *E. coli* lacks a single gene in the signalling pathway (strain UU2685, $\Delta cheZ$), the cells tumble incessantly and are essentially non-motile (C. Huang & Stewart, 1993). With this strain, we first addressed whether PAO1-dependent motility can be re-established by expressing CheZ in response to AHL. Therefore, *cheZ* gene was introduced in UU2685 under the regulation of a LasR-AHL activator responsive promoter, pLasI. As numerous studies reported that over-expression of CheZ abolishes chemotaxis (C. Huang & Stewart, 1993; Scharf et al., 1998), a range of expression level required for motility needed to be carefully regulated. To this end, a degron was employed to destabilize CheZ to reduce basal activity and broaden the responsive range of inducer concentration. Degrons are short amino acid sequences that are specifically degraded by the ClpXP or ClpAP complexes, resulting in an efficient degradation of the fused protein (Flynn, Neher, Kim, Sauer, & Baker, 2003; Shin & Noireaux, 2010). The level of destabilization was first characterized with GFP (Fig 5.2) and subsequently fused with CheZ.

Fig 5.2 Characterization of the efficiency of the degradation tags on GFP expression. (A) GFP expression by the different inducer concentration after 16 hours with degradation tags. (B) Time course of GFP expression with and without degradation tag was compared. GFP tagged with YbaQ was most efficient in lowering the steady-state level of GFP across the range of inducer concentration.

After verification of the functionality of the degradation tags, we wanted to validate its effect on CheZ and hence the workable range of AHL concentration and the tightness of control of CheZ in the circuit design. It has been shown for the *lasQS* sensor which was previously constructed that beyond 10^{-8} M AHL the expression of proteins was similar (Lo, Tan, Hwang, & Chang, 2013) (Fig 3.5). Hence, to demonstrate the range of concentration of AHL that our AHL-dependent motility cell responded to, 10^{-6} , 10^{-9} and 10^{-10} M AHL (equivalent to negligible concentration of AHL) was used to induce CheZ expression. Figure 5.3 A demonstrated that addition of the degon to CheZ resulted in a tight regulation of basal expression, while maintaining specific motility upon AHL induction. No movement was observed for the negative control, Δ CheZ with and without AHL (Fig 5.3 A). CheZ, CheZ-SsrA, CheZ-YbaQ constructs displayed motility throughout the concentration of AHL tested (Figure 5.3 B). Only CheZ tagged with degon was able to minimise leakiness of expression of CheZ with

similar diameter moved as the Δ CheZ (Fig 5.3 B). However, CheZ-YbaQ construct was the most responsive to the changes of AHL with a tight control (Fig 5.3 B).

Fig 5.3 Directed chemotaxis-guided motility of *E. coli* upon induction by commercial AHL. (A) Migration of Δ CheZ cells (UU2685) expressing various CheZ variants on semi-solid media with or without AHL (1nM). (B) Measurement of migration diameter of CheZ reconstituted cells as a function of AHL concentration when cultured at 30°C

However, the above setup only demonstrated the circuit design was able to response to AHL.

Besides the usage of commercial AHL, native AHL containing supernatant from *P. aeruginosa* was filtered and premixed with soft agar which showed similar motility pattern between the constructs as the commercial AHL (Fig 5.4 A).

Fig 5.4 Migration of CheZ variants expressing cells in the presence of supernatant collected from PAO1 or *E. coli* cell cultures in exponential growth phase.(A) Measurement of migration diameter of CheZ reconstituted cells in supernatant of PAO1 and *E. coli* cell cultures of different dilution. They were cultured at 30°C after 16 hours. (B) The migration diameter was averaged from (A) to obtain migration diameter resulted from supernatant of PAO1 and *E. coli* respectively.

However, although our construct accurately responded in the absence and presence of both commercial and native AHL, this may not necessarily justify that the construct responded specifically to AHL. Thus, another set of experiment was essential to demonstrate the specificity of this motility. Besides AHL, AI-2 secreted by *E. coli* is also a quorum sensing molecule and was chosen to be used to validate the specific motility in this work. To confirm the preferential cell motility in the presence of PAO1 (AHL) supernatant over *E. coli* supernatant (AI-2), PAO1 supernatant was replaced with *E. coli* supernatant (Fig 5.4 A & B) and the migration diameter was measured. As expected, our reprogrammed *E. coli*'s motility was triggered preferentially by AHL (Fig 5.4 A & B). Furthermore, directed motility was also demonstrated when the populations of CheZ-YbaQ expressing cells migrate towards the PAO1 supernatant (Fig 5.5). Therefore, the chemotactic features enabled reprogramming in *E. coli* which allowed the population of cells to migrate towards PAO1 where the source of AHL was.

Fig 5.5 Directed migration of reprogrammable *E. coli* towards PAO1. (i) Diagram of plates containing semi-solid media spotted with PAO1 supernatant and Tryptone (growth media) as outlined. *E. coli* cells were plated at the center as shown and grown for 16h at 30°C. (ii~v) Motility of wild-type *E. coli* strain (RP437), $\Delta cheZ$ deleted (UU2685), re-constituted CheZ, re-constituted CheZ-YbaQ. CheZ-YbaQ showed directed motility compared to the control.

5.3.2 Extracellular secretion and characterization of Microcin S against PAO1

Microcin S (MccS) was selected, as it is a recently identified novel antimicrobial peptide that has shown a great killing efficiency against a wide range of *E. coli* strains (Zschüttig et al., 2012) but its killing effect on *P. aeruginosa* was not clear. With a broad range of killing efficiency and a known immunity gene, it was an ideal candidate to be exploited and tested against PAO1 cells. The bactericidal activity of MccS on PAO1 cells was demonstrated by incubating OD₆₀₀ 0.1 of PAO1 with a range of concentration of purified MccS (Fig 5.6 A & B). For each growth assay, the change in OD during exponential growth phase was compared to the corresponding change in the control and the change in growth rate was graphed (Fig 5.6 B). For the first time, we demonstrated that the purified peptide is highly active against PAO1 with an IC₅₀ of 14.7 µg/mL (Fig 5.6 B).

Fig 5.6 Analysis of antimicrobial activity of Microcin S (MccS) against PAO1. (A) Expression and purification of MccS. Lanes 1–3: UU2685 (host cell) – harboring pE8k-pLas-MccS 6XHtagged -without and with AHL induction, and MccS after purification respectively. (B) Various concentrations of purified MccS were tested against PAO1 and the effect on growth rate was compared.

Subsequently, after validating the effectiveness of MccS on PAO1, we wished to further enhance its killing efficiency while maintaining the host cell to be viable by tagging a secretory tag to our peptide. N-terminal fusion of MccS to YebF was implemented for extracellular secretion. YebF is a small, soluble endogenous protein, which can carry fusion proteins in their active states to the medium, as early as 3 hours after induced expression (G. Zhang et al., 2006). The activity of YebF-MccS in extracellular medium after 3 hours induction remained active against PAO1 cells (Fig 5.7). The extracellular medium was collected and concentrated using MWCO (Molecular Weight Cut-Off) filtration unit to selectively collect proteins within 5kDa to 30kDa range as the estimated YebF-MccS is ~20kDa. The activity of the YebF-MccS in the extracellular medium was demonstrated against PAO1. Approximate IC_{50} of secreted protein was 188 $\mu\text{g/mL}$, which indicated that the addition of the YebF to MccS resulted in approximately 10-fold increase in IC_{50} value. As the extracellular medium was concentrated and selected based on molecular weight only, this value is potentially lower if YebF-MccS is purified further.

Fig 5.7 Characterisation of MccS tagged with secretory peptide, YebF against PAO1. Extracellular expression of YebF-MccS fusion protein after 3 hours of induction with 1μM AHL, was concentrated using molecular weight cut-off centrifuge filtration and assayed for its activity against PAO1. Resulting protein concentration of extracellular media containing secreted YebF-MccS was quantified using bradford assay and corresponding effect on cell growth was assayed. Half maximal inhibitory concentration (IC₅₀) of YebF-MccS against PAO1 cells was determined.

Indeed, we had verified that there was a negative effect of MccS on PAO1 and this effect was further enhanced when YebF was tagged to it. We wanted to further confirm that the negative growth rate observed was due to the death of PAO1 cells but not just slowing the growth of PAO1 cells. Should cell death be occurred, rupture of the cell membrane would happen spontaneously. Propidium iodide (PI), being a big molecule, could selectively enter and stain the dead cells red but not the impermeable healthy cells. Hence, cell killing would be verified by Live/Dead cell viability assay, where PAO1 cells treated with YebF-MccS showed significant proportion of cells stained red, whereas the cells treated with control supernatant were mostly stained with

SYTO 9 dye, which stained live cells green (Fig 5.8). Therefore, the extracellular medium containing secreted YebF-MccS was active and functional which caused significant PAO1 cell death (Fig 5.8).

Fig 5.8 Microscopy images of the effect of YebF-MccS on live PAO1 cells. PAO1 cells expressing GFP after treatment with YebF-MccS and *E. coli* supernatant (control) were stained with PI dye to determine dead cells. PAO1 treated with YebF-MccS supernatant showed significant number of cell death.

5.3.3 Cellular ‘Sense and Killing’ against PAO1 cells

We had proven the effective kill of MccS on PAO1 cells and the workability of the sensor (Supplementary figure). Next, we wanted to verify the functionality of the combined sense-kill (*las*QS-YebF-MccS) system; our cells can autonomously sense the presence of PAO1 cells and then initiate PAO1 cell killing, co-culture of the YebF-MccS secreting *E. coli* with PAO1 was set up. PAO1 constitutively expressing GFP was co-cultured with *E. coli* at the indicated starting cell ratio based on OD value. After 12 hours, the resulting GFP fluorescence and viable PAO1 cells were measured.

Significant reduction in growth rate and cell viability of PAO1 was achieved at equivalent starting OD of *E. coli* cells (Fig 5.9). When converted to actual cell number (Supplementary figure), this ratio demonstrated that our engineered *E. coli* cells showed inhibitory ratio (IR₅₀) of 3 *E. coli* cells per PAO1 cell.

Fig 5.9 Co-culture of YebF-MccS expressing *E. coli* with PAO1 cells at different ratio and the resulting effect on growth rate and cell viability was evaluated. GFP expressing PAO1 was used to co-culture with *E. coli* at the given relative cellular ratio. Growth relative to PAO1 after 12 hours of incubation was calculated by taking the arbitrary GFP fluorescence intensity of co-cultured sample relative to untreated PAO1 with GFP expression. Complementary assay using CFU (Colony Forming Unit) was carried out. YebF Control refers to *E. coli* expressing only YebF protein.

5.3.4 Antibiofilm activity of DNaseI against PAO1 biofilm

Besides killing PAO1 cells in planktonic state, we also wanted to target the antibiotic resistant biofilm state of PAO1. Like, killing protein, MccS, we designed the

antibiofilm protein to be expressed only when PAO1 is present. Hence, in our work, our reprogrammed cells were designed to target biofilm matrix of PAO1 by secreting antibiofilm nuclease, Bovine pancreatic DNaseI only on detection of PAO1. To meet this requirement, similarly, DNaseI was tagged to YebF which was coupled to the *lasQS*. To demonstrate the practicability of the system (*lasQS*-YebF-DNaseI) on mature biofilm, the cell containing *lasQS*-YebF-DNaseI were co-cultured with the mature biofilm of PAO1. Fig 5.10 A & B showed that the expression of DNaseI was sufficiently induced by the AHL produced from PAO1, thereby allowing consistent detachment of biofilm. The extent of biofilm detachment (~50%) was not affected when the cell was expressing both YebF-DNaseI and YebF-MccS simultaneously compared to expressing just YebF-DNaseI (Fig 5.10 A). When the viable biofilm cells were counted, only cells expressing both proteins successfully caused significant reduction to both biofilm mass and viable biofilm cells (Fig 5.10 A). This result was also supported by the confocal image that similar reduction in biofilm matrix was observed for cell expressing both YebF-DNaseI and YebF-MccS compared to cell expressing only YebF-DNaseI (Fig 5.10 B). Host cell without DNaseI could not destroy the biofilm matrix (Fig 5.10 B).

Fig 5.10 Analysis of antibiofilm activity of DNaseI against PAO1. (A) The mature biofilm was incubated with the engineered *E. coli* cells for 16 h, and the resulting biofilm was stained with crystal violet and quantified by taking absorbance reading at 595 nm. Viable biofilm cells were also determined by performing CFU counting after 16 h incubation of mature biofilm with *E. coli* cells. (B) Antibiofilm activity of DNaseI was observed under confocal laser scanning microscopy (CLSM). (i) *Pseudomonas* biofilm with green fluorescence was grown on 8-well chambered glass slide for 48 h, which was subsequently treated with the engineered *E. coli* for 16 h, and visualized under CLSM. Scale bar represents 50 μ m. (ii) Images were reconstructed from biofilm Z-stacks using Image J. Scale bar represents 100 μ m.

As our system employed secretion of effector proteins; MccS and DNaseI, it was anticipated that the efficiency in secretion could be the major determinant of the activity of our reprogrammed cells to ‘seek and kill’. Nonetheless, due to the high activity of the antimicrobial peptide, our cells were able to achieve remarkable biofilm degradation and cell killing that is autonomously induced in the presence of PAO1 cells and mature biofilm.

5.3.5 *P. aeruginosa* targeting motile *E. coli* with ‘2-hit killing’ system – Seek and Kill

We had demonstrated successful reprogramming of chemotaxis towards PAO1 (motility module) and the efficacy of MccS and DNaseI secreting cells (killing module) against PAO1 cells in co-culture system. Hence, the 2 modules were integrated with QS device to create the ‘Seek and Kill’ system in *E. coli* against PAO1. In Figure 5.11 & 5.12, we depict the assembled system comprising sensing and motile killer *E. coli* cells that (i) detect QS molecules emanating from PAO1 cells from planktonic and biofilm state, (ii) migrate towards PAO1 cells/biofilm while also expressing antimicrobial and antibiofilm enzymes and (iii) mediate biofilm disruption and cell killing. In Figure 5a, we have placed *E. coli* with integrated ‘Seek and Kill’ system (CheZ-YbaQ & YebF-MccS & YebF-DNaseI) at the top compartment of a transwell apparatus with agar medium. We first checked for specific bacterial migration towards the targeted pathogen by adding supernatant of PAO1 cells by measuring RFP fluorescence (Fig 5.11) after 16 hours incubation (time used to observe directed motility and both ‘2-hit killing’ activities of engineered *E. coli*). Specific migration of *E. coli* was observed with CheZ-YbaQ expressing cells in the presence of PAO1 culture supernatant, while other CheZ variants have shown similar to basal level of migration.

Fig 5.11 Testing the final construct for efficient QS-mediated motility with biofilm-disrupting and cell killing - Seek and Kill system. (A) Schematic depicts AHL-directed cell motility of the reprogrammed *E. coli* (from the inverted transwell insert) towards the PAO1 cells initially seeded on the bottom of the transwell apparatus. Due to the diffused AHL across the insert, the activated *E. coli* will begin to swim vertically towards PAO1 due to AHL-induced CheZ expression. Subsequently, the expression of MccS and DNaseI for secretion will mediate cell killing and disrupt biofilm matrix. (B) Specific motility of reprogrammed *E. coli* across the transwell (Graph of RFP fluorescence collected from the bottom well).

Once the PAO1 responsive bacterial migration was established in the transwell assay, we wanted to establish that our final circuit design could fulfil sense-see-kill function. To evaluate the performance and behaviour of our engineered *E. coli* with integrated system (sense-see-kill) of the 3 devices combined (*las*QS- CheZ-YbaQ- YebF-MccS- YebF-DNaseI), the cell containing this final circuit was tested against PAO1 mature biofilm, its planktonic and biofilm viable cells. The cell viability of PAO1 cells was measured by antibiotic-selective CFU counting. Red fluorescent cells measured indicated dead cells that had allowed PI stain to permeate. The *E. coli* with the integrated system showed approximately 60% reduction in survival cells while others with either motility (CheZ-YbaQ) or killing (YebF-MccS & YebF-DNaseI) modules alone did not result in significant reduction in PAO1 cell survival (Fig 5.12 A). This

result was complemented with the highest percentage of dead cells (~50%) obtained from the Syto9-PI staining (Fig 5.12 A). After validating our reprogrammed cells were able to cause significant reduction in planktonic PAO1, we need to verify its effect on both the mature biofilm matrix and the viable cells within the biofilm matrix. Similar experiment was set-up, except the planktonic PAO1 cells at the bottom of the transwell was replaced with the mature biofilm PAO1 cells. When our integrated ‘Seek and Kill’ *E. coli* was tested against PAO1 biofilm, a significant 60% reduction in PAO1 biofilm relative to control was observed (Fig 5.12 B). Furthermore, 40% reduction in viable PAO1 biofilm cells was observed by our final ‘Seek and Kill’ system.

Fig 5.12 (A) The viability of PAO1 cells was measured after 16h of incubation with reprogrammed *E. coli* on the transwell insert. LIVE/DEAD staining to reflect cell death was performed, and proportion of PI stained (dead) compared to SYTO9 stained cells treated with *E. coli* expressing CheZ with MccS and DnaseI compared to non-motile MccS and DnaseI was compared. (B) The transwell insert with reprogrammed *E. coli* was seeded on the bottom of the transwell containing mature PAO1 biofilm. After 16h incubation, resulting biofilm and viable biofilm cells was analyzed.

Taken together, our integrated system responded to both planktonic and biofilm PAO1, including the viable cells within the biofilm matrix, and exhibit cell migration and

localization that accentuates cell killing activities of our engineered *E. coli* that effectively result in reduction in PAO1 cells and biofilm matrix.

5.4 CONCLUSION AND FUTURE WORK

In this study, we developed a novel integrated system that was able to sense the AHL produced by both planktonic and biofilm PAO1, and exhibit cell migration and localization that enhanced the cell-killing activities of our engineered *E. coli*₂ which led to significant reduction in planktonic and biofilm viable PAO1 cells as well as biofilm matrix. Our sense-seek-kill system was composed of three devices, namely, motility cheZ-YbaQ device, killer YebF-MccS, and degradation YebF-DNaseI device. To enable these devices expression to be dependent on AHL, pLasI that is activated by AHL-LasR complex was coupled upstream to each device. Our integrated system was able to reduce the biofilm planktonic cells by 60%, degrade the mature biofilm matrix by 60%, and reduce the biofilm viable cells by 40%. Thus, our reprogrammed cell could localize in close proximity to the source of AHL, PAO1, on detection of AHL, and secrete MccS to target the free, swimming PAO1, followed by DNaseI that disrupt the biofilm matrix, which caused the biofilm viable cells within the matrix exposed and vulnerable to attack by MccS like the planktonic PAO1 cells.

In the future, using the above circuit design as a platform, we could target other strains by changing the sensing, killing, and degradation module specific to the targeted strains. *S. aureus* uses AIP as the quorum sensing molecule instead of AHL. To tailor for targeting *S. aureus*, circuit design was needed to incorporate a promoter that could detect AIP (G. J. Lyon, Mayville, Muir, & Novick, 2000). Another important aspect that can be modified is the switching of the receptor domain of the killer molecule that can specifically bind to the targeted strain (Gupta et al., 2013). In this way, the killing effect would be more strain-specific.

CHAPTER 6

Conclusions and Future considerations

Pseudomonas aeruginosa is a prevalent nosocomial pathogen and with the emergence of antibiotic resistant bacteria, the number of methods to eradicate *P. aeruginosa* has become more limited. As much as an average of 7,500 compounds for drug discovery is required for one successful drug. Hence, even to get a successful drug is especially costly and time-consuming, which could take up to 15 years. Because of these limitations, it is crucial to have as many available drugs in the pipeline as possible in pharmaceutical companies. Most of the successful drugs are naturally derived. However, most natural products have a primary role that is for something other than the treatment of human disease. Hence, it may be necessary to fully characterize each natural product for particular treatment before dismissing it as an unsuitable drug. In addition, although a particular drug may be functional, it may not be productive and hence costly. Careful design and optimization are required to improve the properties and productivity of the drug. Numerous attempts have been made by organic chemists with the hope of synthesizing functional pharmaceutical products. However, it is often difficult to synthesize these as they usually have complex structures that require a lot of protection and deprotection steps. Versatility of the biological system enables ease in conversion of affordable resources into high-value products such as drugs to treat diseases and agents to remediate polluted sites.

P. aeruginosa secretes and detects the autoinducers, N-acyl homoserine lactone (AHL) as their primary specific signalling molecule involved in the quorum-sensing mechanism. With its high specificity, I exploited the quorum sensing of *P. aeruginosa* as an inducible regulatory system for desirable protein expression through synthetic biology means.

Therefore, the work described in this thesis aimed to develop *P. aeruginosa* quorum-sensing-based genetic circuits that would enable clinically relevant programmable functionalities in *Escherichia coli*: protein release, directed motility, and pathogen killing. Toward this aim, I have developed three systems with a quorum sensing device as a control system: (1) a *P. aeruginosa* quorum sensing-based genetic circuit that enabled cell density-dependent autoregulatory lysis for the release of macromolecules, (2) a probiotic strain with integrated a *P. aeruginosa* quorum sensing device for specific sensing of *P. aeruginosa* and eventually killing of clinical isolates of *P. aeruginosa*, and (3) a genetic circuit that enables engineered *E. coli* to move distinctly towards *P. aeruginosa* and kill the human pathogen.

In chapter 3, I created a *P. aeruginosa* quorum sensing-based genetic circuit that enabled cell density-dependent autoregulatory lysis for the release of macromolecules. The lysis genetic circuit comprised a three-component transducer switch that employed a carbon-depletion-dependent promoter *csiDp* as the transducer, the *lasQS* device as the switch, and the lytic protein colicin E7 to release the macromolecule of interest. Our engineered cell was able to (i) activate only when the stationary phase was reached, which enabled more cell growth and more products produced before extraction; (ii) control activating cell density through varying a range of glucose concentration (0–0.3%, w/v); and (iii) most importantly, a reliable and constant output (lysis) that was independent of the input signal. Finally, the validation of our genetic circuit design was shown from the concentration and quality of plasmid DNA and GFP extracted. Hence, the possibility of reduction in usage of reagents in the industrial field would be able to give a more economical option compared with utilizing costly equipment and reagents, which were sometimes toxic.

In chapter 4, I constructed a probiotic strain with integrated *P. aeruginosa* quorum sensing device for specific sensing of *P. aeruginosa* and eventually killing of clinical isolates of *P. aeruginosa*. The main aim was to extend the idea to implement a clinically relevant and safely engineered probiotic strain with an antiseptic ability to target both planktonic and biofilm states of In7, a clinical isolate of *P. aeruginosa*. Toward this goal, a circuit design was constructed, which enabled the host cell to produce and release In7-targeting proteins that were expressed when AHL secreted by In7 was detected. *E. coli* Nissle 1917 (EcN) was used as the host cell to perform our desired functions. Our data suggested similar observations as previous reports with that level of expression between different host strains varied. In our study, *E. coli* EcN shows up to fivefold reduction in expression level compared with *E. coli* Top10. To compensate for the reduction in expression level, *lasQS*, which sensed AHL, was reconstructed for optimal inducible expression with minimal metabolic burden. I hope that our engineered probiotics could be multifunctional and target the planktonic and biofilm states of In7. I have proven, for the first time, that as low as 1.25 $\mu\text{g/mL}$ of DspB was able to reduce the mature In7 biofilm by 30%. Its activity increased linearly with time. After validation of the DspB activity on the biofilm state of In7, the final annihilation-lytic-AHL responsive EcN was demonstrated with an effective kill of 50% planktonic In7 even when a ratio as low as 1:1 of EcN to In7 was used. For a 3:1 ratio, the killing efficiency was 90%. For the first time, DspB was expressed concurrently with planktonic In7-targeting, S5 pyocin, and I showed that DspB does not hinder the effect of S5 on planktonic In7 and vice versa. Our genetic circuit was able to show significant biofilm inhibition and mature biofilm matrix disruption from the confocal images, with only ~40% of the mature biofilm remaining after treatment. In addition, only ~20% of biofilm viable cells remained after treatment. Last but not

least, after the *in vivo* *C. elegans* model infected with In7 was treated with our engineered probiotics, 30% of *C. elegans* corresponded to a two- to threefold higher survival rate than the control. Taken together, our engineered probiotic had fulfilled the aim of possessing the capability to sense and target both life-forms of *P. aeruginosa* through the regulation of *P. aeruginosa* quorum sensing system integrated in *E. coli*.

In chapter 5, another novel genetic circuit system was created to sense AHL and kill both life-forms of *P. aeruginosa*. While chapter 4 targets In7, I proved another system that could target another type of *P. aeruginosa*, PAO1. An additional module that allowed cell migration was integrated. Thus, besides sense and kill, our new system could sense-seek-kill. This system comprised three devices, namely, motility CheZ-YbaQ device, killer YebF-MccS, and degradation YebF-DNaseI device. The sense-kill system reduced 60% of the planktonic cells, degrading 60% of the mature biofilm matrix and 40% of biofilm viable cells. All in all, the engineered *E. coli* could localize in closer proximity to PAO1, where the source of AHL, in the presence of AHL, secreted MccS microcin that killed planktonic PAO1, followed by the secretion of DNaseI, which disrupted the biofilm matrix and eventually exposed the viable cells within the matrix, making the biofilm viable cells vulnerable to attack by MccS like the planktonic PAO1 cells.

Future work that could be done for the three systems would be to integrate the genetic circuit from plasmid form into genomic DNA. This could stabilize the gene. Unlike genomic DNA, plasmid DNA would be lost, especially when there is no selective pressure like antibiotics. Besides, integration into genomic DNA can minimize horizontal gene transfer. Thus, this allows more versatility and practicability, especially for cell density-dependent autoregulatory lysis circuit design construct. This

would be further discussed in section 6.1. Although I did not integrate the cell density-dependent autoregulatory lysis circuit design construct into the genomic DNA yet, our design was tested in low-copy plasmid, which was a similarly low copy as genomic DNA. Hence, it should be as stable as genomic DNA.

Another consideration for the sense-kill or the sense-seek-kill system is to encompass a bigger range of pathogens, not just specifically In7 or PAO1. This could be done by changing both the quorum sensor and annihilation modules, depending on the targeted species. For example, if the targeted species is *S. aureus*, the quorum sensor should sense AIP (autoinducing peptides) instead of AHL. This would be further discussed in section 6.2. In addition, the receptor and translocation domain of the killer molecule can be switched such that the bacteriocin can specifically bind to the targeted strain. By changing the receptor and translocation domain and retaining the killing domain, the killing effect would be strain-specific since the mode of action of bacteriocin is from the wall of cell membrane.

Last but not least an improvement can also be made in the pathogen seeking killer system. If this system is to apply in other annihilation protein besides MccS, the random diffusion of the selected protein must be much slower than the motility of the engineered cells. Another area of improvement can be made is that my current engineered *E. coli* migrates the fastest at 10^{-9} M AHL which corresponded to the minimal level that promoter *lasI* can detect (Figure 3.5). Hence, a proposed solution for the above mentioned limitations would be that the promoter controlling the transcription of the motility gene, *cheZ*, could be made weaker such that the ratio of *cheZ* to *cheY* would be kept in balance and minimise the chances of overexpression of CheZ abolishes chemotaxis. Thus, this could further broaden the responsive range of inducer concentration. Furthermore, with faster moving engineered cells, this would

outweigh the possibility of fast random diffusion of annihilation proteins. Another consideration is the concurrent expression of MccS and BpDNaseI in my current system. This simultaneous expression of proteins right after the detection of AHL may not be ideal as they may cause a metabolic burden to the cell growth. By implementing a regulatory system that allows differential expression of the modules depending on the morphological states could allow enhanced antimicrobial activity from an optimal expression. Recombinant protein production could be affected by the morphological state of expression host. This would be further discussed in section 6.3.

6.1 INTEGRATION OF THE NOVEL GENETIC CIRCUITS INTO THE GENOME

The advantages of chromosomal integration include genetic stability due to minimised segregation and horizontal gene transfer. Furthermore, metabolic burden arising from plasmid maintenance in cells could be greatly reduced (Westfall & Gardner, 2011; Yadav, Chaudhari, & Kothari, 2011). More importantly, the system would be antibiotic marker free, rendering more applicable to administration as drugs. The spread of antibiotics and resistance markers could pose danger to the environment as well as cause human allergic reaction (Goh & Good, 2008; Jana & Deb, 2005).

The highly cited paper for reference to construct mutant strains is from the Datsenko and Wanner group (Datsenko & Wanner, 2000). They were able to integrate antibiotic marker and therefore replacing the gene at the targeted site. Therefore, similar strategy could also be employed in the integration of my genetic circuits into the genomic DNA of expression host.

6.1.1 Proposed experimental set-up for chromosomal integration

To minimise overloading of expression in the host cells, non-essential genes that are of little or no relevance to the engineered functionality could be identified through literature searches or through trial and error and examine the resultant mutants' viability and its protein expression. To perform gene knockout, a similar approach to Datsenko and Wanner group could be adopted (Datsenko & Wanner, 2000). Briefly, PCR product can be obtained through amplification using pKD13 with 50 nucleotides or more homology to the targeted site at genomic DNA as the primers (includes 25 nucleotides homology to template). The PCR product is transformed into an

electrocompetent expression host cells containing pKD46 (Amp^R) as the λ -Red recombinase plasmid. After which, screening of mutants could be done through kanamycin resistance selection on the agar plate. For the second selection, streak the selected colonies onto a new LB plate at 37°C and subsequently on ampicillin plate to check for loss of pKD46. A third selection is viable only if there are surviving colonies during the second selection, which is to repeat the second selection but at a higher temperature of 43°C. Screening of the correct mutants would be done by colony PCR with 3 sets of primers. After verification that the antibiotic gene is inserted at the targeted site, the kanamycin marker can be removed through the following method. Transform the temperature-sensitive pCP20 into the mutants and new transformants can be selected at 30°C. pCP20 expresses FLP recombinase which targets the FRT-flanked kanamycin cassette. Selected colonies from the transformants would be cultured in LB liquid medium at 43°C overnight. At 37°C, pCP20 will be cured (this is similar to pKD46), so kanamycin gene can be deleted by recombination and the loss of plasmid would occur simultaneously. Continue to culture the mutants until they lose their harbouring plasmids. Last but not least, verification of the loss of pCP20 (Amp^R) and kanamycin resistance cassette should be checked through streaking on the respective antibiotic plates (kanamycin/ampicillin). A summarised methodology of the creation of mutant is illustrated in Figure 6.1.

Fig 6.1 Schematic illustrating the methodology of gene disruption strategy. H1 and H2 refer to the homology extensions or regions. P1 and P2 refer to priming sites (Datsenko & Wanner, 2000).

Once antibiotic-free mutant is obtained, chromosome integration could be done subsequently using the same λ -Red recombination technique (Datsenko & Wanner, 2000). Alternatively, we can use the integrative vector designed by Zucca for targeted integration site in genomic DNA (S. Zucca, Pasotti, Politi, Cusella De Angelis, & Magni, 2013) or Knock-in/Knock-out (KIKO) vectors for rapid integration of DNA genetic circuits constructed previously (Sabri, Steen, Bongers, Nielsen, & Vickers, 2013).

6.2 QUORUM SENSING SYSTEM IN *STAPHYLOCOCCUS AUREUS*

Fig 6.2 Schematic representation of *agr* quorum sensing system of *S. aureus*. (Martin, Hoven, & Cook, 2008)

6.2.1 Literature review of the role of *agr* in *in-vivo*

The regulation of virulence factors in *S. aureus* is growth-phase dependent and *agr* was shown to be of critical importance for the virulence factors in *S. aureus*. This has been demonstrated in the animal studies. The commonly used strain NCTC8325 with a knockout of *agr* operon was tested to be less virulent compared to its wild type NCTC8325 parent strain in a mouse arthritis model (Abdelnour, Arvidson, Bremell, Ryden, & Tarkowski, 1993). *agr* also occupied an important role in a rabbit endocarditis model, based upon the strain RN6390. The model showed that out of 11

animals, 4 developed endocarditis for *agr* mutant while significantly higher mortality was demonstrated with wild type strain (9 out of 10).

In summary, the experiments of the various animal models had portrayed that *agr* has a prominent role *in vivo*. This was supported by majority of the clinical isolates of *S. aureus* being *agr* positive (K. E. Traber et al., 2008). Usually haemolysis is used as an indication of *agr* positive or *agr* negative. The intriguing finding was the existence of disease-causing strains which were either *agr* deficient or *agr* mutant (Fowler et al., 2004). Traber did a study of 146 clinical isolates. Of which, 15% were non-hemolytic (Katrina E. Traber et al., 2008). Other factors besides *agr* locus could be responsible for these disease-causing strains (Katrina E. Traber et al., 2008). The hypothesis was that *agr* was important only during the early stages of an infection since *agr* null strains isolates were also discovered during the infection.

Wright group used a mouse abscess model to verify that *agr* was only activated a few hours later after the injection of *S. aureus* (Jesse S. Wright, Jin, & Novick, 2005). The activation lasted for a few hours before declining. Surprisingly, *agr* activation was again observed 72 hours after injection. This observation could not be explained with the traditional model for *agr* activation. It was postulated that other environmental factors could have influenced the *agr* response. The factors like oxygen concentration and stress response factor could potentially affect *agr* activation. In a low oxygen concentration environment, staphylococcal respiratory response (*srrAB*) would be induced, thus inhibiting the expression of RNAIII (Pragman & Schlievert, 2004). SarA could also be one of the various environmental stress factors influencing the general stress response factor σ^B which in turn influenced the *agr* response (Ambrose L. Cheung, Chien, & Bayer, 1999). There was a decline in virulence of the murine septic

arthritis model (Jonsson, Arvidson, Foster, & Tarkowski, 2004) but strangely not in a murine subcutaneous skin abscess model (Horsburgh et al., 2002) of a σ^B deletion mutant. Last but not least, reactive oxygen and nitrogen species (Chen et al., 2006) can activate SarX which influences MgrA which is a repressor of *agr* (Truong-Bolduc, Dunman, Strahilevitz, Projan, & Hooper, 2005).

agr operon is comprised of 4 genes; AgrB, AgrD, AgrC and AgrA. AgrB is a transmembrane that processes the AgrD to generate the active AIP. It was hypothesized to consist of six transmembrane domains with four of them being hydrophobic and the rest of the two domains involving in the proteolytic cleavage of AgrD being hydrophilic (Martin et al., 2008) (Figure 6.3).

Fig 6.3 Schematic representation of the AgrB (Qiu, Pei, Zhang, Lin, & Ji, 2005)

The length of AIP (AgrD) varies from 7 to 9 amino acids. There are 4 types of AIP produced by *S. aureus* but the majority produce AIP-I (Gholson J. Lyon, Wright, Muir, & Novick, 2002). AIPs contain a thiolactone that is formed through the condensation

of the alpha-carboxyl group of the conserved cysteine (Gholson J. Lyon et al., 2002). AIP-I and AIP-IV has the greatest similarity with only one amino acid difference (Geisinger, George, Muir, & Novick, 2008) at residue 5 with aspartic acid in AIP-I and tyrosine in AIP-IV. This small difference could have contributed to AIP-IV being also a moderate activator of AgrC1 and AIP-I which is a weak activator of AgrC4 (Gholson J. Lyon et al., 2002). It has been shown that the AIPs derived from the different types of AgrD were all agonists for their cognate AgrCs (Figure 6.4) and cross-inhibited the other AgrC groups except AIP-IV which was surprisingly an activator of AgrC1 but AIP-I was a weak activator to AgrC-IV (Gholson J. Lyon et al., 2002). This phenomenon could be explained by the difference of only one amino acid between the AIP-I and AIP-IV; aspartic acid in AIP-I and tyrosine in AIP-IV. Mdowell's group did a structure-function analysis and found out that when the aspartic acid in AIP-I was substituted by alanine, the AIP-derivative became an inhibitor of all four *S. aureus* agr groups (Mdowell et al., 2001). From Lyon's group, they found out that AIP-I activated AgrC-I at 28nM but was unable to activate AgrC-II & AgrC-III and with lesser sensitivity at 26uM to AgrC-IV & AgrC III-IV chimeric receptor (eg. Sensor domain from one AgrC attached to the histidine kinase domain of a different AgrC without changing the signaling characteristic of the domain). It was suggested that both the activation and inhibition involved different binding orientations within the ligand binding pocket of each receptor.

Fig 6.4 Schematic representation of the AgrC with 2 domains; receptor and kinase. The group I and group II chimeras have the polytrophic sensor domains from the group I and group II receptors, respectively, fused to the group IV receptor HK domain (G. J. Lyon, 2001)

Fig 6.5 Schematic representation of AIP-I biosynthesis (Thoendel & Horswill, 2009)

AgrD is a peptide precursor of AIP. It is composed of three parts, namely the amphiphatic N-terminal, the mature AIP in the middle region and a charged C-terminal (Thoendel & Horswill, 2009). AIP is synthesized in 5 steps as shown above (Figure 6.5). Firstly, N-terminal is bound to the cytoplasmic membrane while the AgrB cleaves the charged C-terminal of AgrD. The remaining portion remained bound to AgrB at

Cysteine-84 of AgrB. Internal cyclization takes place when the AgrD cysteine residue attacks the thio-ester bond, thereby forming the thio-lactone ring. Finally, the AIP is exported out of the cytoplasm where the SpsB which is a type I signal peptidase splices out the N-terminal region and releases the mature AIP from the membrane.

The AgrC was predicted to be composed of 2 domains; the sensor and the histidine kinase (HK) domain (Figure 6.6). It is a helix helical transmembrane with three extracellular loops. Interestingly, both the N and the C-terminals are on the cytoplasmic side. The C-terminal, histidine kinase component is conserved in *S. aureus* while the N-terminal is variable which is responsible for the selective binding of different types of AIP (J. S. Wright, 2004).

Fig 6.6 Schematic representation of the 2 domains of AgrC (Geisinger, Muir, & Novick, 2009)

Unlike in AgrD where the difference between the AIP-I and AIP IV is only a single amino acid, the difference in amino acid for AgrC-I and AgrC-IV is 27 amino acids (Geisinger et al., 2008). From Geisinger paper, the team discovered that I171 is a critical amino acid that could affect the specificity of binding either by directly interacting with the peptides or indirectly influencing the ligand-binding pocket (Figure 6.7) (Geisinger et al., 2009). The other important amino acid, the histidine residue was the active site. On the other hand, the activation of AgrA/C was triggered by the R180W, S183F, T197K, L205R,H amino acids which belonged to the sensor domain, as well as M234L, R238H,C,G, Y241C, Q305H,R,E, which belonged to the histidine kinase domain (Geisinger et al., 2009). When these amino acids were mutated, a constitutive activation was observed. Hence these amino acids were found to be essential in the regulation of the activation of AgrA/C (Geisinger et al., 2009). Besides sensitivity, another important parameter, specificity, was determined by the amino acid in the 2nd extracellular loop (Geisinger et al., 2009). In their earlier work, they discovered that the Y100 & A101 amino acids were not important for AIP-1 recognition but important for AIP-IV (Geisinger et al., 2008). Jensen's group also suggested that AgrC loop two has an important role in AIP-1 and IV differentiation (Jensen, Winzer, Clarke, Chan, & Williams, 2008). However, none of the papers suggested the role of the first loop. Hence, from these, we hypothesized that probably the 1st extracellular loop was redundant.

Fig 6.7 Schematic representation of the hexahelical transmembrane of AgrC and the important amino acids in the 2nd loop (Geisinger et al., 2008)

Another interesting fact is that the binding of the ligand, AIP, to the AgrC is correlated to a 2-state model of R and R* with R* being favored by the activating ligand. This means that the receptor acts in an equilibrium of 2 states (Geisinger et al., 2009).

Response regulators of the two component signal transduction system is usually composed of an N terminal receiver domain and a C terminal output domain. Usually the DNA binding region has a structure of helix-turn-helix or sometimes known as HTH (Nikolskaya & Galperin, 2002). One classic example of the response regulator in *S. aureus* is *lyt R* which is involved in the cell autolysis and *AgrA* is one of the members in the *Lyt R* response regulator family. Upon phosphorylation, conformation changes is induced in the molecule, thereby allowing it to bind to a consensus sequence consisting of a pair of direct repeats and consensus sequence ([TA][AC][CA]GTTN[AG][TG]), that are separated by a 12-13 bp spacer region (Nikolskaya & Galperin, 2002). Similar binding sites were identified in front of *algR* and *virR* promoters which are responsible for the pathogenesis of cystic fibrosis in *P. aeruginosa* and gas gangrene in *C. perfringens*. Such a pair of elements has been identified in the P2 promoter and the P3 promoter from the P2-P3 intergenic region of the *agr* operon and RNAIII. Interestingly, the Koenig group discovered that there was a higher binding affinity of the *AgrA* to the P2 promoter than the P3 promoter (Koenig, Ray, Maleki, Smeltzer, & Hurlburt, 2004) . Therefore it was likely that the P3 activation occurred after the auto-activation of the P2 promoter. Another known regulator, *SarA*, was thought to enhance *agrA*-dependent transcription of both the P2 and P3 promoters, either through interacting with the *AgrA* or by displacing *AgrA* in an unknown mechanism (A. L. Cheung, Bayer, & Heinrichs, 1997). Both *sar* (staphylococcal accessory regulator) and *agr* (accessory gene regulator) are the prominent loci that are involved in the synthesis of virulence factor. It has been proposed that *sar* gene product(s) could have interacted with the promoters of the *agr* operon, preferentially P2 promoter, which induced the transcription of RNAII transcript, followed by the RNAIII transcript. The crystal structure of *AgrA* was

hypothesised to be a 10 strand β -fold that intercalates binds around 16 base pairs of one side of the DNA which can be done by insertion of the long loops into the major grooves for a direct contact to the single base of DNA. This is different from the DNA binding proteins that bind at the major groove. (Sidote, Barbieri, Wu, & Stock, 2008).

6.2.2 Applications of Quorum sensing system in *S. aureus*

Fig 6.8 Schematic representation of the potential QS applications (Choudhary & Schmidt-Dannert, 2010)

Although QS phenomenon is usually observed in large bacterial populations, QS is actually triggered when a certain concentration is reached. Hence, even if there is only a little amount of signalling molecules produced by a few cells, in a small confined volume, the threshold concentration could still be reached and activate QS (Boedicker, Vincent, & Ismagilov, 2009). Hence, QS activation is not restricted only to large population which allows greater flexibility in the usage of the QS for applications.

There is a diverse range of applications of QS which includes the production of biochemical, tissue engineering and mixed species fermentations for the engineered quorum sensing systems (Figure 6.8). Another broad application of quorum sensing system is in designing microbial biosensors to identify the relevant bacterial species

present in an environment. The other interesting application is to quench quorum sensing as quorum sensing is often associated with the initiation of biofilm formation and virulence properties. In gram positive bacteria, activated AgrA initiates the transcription of RNA III which behaves as a regulator of QS and regulates the expression of genes involved in initiation of biofilm formation as well as virulence properties (Geisinger et al., 2009).

The creation of a library of QS system components allows the flexibility of mixing and matching with the host system to create a customized biological circuit (Meyer, 2000). This library can be further increased with mutational studies of auto inducers synthases and its receptors. However, it is important to note the restriction of device possibly triggers undesirable responses in the host organism. Also, promoters deriving from one species may not be functional for all species and neither may behave similarly to the species it is originated from. One of the solutions to address such problem is to create a hybrid promoter that is comprised of both the host promoters such as -35 and -10 boxes and heterologous regulatory sequences (F. K. Lee et al., 1999). There are evidences of authors using QS components in their complicated genetic circuits (H.-H. Huang, Camsund, Lindblad, & Heidorn, 2010) which the authors described it as a “plug and play strategy” since it mixes and matches varying input, processor and output modules to come up with customized biological devices. One classic example is the utilisation of AHL to trigger fluorescence. In this manner, a sharp on/off bi-stable switch is being used. With AHL concentration falling below threshold, no GFP would be detected. Hence, target gene expression would be highly reliant on the input concentration. Instead of antibiotic inducible like some of the promoters, this provides a new type of inducible promoter whereby it is cell-density dependent since a higher cell density would induce a higher expression of the signalling molecules. This

application would be useful in designing environmental biosensor as well as producing toxic gene products. QS-based circuits can also be used to control target gene expression of certain cell densities of the different cells involved through microbial consensus consortium.

Most of the engineered QS systems are derived from gram negative bacteria which secrete AHL. Unfortunately, in the event of applying to multiple AHL systems, there is a high possibility of crosstalk between the AHL signals and receptors even among the same species. On the other hand, the gram positive AIP system is much more species-specific which could provide a better control of the system. However, AIPs are generally larger than AHLs which could pose an issue of a slower diffusion rate on the solid medium.

6.2.3 Proposed construct for Quorum sensing system in *S. aureus*

As mentioned earlier, we proposed to construct a quorum sensing system which enables the engineered cell to sense *S. aureus* (especially group I which is the majority of *S. aureus* out of the four different types) through the presence of AIP. This device could substitute the Quorum sensing system from *P. aeruginosa* from my genetic circuit design earlier to sense *S. aureus* instead of *P. aeruginosa*.

Fig 6.9 Schematic representation of the proposed QS device incorporated to *E. coli* to detect *S. aureus*

In this construct (Figure 6.9), there would be three modules; the *agrC* module which forms the transmembrane complex responsible for binding with the extracellular AIP, the *agrA* module which forms the activator of the promoter of the *agr* operon and lastly the reporter module which translates the input (signalling molecule) to a readable output. The AgrC may require a signal peptide to be directed to the right location to form loops at the membrane. Hence, I propose to use the signal peptide, Lpp' derived from *E. coli* since our host cell is *E. coli*.

If this designed construct could not work as well as intended, we may perform the following: first, we can use gram positive bacteria besides *S. aureus* as our working organism for the detection of *S. aureus* since they would have a more similar behaviour in comparison with *E. coli*. The condition of selection of gram positive bacteria as host cell is it must be GRAS (Generally Recognized As Safe; a term used by FDA) organisms. For example, lactic acid bacteria can be used as the host cell to detect the presence of *S. aureus* in the gut. Alternatively, *Lactobacillus* could also be used as the expression host. *Lactobacillus* had been engineered for mucosal delivery of therapeutic molecules (X. Liu et al., 2006). Another viable option is to clone cofactors

like *sarA* which is absent in *E. coli* but may have a prominent role in the interaction with activated AgrA in order for the promoter of *agr* operon to be triggered.

Additionally, on top of the sensing module for *S. aureus*, we could create a concerted response against *S. aureus* by incorporating a killing device after the sensing device. Future work like combining more inputs via an AND gate could provide a higher specificity and better control of the system.

6.3 RECOMBINANT PROTEIN PRODUCTION IN *E. COLI*'S DEPENDENCY ON CELL PHYSIOLOGY

Overexpression or high level of gene expression may affect cell physiology and cause the host to be stress-sensitive. On the other hand, lower cellular activity incurred due to the limitation of the nutrients and oxygen especially when cells are in high density. A balance between the cell growth and gene expression is necessary to optimise the recombinant protein productivity (W. E. Bentley, Mirjalili, Andersen, Davis, & Kompala, 2009; Boor, 2006; Tamarit, Cabiscol, & Ros, 1998). To improve recombinant protein productivity, proper identification of crucial genes that affect cell physiology under various stressful conditions is critical for cell physiology enhancement. Systematic approach by transcriptomic and proteomic analyses could be applied to identify the key genes (Aldor et al., 2005; Haddadin & Harcum, 2005). For those up-regulated genes under particular stressful condition, measures could be applied to down-regulate these genes through antisense for gene silencing (Kim & Cha, 2003). On the contrary, for the down-regulated genes, these genes should be co-expressed in greater amounts so as to enhance its natural response to counteract the physiological stresses caused by recombinant protein production (Tolia & Joshua-Tor, 2006). A summary of the methods to improve cell physiological is shown in Figure 6.10.

Fig 6.10 A summary of methods to improve the cell physiology under stress conditions.

One important method to improve cell physiology is to manipulate the stress-responsive genes. To tackle the misfolded proteins that possess toxicity, co-expression of proteases could help in degrading these proteins and hence, improving cell physiology (Pan, Hsiao, Weng, Wu, & Chou, 2003). Co-expression of heat-shock proteins or sigma factors could also bring benefits to the cell physiology (Thomas & Baneyx, 1996). Those proteins which are down-regulated under stressful conditions should be up-regulated to improve the cell physiology and hence the recombinant protein production. This is a similar strategy to that of identifying the protein in the rate limiting step within a biosynthetic pathway before subsequently overexpressed. (J. H. Choi, Lee, Lee, & Lee, 2003; Han, Jeong, Yoo, & Lee, 2003). This would in turn, significantly improve the yield of desired end product. On the other hand, suppression of the genes up-regulated when stationary phase occurred could enhance the cell physiology. The reason is when cells are experiencing stationary phase, its metabolic

and cellular activities are minimised, causing recombinant protein production to be unfavourable (K. J. Jeong & Lee, 2003).

Another strategy to improve the cell physiology is to manipulate the folding effectors by co-expression of the folding accessory proteins so as to improve the target proteins' solubility, structural stability, translocation/secretion efficacy or di-sulphide bond formation (Narayanan & Chou, 2008; Sandee, Tungpradabkul, Kurokawa, Fukui, & Takagi, 2005). Alternatively, manipulation of the stationary phase genes could also be a possible solution. This includes knocking out the stationary phase genes, eg. *rmf* gene that is involved in encoding ribosome modulation factor in ribosome multimerization (Imaizumi, Koseki, Matsui, & Kojima, 2006; Imaizumi et al., 2005). Mutant strains with physiological advantage for recombinant protein production could also be screened for improving their industrial application (Madan, Kolter, & Mahadevan, 2005; Sonderegger, Schumperli, & Sauer, 2005).

A proposed future work is to identify the genes under stress conditions in my experimental set-up through microarray and employed either the manipulation of stationary genes strategy to knock out stationary phase genes or stress-responsive genes to further improve the efficiency of cell-killing as well as biofilm degradation through further enhancing the recombinant protein production. This is especially critical when the nutrients or oxygen availability may be lacking in the gut, hence implementing such strategies could further assist to bring the engineered *E. coli* one step closer to becoming an effective drug in combating infectious diseases. Subsequently, using the methodology mentioned earlier in section 6.1, I can integrate my genetic circuits into these knockout strains. This would enable a creation of viable cell with stable genetic properties that can perform its functionalities effectively.

Appendixes

Supplementary figures

FigS1 Characterization of constitutive promoters and *lasQS* in *E. coli* and their effect on cell growth. (A) Constitutive expression of GFP was averaged out and relative to the reference promoter *rrsBp*. The cell growth was monitored concurrently in Top10. Growth rate was inversely proportional to promoter strength. (B) Similar trend of the order of strength of σ^{70} promoters was observed in EcN. In decreasing promoter strength: P_{J23102} , P_{J23108} , P_{J23105} , P_{J23113} . (C) Measurement of customized quorum sensor device (P_{con} LasR- P_{Las} GFP) with different σ^{70} promoters to validate the correct promoter to use. (i) Circuit design of customized quorum sensor to verify the best promoter to use. (ii) RFU/OD was the highest with the construct containing P_{J23108} than the rest of the constructs. (D) Cells were exposed to a range of concentration of AHL. Similar RFU/OD was obtained for concentration above 10^{-8} M.

FigS2 Characterization of the lysis profile in *E. coli* with different strength of ribosomal subunit (RBS) and *E. coli* host strain and the design of different configuration of the 3 genes; DspB, S5 and E7. (A) With the intention of delaying the lysis time to have more inducible protein (S5, DspB) accumulation, since, promoter was fixed, the RBS was the only parameter that can be manipulated. Hence, (i) RBS3 was changed to a (ii) weaker RBS4 and characterized in Top10 (P_{J2310X} LasR- P_{Las} S5 RBS X E7). E7 and S5 share the same transcript. Both RBS was obtained from BioBrick registry. However, the lysis time was similar at around 1.5 hours, but only the sensitivity was noticeably changed by which the construct containing P_{J23105} and RBS4 was not able to lyse Top10. (B) Choosing the candidates with RBS3, due to expression in lower expression-host strain, EcN, the lysis was completely removed for all the different promoters. Up to 5-fold difference of protein expression in EcN compared to Top10. Hence, E7 could not be placed behind S5 (with any RBS strength coupled to E7) for expression in EcN as RBS3 was only 1/3 of the highest strength RBS5 which could not obtain a successful lysis (P_{J23108} LasR- P_{Las} S5 E7). S5 (~1.5k base pair) being too long could be the limiting E7 transcription. (C) We had 3 genes (S5, DspB, E7) that were necessary to be transcribed by P_{Las} promoter. It was important to consider the number of genes to be transcribed under the same P_{Las} promoter; 1, 2 or 3. Since S5 and E7 cannot share same promoter, all 3 genes sharing one P_{Las} promoter was not possible.

FigS3 High throughput screening of best configuration (among remaining two P_{Las} promoters constructs) through co-culturing of planktonic *P. aeruginosa* with engineered EcN. The co-culture was done in 96 microplate well with shaking over a time frame of 5hours. RFU was measured, corresponding to the surviving In7 cells which contained constitutively expressed GFP in plasmid. RFU fold on the vertical axis indicated the RFU measured at time, t hours with respect to 0 hour. The best construct in EcN is the construct with J23108 promoter and with terminator (108 S5 DspB E7) was labelled as Nissle-SDE.

FigS4 The lysis profile of the 3 different configurations and the growth curve of *P. aeruginosa*, In7 with different starting OD. (A) Circuit design of the 3 final constructs (SE, SDE, 3SED) for verification of its effect on planktonic *P. aeruginosa*. (B) Time-delay element was expected by transcript configuration for final construct as from the the killing effect of Nissle-SDE was higher compared to Nissle-3SED against In7 (Fig5A(ii)). The lysis by Nissle-3SED was 1 hour slower than the lysis by Nissle-SDE, giving more S5 and DspB proteins accumulation. (C) Growth profile of In7 with different starting OD at 0.2 and 0.4. (D) Correlation of OD with CFU of In7.

FigS5 The plasmid map of the plasmids used in this thesis.

FigS6 10^{-3} -fold diluted supernatant (OD 1.0) is equivalent to $\sim 10^{-7}$ M AHL. Therefore, estimated AHL concentration present in supernatant is as high as 10^{-4} M. OD 0.1 at 600nm of *P. aeruginosa* is equivalent to 3.2×10^5 CFU/mL. OD 0.1 at 600nm of *E. coli* is equivalent to 2.0×10^5 CFU/mL. At fixed OD, *E. coli* has less number of cells than *P. aeruginosa*.

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