

PROGRAMMING MICROBES TO TREAT SUPERBUG INFECTION

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Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research done by me and has not been submitted for a higher degree to any other University or Institute.

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Blessings in Him

Abstract

Superbug infection is one of the greatest public health threat with grave implications across all levels of society. Towards a new solution to combat infection by multi-drug resistant bacteria, this thesis presents an engineering framework and genetic tools applied to repurpose commensal bacteria into “micro-robots” for the treatment of superbug infection. Specifically, a prototype of designer probiotic was developed using the human commensal bacteria *Escherichia coli*. The engineered commensal was reprogrammed with user-specified functions to sense superbug, produced pathogen-specific killing molecules and released the killing molecules via a lytic mechanism. The engineered commensal was effective in suppressing ~99% of planktonic *Pseudomonas* and preventing ~ 90% of biofilm formation. To enhance the sensing capabilities of engineered commensal, genetic interfaces comprising orthogonal AND & OR logic devices were developed to mediate the integration and interpretation of binary input signals. Finally, AND, OR and NOT logic gates were networked to generate a myriad of cellular logic operations including half adder and half subtractor. The creation of half adder logic represents a significant advancement of engineering human commensal to be biological equivalent of microprocessor chips in programmable computer with the ability to process input signals into diversified actions. Importantly, this thesis provides exemplary case studies to the attenuation of cellular and genetic context dependent effects through principles elucidated herein, thereby advancing our capability to engineer commensal bacteria.

List of Abbreviations

3OC12HSL	N-3-oxododecanoyl homoserine lactone
5'UTR	5' untranslated region
λ CI	Lambda CI repressor
AHL	Acyl homoserine lactones
AI-2	Autoinducer-2
Amp ^R	Ampicillin resistance
AND	A logic output that is "true" only if both of the inputs are "true"
bp	Base pair
C4HSL	N-butanoyl-L-homoserine lactone
CF	Cystic fibrosis
Cm ^R	Chloramphenicol resistance
CRISPR	Clustered regularly interspaced short palindromic repeats
DNA	Deoxyribonucleic acid
GFP	Green fluorescent protein
kb	Kilobase
NOT	A logic gate which implements logical negation
OD	Optical density
OR	A logic output that is "true" if either or both of the inputs are "true"
pBAD	Arabinose promoter from <i>Escherichia coli</i>
pHrpL	AND gate promoter from <i>Pseudomonas syringae</i>
pLuxR	Quorum sensing promoter from <i>Vibrio fischeri</i>
pRHAB	Rhamnose promoter from <i>Escherichia coli</i>
Rbs	Ribosome binding site
RFP	Red fluorescent protein
RFU	Relative fluorescence unit
RNA	Ribonucleic acid
T3SS	Type III secretion system
T7RNAP	T7 RNA polymerase
TF	Transcription factor
VSM	Virulence secondary metabolite
XOR	A logic output that is "true" if either, but not both, of the inputs are "true"

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Chapter One

1. INTRODUCTION

1.1 Background and Motivation

One of the biggest public health threats that the world is facing today is the rapid emergence of superbug infection. Superbugs are bacterial pathogens that gained tolerance to commonly-used antibiotics and continue to proliferate in the presence of inhibitory concentration of the antibiotics¹. In the most recent and biggest study by far, the World Health Organisation (WHO) analysed data from 114 countries and acknowledged that the superbug crisis may be worse than the 1980s Aids epidemic – which had claimed over 25 million lives worldwide and “threatens to turn the clock back on modern medicine”². Two classes of superbug exist; in the first class, commensal bacteria including *Staphylococcus aureus* and *Enterococci* which naturally reside in the human body, acquire virulent and antibiotic-resistant genes through horizontal gene transfer and convert into infectious pathogens³. Opportunistic bacteria that originate from the surroundings and cause infection among immunocompromised patients such as those undergoing cancer chemotherapy, infected with HIV, infants or elderly patients, form the second class of superbug pathogens. Due to protracted exposure to rigorous environment, opportunistic bacteria are adapted with multi-drug resistant genes and have been suggested to be the evolutionary origins of antibiotic

resistance in clinical pathogens⁴. While antibiotics had been effective in controlling the spread of bacterial infection in the early 1960s, it is evident that the current development of antibiotics cannot cope with the speed at which bacterial pathogens evolved against our existing arsenal of antibiotics.

Increasingly, the risk of common infection and minor injuries exacerbating into critical health conditions have been frequently observed across all levels in society, pronouncing the need for new approaches to combat infections caused by multi-drug resistant bacteria. Contemporary treatments against superbug infection include antibiotic chemotherapy and bacteriophage therapy. In antibiotic chemotherapy, a combinatorial treatment involving multiple antimicrobial agents is usually preferred over monotherapy due to the rapid acquisition of drug tolerance among opportunistic bacteria. This approach, however, promotes unspecific killing of bacteria and upsets a healthy human microbiome and immune system, thereby increasing one's vulnerability to infection by other pathogens. Phage therapy involves strain-specific bacteriophages that invade and destroy the cellular integrity of pathogens⁵. The therapeutic potential of employing virus in bacterial infection, however, is limited, as a directed treatment cannot be re-employed after the infected host develops specific antibodies against the introduced virus⁶. Bacterial pathogens also commonly acquire immunity to phage attacks by (1) altering cell surface receptors, (2) producing masking proteins and exopolysaccharides to reduce phage adsorption⁷, or (3) deactivating viral DNA through CRISPR spacers in similar fashion as RNA interferences among eukaryotic organisms⁸. Nonetheless, phage therapy remains as one of the most promising alternatives

to the control of superbug infection among immunocompromised patients as exemplified in several recent studies by *Lu* and colleagues using phage synthetic biology⁹⁻¹³.

Probiotics modulate inflammatory responses¹⁴ and protect humans against infection through a myriad of mechanisms including deactivation of toxins, inhibition of pathogens in gut, urinary and respiratory tracts and activation of innate immune system¹⁵⁻¹⁷. In other studies, probiotics have also been demonstrated to positively regulate metabolic diseases¹⁸, food allergies¹⁹, neurodevelopmental disorders²⁰ and cancer treatments²¹. Drawing inspiration from nature, designer probiotics may prove to be a potent weapon in the fight against superbug infection if augmented with the right set of genetic tools and functions. The advantages of using probiotics as prophylactic and therapeutic measures for infection control are multi-fold. Firstly, probiotics are known to survive transition across the gastrointestinal tract and can serve as excellent delivery vehicle. Secondly, the designer probiotics presented in this thesis only targeted *Pseudomonas* pathogen while leaving the rest of the gut microbiota intact. This is crucial to maintain a healthy immune function and prevent the occurrence of secondary infection by other pathogens. Finally, the designer probiotics can be genetically tailored to incorporate kill-switches that prevent their survival beyond target host or to ameliorate additional human health complications such as insulin resistance and obesity. In all, designer probiotics are practical means to precision medicine.

Synthetic biology aims to engineer genetically modified biological systems that perform novel functions that do not exist in nature, with reusable, standard interchangeable biological parts. The use of these standard biological parts enables the exploitation of common engineering principles such as standardization, decoupling, and abstraction for synthetic biology²². With this engineering framework in place, synthetic biology has the potential to make the construction of novel biological systems a predictable, reliable, systematic process. While the development of most synthetic biological systems still remains largely *ad hoc*, recent efforts to implement an engineering framework in synthetic biology have provided long awaited evidence that engineering principles can facilitate the construction of novel biological systems. Thus far, synthetic biology principles have been successfully applied to develop novel biological systems of various applications, such as drugs and biofuels production²³⁻²⁷, microbiota restructuring²⁸, and even cancer therapy²⁹. Given the stalled development of new antibiotics and the increasing emergence of multi-drug-resistant pathogens, using synthetic biology to design new treatment regimens for infectious disease could address an urgent need.

1.2 Objectives and Milestones

The overall aim of this thesis was to develop a framework for the rational engineering of commensal bacteria with human-specified functions. Specifically, commensal bacteria were genetically reprogrammed into “micro-robots” and equipped with novel, regulated functions for the prevention and treatment of superbug infection. Studies described in this thesis were independently performed in Nanyang Technological University (Singapore)

and Imperial College London (United Kingdoms). Specific objectives, tasks and milestones are as follows:

- I. Genetic engineering of commensal microbe to sense and eradicate a common superbug pathogen, *Pseudomonas aeruginosa*. This proof-of-concept study would serve as an exemplary reference to how the synthetic biology engineering framework could be harnessed to develop designer probiotic against specific superbug infection.
- II. Development of genetic interfaces that could integrate multiple input signals to produce specific outputs for enhanced biosensing capabilities. This proof-of-concept study entailed the execution of digital operation (AND & OR binary logic) in whole cell biosensor to improve the specificity and sensitivity of detecting dual input signals. The design framework established herein would significantly simplify the engineering of digital operation in living cells and can be applied in other related projects.
- III. Programming autonomous decision-making in commensal microbe with biological half adder. This task built on the networking of logic devices developed in Task II to exhibit advanced half adder logics. Half adders are basic units in microprocessor chips with programmable decision-making functions. The design principles established in this study, in particular causes, effects and solutions to genetic context dependency, would be of significance usefulness for future design of complex genetic devices and circuits.

1.3 Thesis Outline and Organisation

Superbug infection is one of the greatest public health threat with grave implications across all levels of society. Towards a new solution to combat infection by multi-drug resistant bacteria, this thesis presents an engineering framework and genetic tools applied to repurpose commensal bacteria into “micro-robots” for the treatment of superbug infection. In Chapter 1, the thesis’s background information, motivation, and defining objectives are described and discussed.

Chapter 2 presents the literature review of the biological properties of the model superbug targeted in this thesis, *P. aeruginosa*. A comprehensive review of synthetic biology concept in medical biotechnology is discussed. The chapter also presents an overview of synthetic genetic circuits and logic devices that have been developed to date.

Chapter 3 presents the detailed methods that were performed in the studies outlined in this thesis. This includes the protocols that were performed to design, assemble, characterise and evaluate the effectiveness of the engineered microbe in sensing and eradicating the model superbug, and those which were used to develop and characterise genetic logic gates that enabled engineered *E. coli* to function as biological microprocessor.

Chapter 4 presents the design principles that were applied to engineer commensal *E. coli* into a therapeutic microbe against *Pseudomonas* infection and the corresponding results (Task I). Specifically, the commensal bacteria

were reprogrammed with user-specified functions to sense superbug, produced pathogen-specific killing molecules and released the killing molecules via a lytic mechanism.

To enhance the sensing capabilities of engineered commensal, genetic interfaces comprising orthogonal AND & OR logic devices were developed to mediate the integration and interpretation of binary input signals. Chapter 5 presents a novel forward engineering approach to enable AND & OR digital operations in synthetic biological systems (Task II). As a proof of concept study, the genetic interfaces developed in this chapter were systematically characterised using prebiotic sugar inducers, arabinose and rhamnose.

Chapter 6 presents the engineering principles and challenges faced in the integration of disparate bio-logical devices to repurpose *E. coli* with half adder computational function (Task III). The half adder is an advanced digital logic operation that demonstrates both AND & XOR outputs from binary inputs. A half subtractor was also developed to illustrate the flexibility of the synthetic biology approach adopted in this study.

Chapter 7 presents a summary of novel contributions of this thesis to the scientific community. Additional viewpoints on possible future plans to advance the development of designer probiotics in terms of introducing new genetic functions (engineered cell motility and biofilm dispersal), and bringing the engineered commensal bacteria into a next phase of pre-clinical animal testing

are discussed. Appendix and references acknowledged in this thesis are presented in Chapter 8 and 9 respectively.

1.4 List of Publications

Saeidi Nazanin, **Choon Kit Wong*** et al. Engineering microbes to sense and eradicate *Pseudomonas aeruginosa*, a human pathogen. *Mol Syst Biol* **7** (2011).
*co-first author

Choon Kit Wong et al. in Engineering and Analyzing Multicellular Systems, Vol. 1151. (eds. L. Sun & W. Shou) Springer New York, 117- 133 (2014).

Choon Kit Wong et al. Isolated nucleotide molecule and method of sensing and killing pathogenic microorganism. US Patent 9,044,020 B2. (2015)

Adison Wong et al. Layering genetic circuits to build a single cell, bacterial half adder. (2014). *BMC Biol* **13** (2015)

Chapter Two

2. LITERATURE REVIEW

In this chapter, the pathogenic properties of *Pseudomonas aeruginosa* (*P. aeruginosa*) are reviewed with special emphasis on their multi-drug resistant and quorum sensing properties driving virulence behaviour. Next, an overview of pyocins – antimicrobial peptides derived from environmental derivatives of *P. aeruginosa* and specific against clinical isolates of *P. aeruginosa* – are introduced. A comprehensive literature review on the field of synthetic biology is then presented. Specifically, the core engineering principles and biotechnological applications associated with synthetic biology, and the challenges of engineering synthetic gene circuits are discussed. Finally, the concept of analog and digital genetic circuits, and how digital circuits can be programmed to display digital operations are explained.

2.1 *Pseudomonas aeruginosa* as an Opportunistic Pathogen

P. aeruginosa is an opportunistic, gram-negative bacterium, commonly associated with causing chronic, nosocomial infections among cystic fibrosis and immune-compromised patients. Among hospital acquired infection, marrow transplant and neutropenic patients with compromised immune systems are gravely vulnerable to *P. aeruginosa* associated outbreaks of pneumonia and septicaemia, accounting for an estimated mortality rate of

30%³⁰. Cystic fibrosis (CF) patients who lack the ability to regulate mucus due to mutation in the gene coding for protein cystic fibrosis transmembrane conductance regulator are also lethally susceptible to *P. aeruginosa* infection³¹. Bacteria colonization along the respiratory tracts developed biofilms, leading to breathing difficulties and high fatalities among CF patients. Contemporary treatments against *P. aeruginosa* infection include chemotherapy using a combination of potent antibiotics and phage therapy with strain-specific bacteriophage that destroy cellular integrity of the pathogen^{5, 32}. Such methods are however ineffective in due course as (i) drug exposure incites mutation in the antibiotic resistance genes overtime, allowing *P. aeruginosa* to develop resistance against synthetic antibiotics and prevent vital metabolic activities from being affected by its introduction, and (ii) phage directed treatment cannot be employed a second time after infected host developed immunity against the introduced virus⁶. With the pace of spontaneous mutation far exceeding the development of antibiotics, acquired multidrug resistance in *P. aeruginosa* has presented an unparalleled challenge to medical treatments^{33, 34}.

Clinical treatments to infection initiated by *P. aeruginosa* remain a central issue among medical researchers due to the pathogen's enhanced resistance to numerous antibiotics. Several mechanisms have been proposed to elucidate its innate defences against antibiotics, for example its low-permeability cellular envelope against extracellular antibiotics, the presence of multidrug efflux systems for drug rejection, the expression of an antibiotic

deactivation protein encoded by *ampC* gene and horizontal gene transfer of plasmid, transposons or phages harbouring antibiotic resistance cassettes^{35, 36}.

Table 2-1. Antibiotic efflux pumps. The table is adapted from ^{35, 36} with permission.

Efflux pump	Substrate
MexAB-OprM	β -lactams, fluoroquinones, chloramphenicol, macrolides, novobiocins, sulphonamides, tetracycline, trimethoprim
MexCD-OprJ	Anti-PA PCN, chloramphenicol, CPM, fluoroquinones, macrolides, MERO, novobiocins, trimethoprim
MexEF-OprN	carbapenems, chloramphenicol, fluoroquinones, trimethoprim
MexJK	erythromycin, tetracycline
MexGHL-OpmD	fluoroquinones
MexVW	chloramphenicol, erythromycin, fluoroquinones, tetracycline,
MexPQ-OpmE	chloramphenicol, fluoroquinones, macrolides, tetracycline
MexMN	chloramphenicol

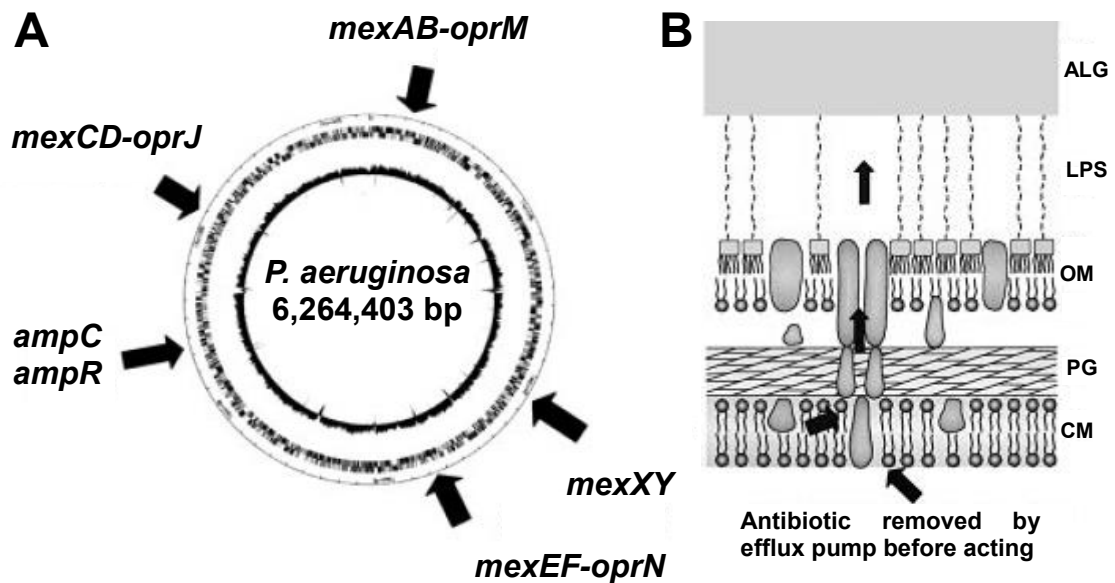


Figure 2-1: Multidrug efflux pumps of *P. aeruginosa*. (A) Chromosomal genes encoding for multiple efflux system and beta-lactamase. (B) Schematic of efflux pumps removing antibiotics from the inner cytoplasm and periplasm out of the cell. CM: cytoplasmic membrane, PG: peptidoglycan, OM: outer membrane, LPS: lipopolysaccharides, ALG: alginate. The figure is reproduced from ³⁵ with permission.

Figure 2-1 shows the genomic mapping and graphical illustration of multiple efflux system in PAO1³⁵. Each multidrug efflux pump is composed of three modules; an antiporter embedded across the cytoplasmic membrane, a protein linker and an outer-membrane porin for expulsion of molecules with toxic effects on *Pseudomonas* viability. Other than polymyxins, all other classes of antibiotics are susceptible to the effects of multidrug efflux pumps. Although the genes are encoded in all isolates of *P. aeruginosa*, their expressions are tightly controlled by regulatory repressors in an antibiotic-free environment to preserve energy for other pivotal cellular activities. Nonetheless, drug exposure may incite mutation in the antibiotic regulatory genes, leading to enhanced expression of drug efflux systems and allowing *P. aeruginosa* to develop resistance against novel antibiotics.

The bacterial community in a biofilm is protected by an excreted matrix of extracellular polymeric substance (EPS) composed of polysaccharides and proteins. The development of a biofilm impedes drug delivery to target sites of infection and greatly reduces the efficacy of antibiotics. Three possible mechanisms of antibiotic resistance in biofilms are shown in the **Figure 2-2**³⁷. The first defence mechanism (yellow) is slow or incomplete penetration of the antibiotic into the biofilm after being deactivated in the surface layers. The second mechanism³⁸ proposes that a subpopulation of *P. aeruginosa* in biofilm differentiates into a highly protected phenotypic state which is invulnerable to antibiotics. The third protective system³⁹ which occurs deep within the biofilm illustrates a nutrient depleted zone rich in waste concentrations where antibiotic effects are nullified. These mechanisms allow *P. aeruginosa* to persist on infected surfaces and cause recurrent infections. *P. aeruginosa* is highly adaptable to differing host environment. In addition to its potential to exploit diverse carbon sources ranging from glucose to diesel for growth and metabolism, it is also able to survive in anaerobic conditions by utilizing nitrate or arginine as a respiratory electron acceptor. These strategies are critical to its survival in challenging conditions, such as in cystic fibrosis infections where oxygen diffusion is restricted.

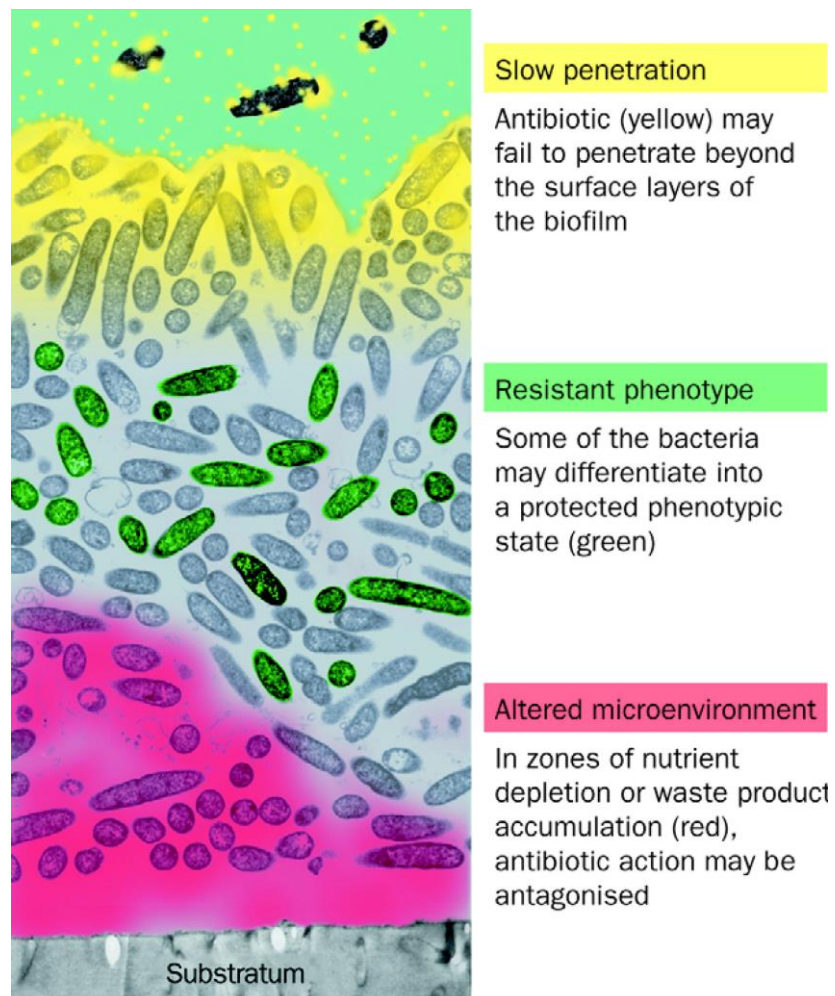


Figure 2-2: Antibiotic resistance of bacteria in biofilms. (1) The biofilm matrix is an obstacle to diffusion and causes the inactivation of antibiotics. (2) A sub-population of *P. aeruginosa* may differentiate into antibiotic-resilient phenotype and co-exist with antibiotic-sensitive cells. The figure is reproduced from ³⁷ with permission.

2.2 Quorum sensing in *P. aeruginosa*

Quorum sensing (population sensing) is an organic chemical signalling cascade that regulates a myriad of physiological activities such as cell motility, virulence, biofilm formation and growth. This sensing mechanism is mediated by various diffusible, chemical signals known as autoinducers that are putatively produced by the synthase gene of the bacteria. The extracellular concentration of signalling molecules increases as a function of cell density and is permeable to cell membrane, hence allowing an equilibrium concentration to be established within cells and the local environment. Upon attaining a threshold concentration of chemical signals, the inducers bind to specific quorum sensing promoters or associated proteins within cell and elicit expressions or repressions of multiple genes, including those that are functional for production of autoinducers. Some common examples of autoinducers are acyl homoserine lactones (AHLs) that direct gene expression in gram-negative bacteria³⁸, oligo-peptides in gram-positive bacteria⁴⁰ and autoinducer-2 (AI-2) that are recognised by both categories of bacteria⁴¹.

Both *E. coli* and *P. aeruginosa* are gram negative bacteria which utilise acyl homoserine lactones as the primary quorum signalling molecules. The first and best characterised quorum sensing model of gram-negative microbe is the autoinduction of bioluminescence in marine bacterium *Vibrio fischeri*⁴². Bioluminescence in *V. fischeri* involves expression of *luxR* and *luxI* genes from the *lux* operon which are critical in the production of cellular communication proteins: LuxI AHL synthase and LuxR cofactor. The synthase is then responsible for the production of autoinducers via the fatty acid biosynthesis

pathway using S-adenosylmethionine substrate transported by acylated acyl carrier protein (**Figure 2-3**)⁴³. Similar production mechanisms are also present in other gram-negative bacteria, with each LuxI homolog producing AHL that differs in either length or functional groups (hydroxyl, carbonyl or hydrogen atoms) on the acyl side-chain. Thus, with each bacterium possessing disparate synthase sequence, a high level of specificity can be achieved during intercellular quorum communication. A list of common AHLs with its associated species is shown in the **Table 2-2**^{44, 45}.

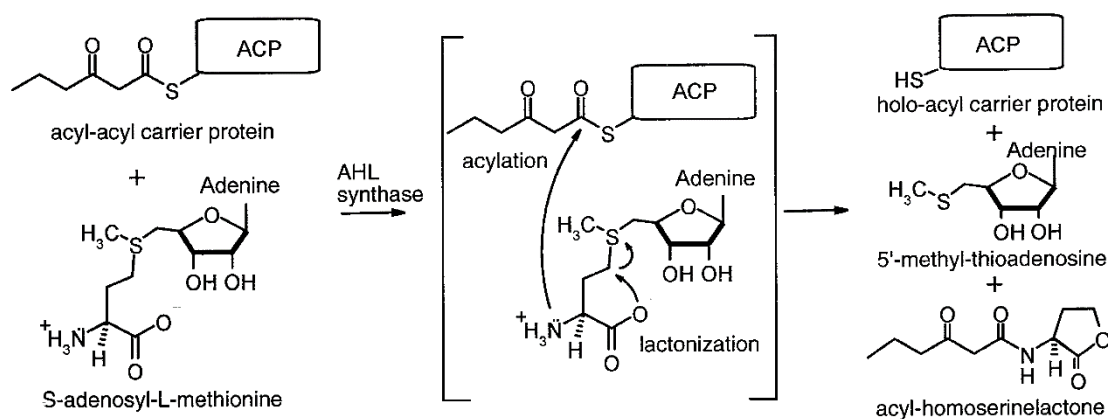
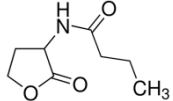
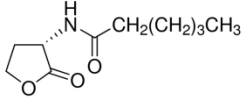
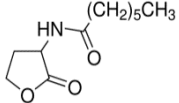
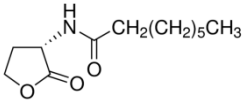
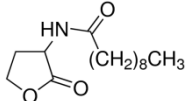
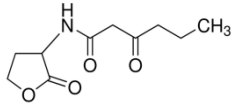
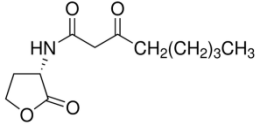
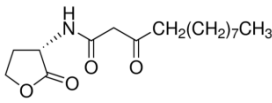


Figure 2-3: Biosynthesis of AHL. Two substrates acyl-acyl carrier protein and S-adenosyl-L-methionine is catalysed by AHL synthase to undergo acylation and lactonization reaction. After reaction is completed, the product AHL and regenerated cofactors 5'-methyl-thioadenosine and holo-acyl carrier protein are released. The figure is reproduced from ⁴³ with permission.

Table 2-2. Autoinducer homoserine lactone in Gram-negative bacteria. The table is adapted from ^{44, 45}

Full Name	Molecule Abbreviation	Species	Chemical formula
Butyryl-homoserine lactone	C ₄ HSL	<i>P. aeruginosa</i>	
Hexanoyl-homoserine lactone	C ₆ HSL	<i>C. violaceum</i> <i>R. solanacearum</i>	
Heptanoyl-homoserine lactone	C ₇ HSL	<i>E. psidii</i>	
Octanoyl-homoserine lactone	C ₈ HSL	<i>B. cepacia</i> , <i>V. fischeri</i>	
Decanoyl-homoserine lactone	C ₁₀ HSL	<i>C. violaceum</i> <i>B. pseudomallei</i>	
3-oxohexanoyl-homoserine lactone	3OC ₆ HSL	<i>V. fischeri</i> <i>Y. pseudotuberculosis</i>	
3-oxooctanoyl-homoserine lactone	3OC ₈ HSL	<i>A. tumefaciens</i>	
N-3-oxododecanoyl-homoserine lactone	3OC ₁₂ HSL	<i>P. aeruginosa</i>	

Two *lux*-homolog quorum sensing systems are present in *P. aeruginosa*: *las* system in combination with 3OC₁₂HSL and *rhl* system with C₄HSL. The *las* system involves a transcriptional activator LasR and autoinducer synthase LasI, and regulates the production of virulence factors such as elastase, LasA protease, exotoxin A⁴⁶, and secretory proteins encoded by *xcpP* and *xcpR*

genes⁴⁷. This quorum sensing system is positively regulated by factors GacA⁴⁸ and Vfr that is required for the transcription of LasR protein⁴⁹, and negatively regulated by RsaL that suppresses transcription of LasI synthase⁵⁰. The *rhl* system comprises of RhIR transcriptional regulator and RhII synthase which generates C₄HSL with some subtle production of C₆HSL. It is activated after initiation of *las* system and controls the expression of *rhlAB* operon that encodes for rhamnolipid synthase to synthesize rhamnolipid, a biosurfactant that emulsify hydrocarbon substrate for utilization by the cells⁵¹. Interestingly, it has also been shown that the expression of *rhlAB* operon is repressed by other regulatory components during the exponential phase even in the presence of C₄HSL and RhIR⁵², suggesting that genes regulated by *rhl* system also involve *las* system for complete expression⁵³. The *rhl* quorum sensing system is known as vsm (virulence secondary metabolites) as it also regulates the expression of virulence factors LasA, LasB and pyocyanin^{54, 55}.

Uptake and secretion of C₄HSL and 3OC₁₂HSL differs due to disparate molecular length and hydrophobicity of substituent groups; C₄HSL is freely diffusible through cell membrane while 3OC₁₂HSL requires an active efflux system⁵⁶. Studies have shown that the combination of autoinducer and its respective R-proteins are highly specific in terms of transcriptional activity⁵⁷ i.e. C₄HSL will not activate LasR protein and vice versa and the resultant activator complex are sensitive to only specific promoters. It is however important to note that neither system is completely independent of each other. **Figure 2-4** illustrates cellular communication cascade in *P. aeruginosa* and the interdependency between lasR/3OC₁₂HSL and rhIR/C₄HSL quorum sensing

systems⁴⁶. Here, *las* system not only exerts control as an upstream transcriptional regulator to RhIR expression, but is also able to monitor activity of *rhl* system via post-translational competitive binding of 3OC₁₂HSL to RhIR protein⁵⁸.

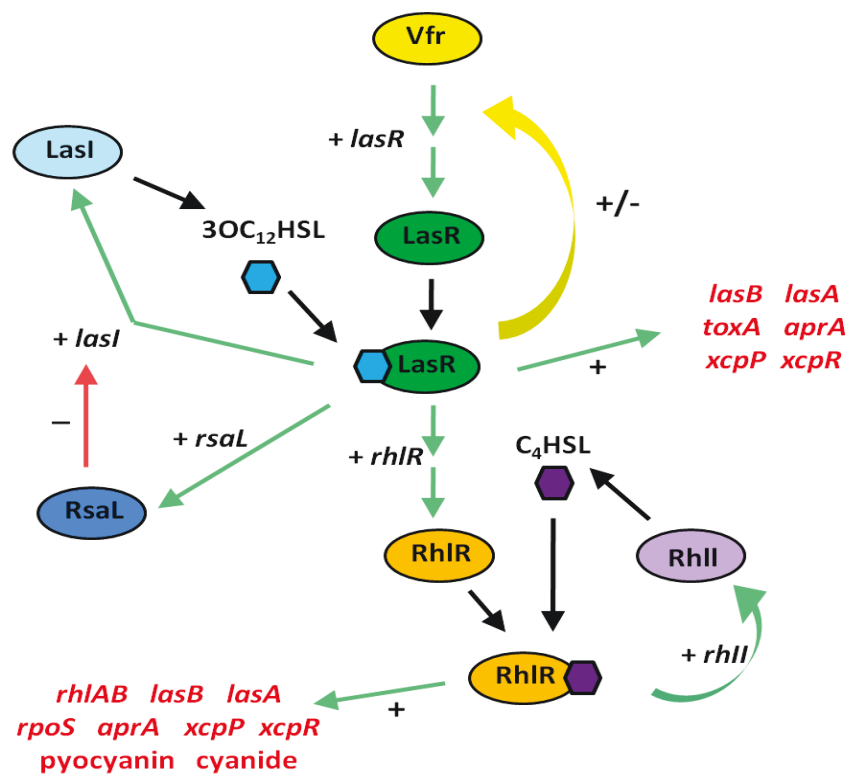
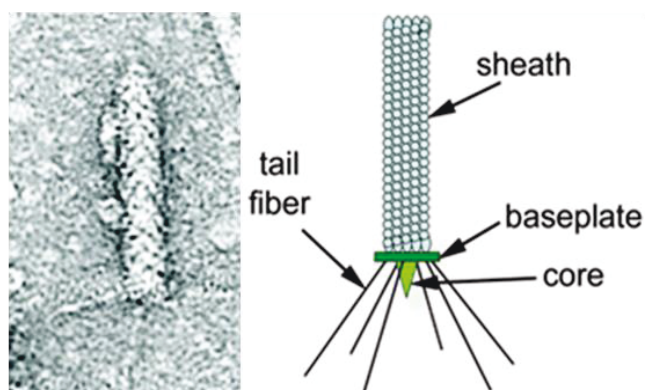


Figure 2-4: Quorum sensing network in *P. aeruginosa*. The figure is adapted from⁴⁶ with permission.

2.3 Pyocins of *P. aeruginosa*

Pyocin treatment strategy was inspired by bacteria's most primitive attempt in securing its survival in a competitive environment alongside other bacteria strands by means of ribosomally-synthesized antibacterial peptides or bacteriocins. First discovered by *Francois Jacob* in 1954, pyocins are narrow-spectrum bacteriocins produced by clinical isolates of *P. aeruginosa* and termed after the species former name: *Pseudomonas pyocyanea*⁵⁹. Bacteriocins are specific and effective against proximal species and thus have garnered attention in the medical engineering application as the new generation antimicrobial molecules⁶⁰. Most discoveries associated with natural pyocin production have been derived from studies of *P. aeruginosa* strand PAO1. Pyocins can be classified into 3 types⁶¹. The R and F types are distinguished by their rod like morphology, bearing similarity to bacteriophage tails that have evolved and specialised as antibacterial peptide (**Figure 2-5**). With both classes of pyocin being resistant to protease and nuclease activities, the only distinct difference between R and F type hinges upon the higher degree of flexibility encountered in the F type. The S type was first discovered by Ito and Kageyama with an isolate of *P. aeruginosa* that was known to produce R type pyocin⁶². The isolate was treated with anti-R serum and spotted for inhibition studies. Results showed broader inhibition zone, confirming that the same *P. aeruginosa* isolate was capable of secreting a plethora of pyocins. Studies by Smith estimated that R and F type pyocins are synthesized by 90% of all *P. aeruginosa* strains and S type by 70% of them⁶³.

A SEM & Graphic illustration of Pyocin R2



B Illustration of R & F type Pyocin

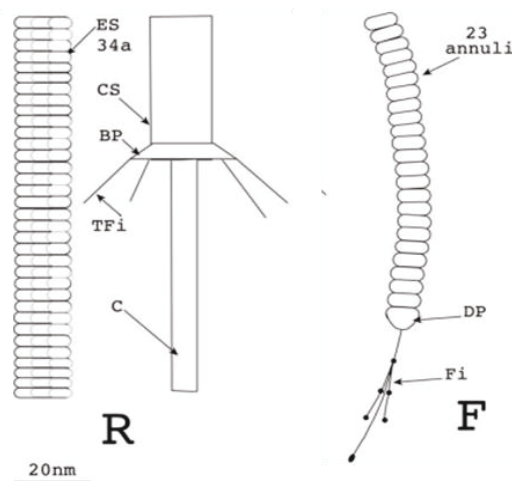


Figure 2-5: Pictorial representation of R & F type pyocins. (A) SEM image and graphical illustration of R2. (B) Comparison between contractile, inflexible phage-tailed R type pyocin and non-contractile, flexible F type. The figure is adapted from ^{61, 64} with permission.

S type pyocins are soluble and responsive to protease activities as opposed to R and F types. To date, seven S type pyocin have been discovered and characterised. Protein purification revealed that several S type pyocin comprises of two protein molecules associated in complex, with parallel structural and functional features similar to DNase based colicins. The larger protein has been identified as the functional protein for killing while the smaller

protein is regarded as the immunity protein that confers the host cell with defence mechanism against its own pyocin^{65, 66}. Pyocin S1, S2, S3 and AP41 initiate cell death in other *P. aeruginosa* strains through endonuclease action of the C terminus of the larger protein, with the killing activity of S1 and S2 larger than AP41⁶⁷.

Genomic analysis of S4 and S5 coding sequence predicted ternary structures that are analogous to tRNase and pore forming proteins⁶⁸. Pyocins, in particular pyocin S5, present great potential in the treatment of *P. aeruginosa* infection for the following reasons: (1) pyocin S5 is soluble and can be readily overexpressed in engineered probiotic *E. coli* without compromising survivability of the producing host bacteria. (2) Pyocin S5 acts by forming pores on the membrane of *P. aeruginosa* and does not need to be translocated into the pathogen interior, thereby avoiding the mechanism of antibiotic efflux pumps. (3) Unlike other bacteriocins such as colicin, the immunity genes of pyocins are encoded in the chromosome of pyocin-producing *Pseudomonas* instead of plasmid vectors as encountered with most bacteriocins. Consequently, development of pyocin resistance by lateral gene transfer has not yet been encountered to date. (4) Pyocin bactericidal activities are specific to *P. aeruginosa* and will not target other commensal bacteria that are vital to human health⁶⁹. Together with its characteristics of being soluble and small at 498 amino acids, S5 is an ideal pyocin for treatment against *P. aeruginosa* infection.

Table 2-3. S-type pyocins from PAO1.

S Type Pyocin	Mode of Killing	Reference
S1	DNase	65
S2	DNase	65
S3	DNase	70
S4	tRNase	68
S5	Pore forming	68, 69
AP41	DNase	71

2.4 Designing Biological System through Synthetic Biology

Synthetic biology is the application of engineering science to the systematic design of biological systems – using the design, build, test and analyse paradigm⁷². This enables the artificial design and assembly of functional biological systems that can be readily applied towards solving contemporary problems facing the world. Thus far, synthetic biologists have demonstrated that this design framework can be applied to advance applications in energy, environment and healthcare. Particularly, novel biological systems have been constructed to produce isoprenoid- and alkaloid-based drugs^{26, 27} and biofuels²³⁻²⁵, to restructure the gut microbiota²⁸, and to treat cancer²⁹.

One global crisis that has been partially relieved through synthetic biology-driven technology is the control of malaria caused by the protozoan

Plasmodium. Malaria is estimated to cause annual deaths of more than 3 million with worldwide contagion of 500 million. Although the drug artemisin has been proven to be effective in the treatment against malaria, current methods of production are too expensive to be considered economically feasible in ameliorating the effects felt in developing Sub-Saharan Africa. In 2006, *Keasling* and colleagues successfully produced artemisin precursors in metabolically engineered yeast²⁶. This biotechnology allows the cost of artemisin to be drastically reduced from US\$2.40 to US\$0.25 per dose. Synergetic applications of synthetic biology and systems metabolic engineering have also lead to the advancement of biofuel research by incorporating novel metabolic pathways for fuel production into an engineered microbe in 2010²³.

Disruption to a healthy gut flora has been linked to the pathogenesis of inflammatory bowel disease, chronic diarrhoea, colitis and other irritable bowel syndromes⁷³. One established method to restore a healthy gut flora is through faecal microbiota transplantation of stool bacteria harvested from healthy donor⁷⁴. In 2014, a novel method to engineer the human microbiome with CRISPR-Cas technology was introduced (**Figure 2-6**)²⁸. CRISPR-Cas is an adaptive prokaryotic defence strategy to reject undesired mobile genetic elements⁷⁵. By redesigning the spacer sequences of CRISPR RNA (crRNA) to complement specific genetic signature, the cas9 endonuclease was guided, with the support of trans-activating CRISPR RNA (tracrRNA) to introduce double strand break in target genes that encoded for antibiotic resistance. This microbiome engineering technology successfully attenuated

Enterobacteriaceae and enterohemorrhagic *E. coli* infections in *G. mellonella* infection model.

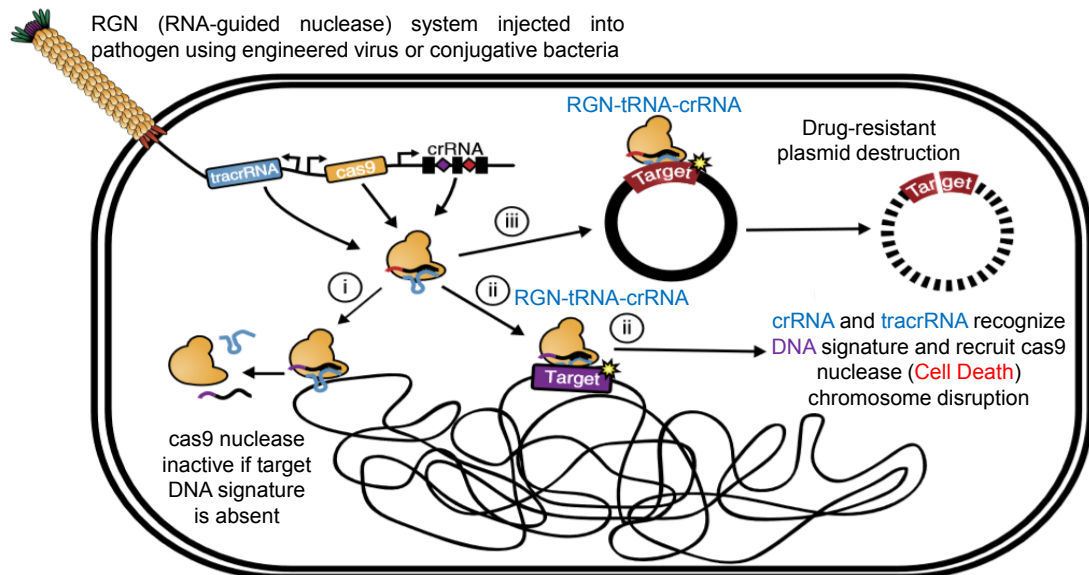


Figure 2-6: Engineering of human microbiome through selective destruction of pathogenic genetic signature. The figure is adapted from ²⁸ with permission.

The construction of functional biological systems involves development of standard biological parts, popularly termed as “Biobricks”. Biobricks are functional modular DNA sequences that are held in circular standardised plasmid vectors. Standardisation enables synthetic biologists to ligate various Biobrick parts together to form biological “devices” which are capable of performing a user-defined functions such as production of certain recombinant protein. Finally, a myriad of biological devices could be incorporated simultaneously into microbe cell chassis to perform more sophisticated tasks. This approach can be applied to generate novel biosensors for any molecules and the coupling of multiple environmental cues through design of biological logic gates pronounces specificity of the detection system. Thus, by regulating

the production of functional proteins under transcriptional or translational sensing elements, living cells can be remodelled into powerful micro-robots for a myriad of constructive applications.

Biobricks can be broadly classified into 4 categories by its function: promoter, ribosomal binding site (RBS), protein coding sequence and terminator. The promoter allows RNA polymerase to bind and initiate transcription upon detection of the transcription start site. Ribosomal binding site encodes genetic information for the binding of ribosomal subunits on mRNA transcript to activate translation. Protein coding sequences code for functional proteins that is largely associated with the application of interest. Terminator sequences stop transcription by forming a hairpin secondary structure that disrupts the DNA-mRNA-RNA polymerase ternary complex. Biobricks are standardised by designing each part to be flanked by precise “prefix” and “suffix” sequences. The prefix consists of restriction sites EcoRI, NotI and XbaI while the suffix consists of restriction sites SpeI, NotI and PstI. XbaI and SpeI are isocaudomeric, possessing different restriction enzyme recognition sites but generate similar termini upon cleavage (**Figure 2-7**). The introduction of XbaI and SpeI in prefix and suffix sequences enable biobricks, cleaved individually by XbaI and SpeI, to anneal and form longer composites and devices⁷⁶. In 2010, another standardised assembly method – the BglBrick assembly standard, was created using a different set of isocaudomeric restriction sites (EcoRI, BglII, BamHI and XhoI)⁷⁷. By comparison, the BglBrick assembly involves restriction enzymes that are unaffected by overlapping *dam* or *dcm* methylation and cut as effectively as the Biobrick restriction sites. However

unlike Biobrick assembly, the Bglbrick assembly creates innocuous in-frame scar sequences “ggatct” that encodes for glycine-serine which is more suitable for the construction of fusion proteins.

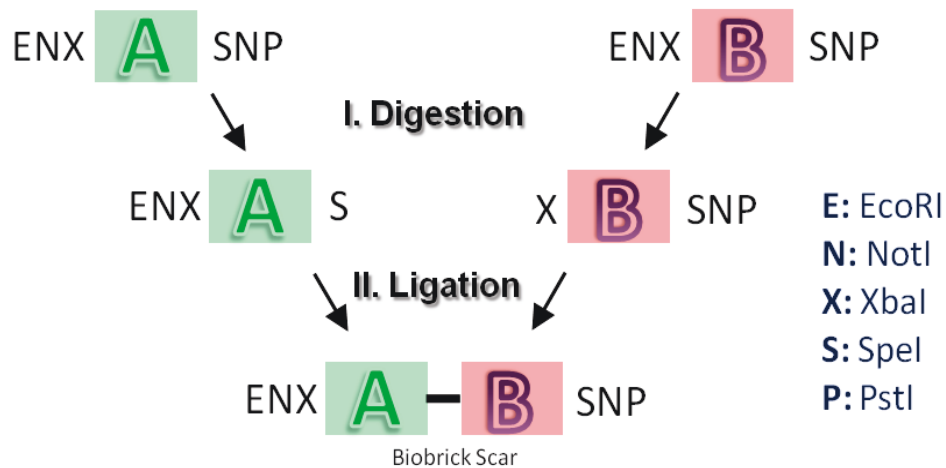


Figure 2-7: Biobrick assembly framework. This figure is adapted from the Registry of Standard Biological Parts (<http://parts.igem.org>).

The key design principles of synthetic biology are standardisation, characterisation, abstraction and decoupling⁷⁸. While standardisation allows the architecture of intricate systems from modular units, characterisation provides designers with critical information to optimise operational performance, durability and safety. The significance of characterisation is even more pronounced in engineering industries, such as the characterisation of surface catalysts to enhance reaction selectivity or the development of material safety data sheet to enforce process safety. By comparison, characterisation of standard biological parts aims to deliver the same purpose – to describe the intrinsic properties of discrete biobrick parts appropriately so as to enable controlled and predictable performance of synthetic biological

systems. Decoupling and abstraction hierarchy assumes that the development of a synthetic biological system can be redefined and modelled computationally in terms of system complexity. That is, an overall system is composed of several independent sub-systemic biological devices which are in turn assembled from orthogonal and modular biological parts. This engineering approach allows synthetic biologists to rationally design and construct biological systems without regards to specifications at other levels. Recent advents in synthetic biology have provided a unified foundational methodology for conducting characterisation experiments of biobrick parts^{76, 79}. Published works at present include the measurement of promoter activity for constitutive and inducible promoters, functional biological logic gates and quorum sensing elements, all of which are strategic tools in either transcriptional control or inter cellular communication.

2.5 Synthetic Gene Circuits and Logic Devices

One key aspect of synthetic biology is the engineering of biological networks⁸⁰. Genetic circuits are evolutionarily adapted by cells to coordinate diverse biological properties including metabolism and growth^{81, 82}, cell cycle⁸³, biofilm differentiation⁸⁴, cell-cell signalling⁸⁵, motility⁸⁶, virulence^{87, 88}, competitive fitness^{89, 90}, and molecular transport^{91, 92}. By and large, our current knowledge of genetic circuits and network topologies are acquired through a top-down systems biology approach which involve the genetic perturbations of model systems complemented with *de novo* mathematical and computational modelling⁹³. While these approaches have been effective in presenting a holistic understanding of cellular interaction maps, questions such as why

certain genetic architectures are preferred or ways to modify the sensitivity and specificity of input-output relationships of genetic circuits remain unanswered. Synthetic biology provides an alternative approach to understanding biology through the bottom-up design and assembly of simple, well-characterised genetic components into synthetic gene circuits. In the words of esteemed Nobel Prize winner *Richard Feynman* (1988), “*what I cannot create, I do not understand*”. Thus far, the synthetic biology design framework has been successfully applied to construct genetic circuits that enabled operations of genetic toggle switches⁹⁴⁻⁹⁶, oscillators^{95, 97-99}, bandpass filter¹⁰⁰, genetic amplifier¹⁰¹⁻¹⁰³, genetic clocks^{104, 105}, edge detector¹⁰⁶, pattern generators^{107, 108}, cellular counter¹⁰⁹ and memory devices^{101, 110}.

Design of robust and functional genetic circuits are more challenging as compared to conventional genetic engineering that requires minimal genetic manipulation^{111, 112}. In several studies, synthetic gene circuits failed to operate as desired when the preliminary design conditions were imbalanced^{94, 95, 108, 113}. That is, for coupled genetic circuits to generate the desired response at the system level, component regulators have to be precisely tuned to synchronise their behaviour with other modules of the system. This obstacle may be resolved through optimisation of genetic circuits with accurate modelling, parts libraries and directed evolution, although directed evolution is difficult to implement for oscillatory circuits without definite ON-OFF digital performance¹¹⁴⁻¹¹⁸. Second, modules that make up the genetic circuits have to be orthogonal and function independently when combined. In this respect, genome ‘parts mining’ have been applied to obtain novel biological parts from

unrelated microorganisms^{119, 120}. These biological parts are then extensively characterised or mutagenised to be orthogonal to chassis and other parts. Third, coupled genetic circuits may be subjected to “retroactivity” if component regulators at low quantity are sequestered to high-affinity promoters in downstream circuitry, resulting in changes to the dynamics of upstream modules⁷⁸. This effect can be significant in the case of oscillatory systems or in systems with both parallel and serial circuits. *Del Vecchio* and colleagues analysed the input-output dynamic characteristics of transcription regulators and suggested that the implementation of a feedback amplification loop to the upstream generation of the regulators may attenuate the effect of retroactivity^{121, 122}. Fourth, the performance of synthetic gene circuits can be affected by environmental, cellular and genetic context dependent effects including nutrient availability, aeration, toxic interactions, nitrogen and carbon metabolism, resource allocations and genetic architecture¹²³⁻¹²⁶. In this respect, orthogonal genetic modules can be independently characterised in the same cellular and environmental context before being interfaced together to enable better predictability of the engineered biological system¹²⁷. Additionally, RNA processing enzymes have been shown to be effective buffers against 5' untranslated region genetic-context dependent effects^{128, 129}. Nonetheless, much awaits to be discovered in this largely unexplored domain of synthetic biology.

Synthetic biological systems can be designed to perform analog or digital computation, or a combination of both as observed in natural systems^{130, 131}. Analog genetic circuits enable the processing of graded information with less

devices and lower consumption of cellular resources⁸⁰. By comparison, digital genetic circuits are significant in situations when decision making is required¹³². *Daniel et. al.* presented the first synthetic analog circuit by rewiring the amount of transcription factors to be logarithmically linear over broad range of inducer concentration (**Figure 2-8**). This was achieved through two features in the engineered circuit: (1) A positive feedback loop on a low copy plasmid to produce transcription factor (TF), thereby offsetting the saturated binding of inducer to TF, and (2) exacting the expression of output reporter under the control of TF in (1) on a high copy plasmid, thereby offsetting the saturation of promoter DNA binding sites. That is, the availability of DNA binding sites was designed to be in excess of inducer-TF complex and the availability of TF was designed to be non-saturating over the range of inducer added. In contrast, a control circuit without positive feedback loop to offset saturated inducer binding displayed nearly digital behaviour. Finally, the authors demonstrated that basic log-linear modules could be linked in various manner to achieve advanced analog operation, including multiplication, negative log and power law functions. Notably, these were achieved with only two transcription factors in each circuit, saving precious cellular resources.

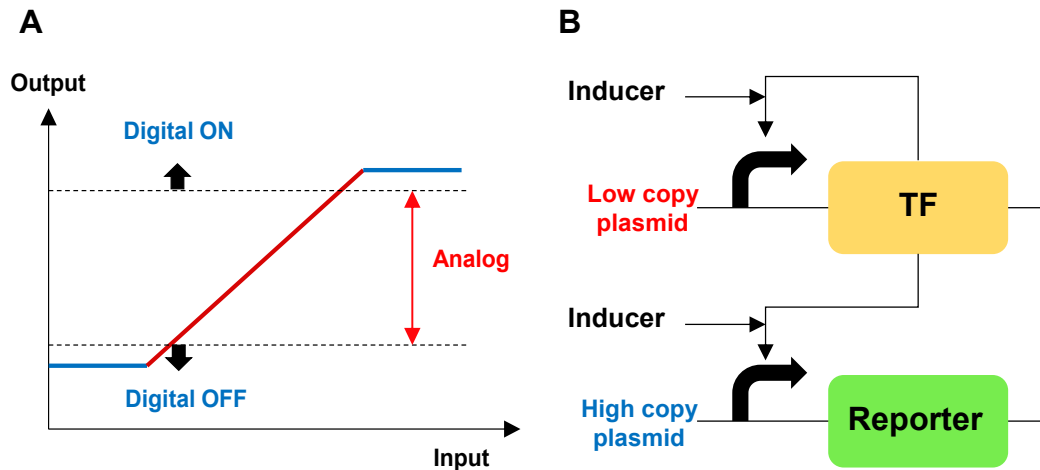


Figure 2-8: Synthetic gene circuits. (A) Graphical representation of analog and digital outputs. Analog genetic circuits process information over wide range of inputs while digital circuits discretize minima and maxima outputs as OFF and ON logics. (B) Simplified schematic of analog genetic circuit designed by *Daniel et. al* (2013). The figure is adapted from ¹³² with permission.

Digital genetic circuits are commonly associated with genetic switches and logic gates. A biological logic gate is a genetic interface that integrate and process input signals from two or more inducible genetic devices into a specific output signal (control of a cellular response). In 2007, the first biological AND gate was developed by *Anderson et. al.* to enhance the specificity of whole-cell biosensor¹³³. In this design, an input switch device (activated by input A) was used to regulate the expression of T7 RNA polymerase (T7RNAP) modified with two amber stop codons in the middle of their genes. Because T7RNAP are viral RNA polymerases, they are orthogonal to intrinsic genetic networks in host bacteria systems and are strong transcription drivers. A second input switch (activated by input B) was used to regulate the expression of *supD* which enabled the proper translation of T7RNAP. In particular, SupD tRNA mediates the translation of amber stop

codons into serine residues instead of stopping translation. Therefore, in the presence of both input A and B, functional T7RNAP would be produced to trigger the downstream expression of green fluorescent reporter (GFP) placed under the control of cognate pT7 promoter. In the presence of only input A, T7RNAP translation would be terminated prematurely. Likewise, the presence of input B alone would not permit the expression of T7RNAP transcript. Thus in both situations with only singular input, GFP was not observed.

Advances in structural and molecular biology studies of T7RNAP revealed that nicked N- (1-179) and C-terminal (180-880) fragments of T7RNAP could be non-covalently refolded into a functional protein during transcription initiation and that the binding specificity of T7RNAP to pT7 promoter library was dictated by a core region in the C terminus¹³⁴⁻¹³⁷. In 2013, *Shis* and *Bennett* exploited these properties to develop three transcriptional AND gates that were comparatively orthogonal to one another using mutant split T7RNAP¹³⁸. Importantly, this study shows that proteins which participate in DNA transcription could be engineered by fragmentation and mutagenesis to generate orthogonal logic gates, thereby expanding our capacity to program biological systems.

In 2014, *Schaerli* et. al. noted that simply relying on the non-covalent assembly of split T7RNAP led to reduced transcription activity and adopted a different approach to engineer split T7RNAP AND gate. Using a split-intein splicing strategy that had earlier been demonstrated for engineering logic gates in mammalian cells^{139, 140}, the split fragments of short-lived T7RNAP were fused

to *Npu* and *Ssp* DnaE inteins from *Nostoc punctiforme* and *Synechosystis* spp. to develop intein-spliced T7RNAP AND logic gate. In the presence of two inputs, split T7RNAP fragments generated from independently regulated genetic switches undergo post-translational protein splicing to restore a covalently repaired T7RNAP, resulting in gene expression from pT7 promoter.

Biological AND gates can also be designed using promoters that require specific chaperone-transcription activator pair to elicit DNA transcription. In this respect, two studies have separately explored the genetic regulatory systems in pathogenic bacteria type III secretion system (T3SS) to develop modular and orthogonal AND logic gates in layered genetic circuits^{120, 127}.

In 2011, *Wang et. al.* developed a functional AND logic gate in *E. coli* using codon-optimised chaperone and transcription activator isolated from the hyper sensitivity and pathogenicity regulon (σ 54-dependent HrpRS transcription system) in *P. syringae*¹²⁷. To achieve this, a library of synthetic gene circuits were assembled and extensively characterised to optimise the production rate of HrpR and HrpS transcription activators, thereby enabling near-digital performance of the biological AND gate. The authors also introduced a NOT gate module downstream of the HrpRS AND gate module to generate NAND logic outputs, thus presenting a modular approach to engineer genetic circuits.

Elsewhere, *Moon et. al.* looked to T3SS genomic clusters in *Salmonella typhimurium*, *Shigella flexneri* and *P. aeruginosa* for novel biological parts to develop three modular AND gates¹²⁰. To mitigate potential crosstalk between

AND gate modules and expand the dynamic range one of the AND gate promoter, directed evolution was applied to SicA chaperone and -10 region of *pipaH* promoter gene using saturation mutagenesis. The authors demonstrated orthogonality and robustness of the AND gate modules by layering them all together to perform 4-input AND logic operation, one of the finest synthetic gene circuits ever created in prokaryotic cell. Impressively, the 4-input AND digital circuit was built with 11 regulatory proteins and 3 AND gate modules that assimilated and processed input signals from 4 genetic switches. Genetic circuits with OR logic operations can be designed either by placing inducible promoters in tandem arrangement, or by regulating the expression of target gene in discrete expression cassettes.

In 2011, *Tamsir et. al.* successfully created three OR logic modules out of pBAD, pTET and pLAS promoters by positioning any two out of three inducible promoters in tandem¹⁴¹. To demonstrate versatility of the engineered OR gates, genetic NOT logic modules comprising of λ CI repressor and p λ CI promoter were then coupled downstream of OR modules to achieve single cell NOR logic operation. Using quorum sensing as chemical “wires” to mediate signal transduction between cells, NOR and NOT gate modules in 4 different cell population were connected into a synthetic cellular consortia with XOR computing power (**Figure 2-9**). Importantly, the work demonstrated how synthetic biology principles could be applied to program distributed computations in single cells, which could then be networked to meet higher-ordered objectives through cell-cell communication and division of labour.

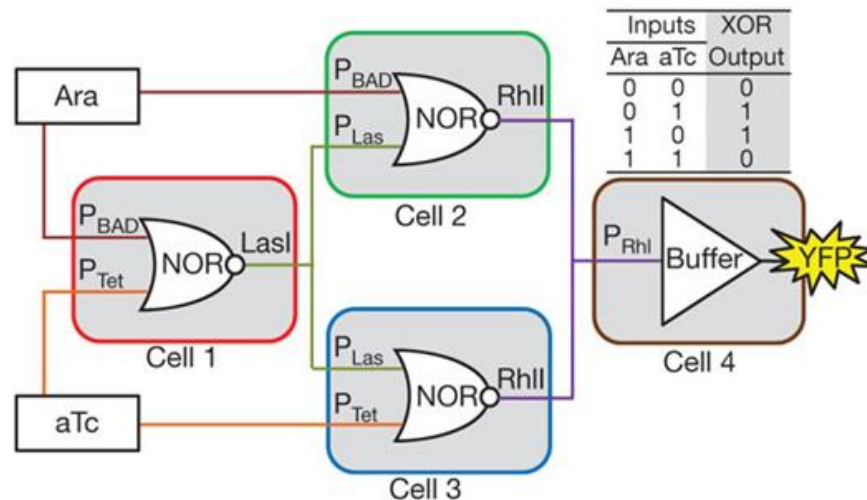


Figure 2-9: Simplified schematic of multi-cellular computing system connected by quorum sensing communication. The figure is reproduced from ¹⁴¹ with permission.

Alternatively, unidirectional recombinases can be used to program digital performance in prokaryotic cells with memory. In 2012, *Siuti et. al.* successfully programmed *E. coli* cells to perform up to 16 different types of two-input Boolean logic functions with only Bxb1 and phiC31 serine recombinases¹¹⁰. Specifically, inducible switches were used to regulate the expression of the Bxb1 and phiC3 recombinases, the production of which would catalysed irreversible site-specific DNA inversion. By flanking constitutive promoters, gene coding sequences and terminators with specific inversion sites recognised by target serine recombinases, robust and efficient DNA inversion could be effected. Cells programmed by serine recombinases retained DNA-encoded memory for at least 90 cell generations, providing a resourceful platform for therapeutic and diagnostic biotechnology¹⁴².

Chapter Three

3. METHODOLOGY

Synthetic biology principles have been applied to program human commensal microbe with novel molecular functions in simple plug and play mode.

In Section 3.1, the methods that were performed to assemble, characterise and evaluate the effectiveness of the engineered microbe in multi-cellular systems will be discussed. Specifically, the respective genetic modules that enabled the engineered *E. coli* to sense, lyse and release antimicrobial peptide pyocin S5, and the bactericidal activity of pyocin S5 were independently characterised. The effectiveness of the engineered *E. coli* in controlling the population of a clinical isolate of *P. aeruginosa* when both bacteria population were cultured together were also studied.

Section 3.2 describes the methods which were used to develop and characterise genetic logic gates that enabled engineered *E. coli* to function as biological microprocessor, including AND, OR, IMPLY, XOR, half adder and half subtractor logic operations. The logic gates developed in this study enabled *E. coli* to process multiple biosensing input signals and elicit the desired molecular responses, thereby allowing greater specificity in diagnosis and treatment.

Lastly, Section 3.3 describes a list of troubleshooting tips that can be applied to overcome certain experimental difficulties, such as cloning genetic parts with rich hairpin structures or to obtain microscopic images of better resolution. Part of this chapter was published in Springer Protocols, Humana Press¹⁴³. Additional supplementary information is presented in appendix A.

3.1 Therapeutic Microbe against *Pseudomonas* Infection

3.1.1 Design of Therapeutic Microbe against *P. aeruginosa*

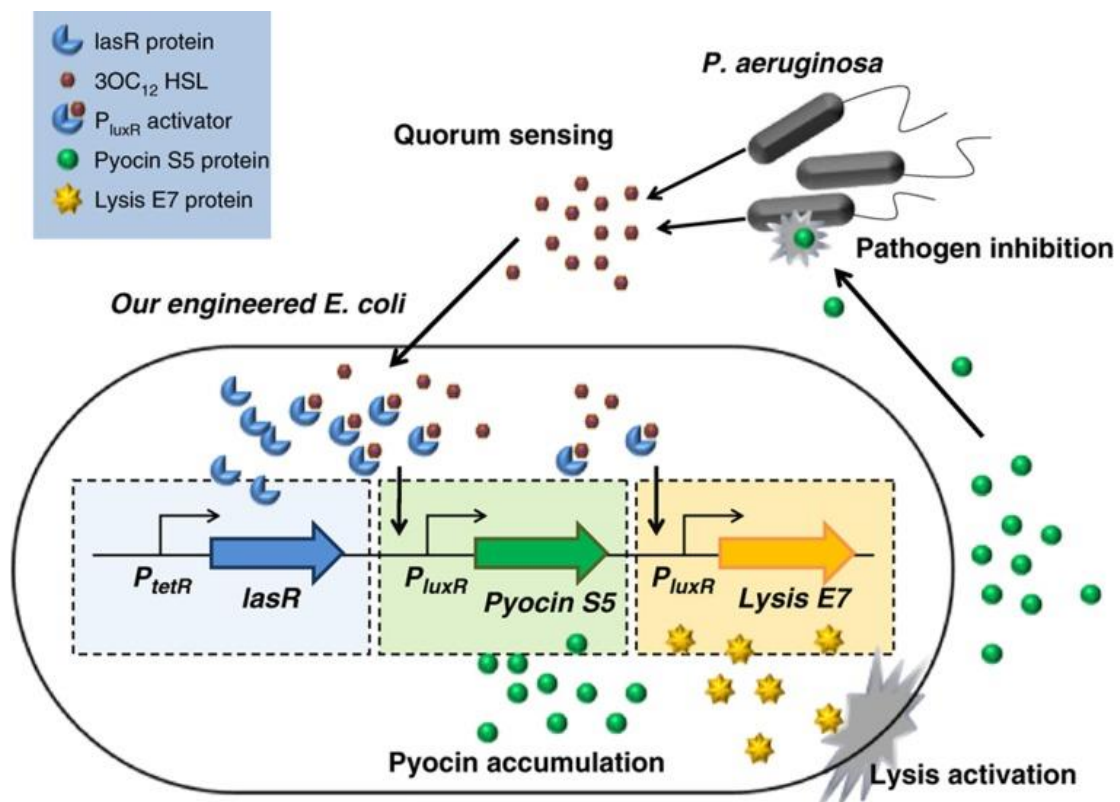


Figure 3-1: Schematic of *Pseudomonas* Sense and Kill system. 3OC₁₂HSL signalling molecules are intercepted and recognised by the sensing device of the engineered *E. coli*, leading to subsequent expression and production of Pyocin S5 and E7 lysis protein. Accumulated pyocin S5 are released into the extracellular environment when *E. coli* host lysed itself, resulting in the eradication of *P. aeruginosa*. The figure is reproduced from ¹⁴⁴ with permission.

To expand the ability of gut microbes to defend against *P. aeruginosa* infection, commensal bacteria could be repurposed with novel functions that enabled them to sense quorum sensing molecules from *P. aeruginosa*, synthesize killing molecules which are specific to the pathogen, and release the accumulated killing molecules through self-lysis to eradicate the pathogen¹⁴⁴. Thus, to develop a synthetic biological system with such high level specifications, *E. coli* was genetically reprogrammed with sensing, killing and self-lysis modules. **Figure 3-1** shows a schematic of the sensing and killing genetic system.

The sensing device was designed based on the more dominant Type I quorum sensing mechanism of *P. aeruginosa*. The *tetR* promoter, which is constitutively on, produces a transcriptional factor, LasR, that binds to AHL 3OC₁₂HSL. The *luxR* promoter, to which LasR-3OC₁₂HSL activator complex reportedly binds, was adopted as the inducible promoter in the sensing device¹⁴⁵. Importantly, previous studies had estimated extracellular concentration of 3OC₁₂HSL to be between the ranges of 1E-06 to 1E-04 within proximity to the site of infection^{146, 147}.

We utilised E7 lysis protein to lyse the *E. coli* chassis. The E7 lysis protein is a key component of the SOS response system in colicin-producing cells and functions to export bacteriocins into the extracellular space under stressful environmental conditions¹⁴⁸. Recent studies have shown that the E7 lysis protein is effective in causing inner membrane damage and maybe associated with the activation of outer membrane phospholipase A (OMPLA) for outer

membrane modification¹⁴⁹. Formation of the LasR-3OC₁₂HSL complex, which binds to the *luxR* promoter, activates the killing and lysing devices, leading to the production of pyocin S5 and E7 lysis proteins within the *E. coli* chassis. Upon reaching a threshold concentration, the E7 lysis protein perforates membrane of the *E. coli* host and releases the accumulated pyocin S5. Pyocin S5, which is a soluble protein, then diffuses toward the target pathogen and damages its cellular integrity, thereby killing it.

3.1.2 Gibson Assembly

Gibson assembly is an isothermal ‘multi-pot’ cloning technique that harnessed the collective enzymatic actions of T5 exonuclease, Phusion DNA polymerase and Taq DNA ligase to assemble genetic constructs of up to several hundred kilobases. Originally developed for the assembly of synthetic genomes, it is now widely used for fast and efficient pathway reconstruction¹⁵⁰. In this demonstration, the final system (QS-S5-E7) was constructed using a customized protocol of Gibson assembly technique (**Figure 3-2**).

1. An *in silico* design of the overall genetic construct was performed on a plasmid drawing software.
2. Primers for PCR amplification of linear inserts and vector were designed and synthesized to achieve fragment sizes of 300 bp – 5 kb (see **Note 2**). Of note, primers were designed to incorporate between 20 bp – 40 bp of overlapping sequences. Spacer sequences were added where necessary (see **Note 3,4**).

Table 3-1. PCR reaction composition

Initial Concentration	Volume per 40 μ l reaction	Final amount in 40 μ l
Sterile ddH ₂ O	up to 40 μ l	
Phusion PCR buffer (5 $\times$)	8 μ l	1 $\times$
DMSO (100%)	2 μ l	5 $\times$
dNTP mix (40mM)	0.8 μ l	0.8 mM
Forward primer (10 μ M)	0.8 μ l	0.5 μ M
Reverse primer (10 μ M)	0.8 μ l	0.5 μ M
DNA template (1-5 ng)	variable	5-20 ng
Phusion High-Fidelity DNA polymerase (2U μ l ⁻¹)	0.4 μ l	1 U

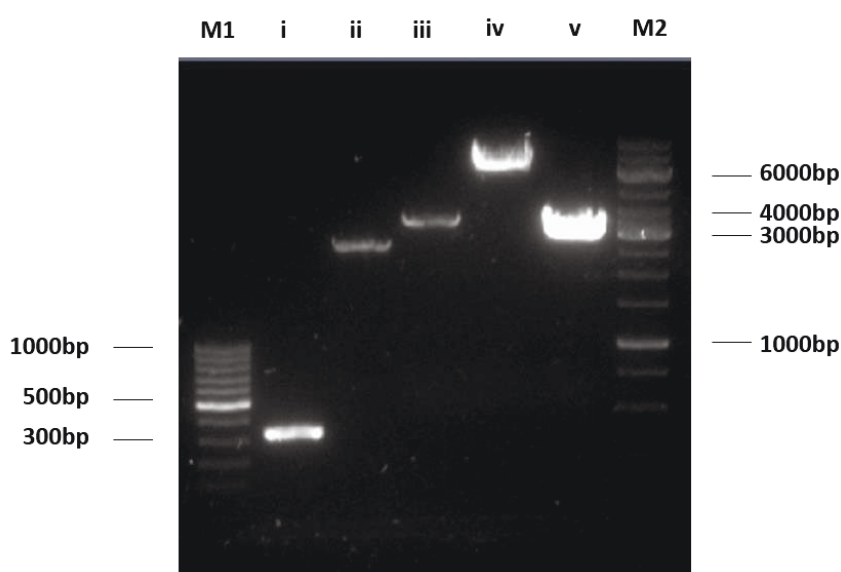


Figure 3-2: Gibson assembly of Sense-Kill system QS-S5-E7. The image shows gel electrophoretic analysis of the final assembled construct and the associated PCR-verified modules: (i) E7 insert ~320 bp, (ii) QS-S5 ~2.8 kb, (iii) linearized vector ~3.5 kb, (iv) linearized QS-S5-E7-vector ~6.6 kb and (v) double digested fragments of QS-S5-E7 ~3.1 kb and linearized vector ~3.5 kb. Electrophoretic separation was performed with approximately 300 ng of DNA on a 0.8% w/v agarose gel at 120 V for 30 min. (M1) NEB 100 bp and (M2) NEB 1 kb DNA ladder.

3. PCR reaction tubes were set up on ice as described in **Table 3-1**. Phusion High-Fidelity DNA polymerase were used for the amplification of long DNA fragments.
4. PCR reactions were performed in a thermocycler with cycling conditions as described in **Table 3-2**. Of note, successful PCR products were generated using a two-cycle PCR approach. During the first cycle, DNA template was enriched for 5 cycles by using the T_m which overlapped with the source DNA template to determine the annealing temperature. The second cycle adopted a higher T_m of the enriched template for more specific priming of target amplicons. Here, I demonstrate the amplification of three DNA fragments using source templates from open plasmid repository, with sizes of 2.8 kb (QS-S5), 320 bp (E7) and 3.5 kb (vector).
5. The amplified DNA fragments were separated on a 0.8% w/v agarose gel by gel electrophoresis (85 V, 45 min).

Table 3-2. PCR cycling conditions

Cycle No.	Denaturation	Annealing	Extension
1	98 °C, 30 s		
2 – 6 (1 st cycle)	98 °C, 10 s	$T_{m1} + 3\text{ °C}$, 30 s	72 °C, 15 s/kb
7 – 26 (2 nd cycle)	98 °C, 10 s	$T_{m2} + 3\text{ °C}$, 30 s	72 °C, 15 s/kb
27			72 °C, 5× of extension

7. DNA bands of interests were visualised on a blue light transilluminator and isolated with a sterile scalpel. The isolated DNA fragments were then purified with QIAquick Gel Extraction Kit according to vendor's instruction and analysed with Nanodrop. Of note, good DNA concentrations above 20ng/μl were ideal for efficient Gibson assembly reactions (see **Note 5**). Purified DNA fragments were kept in ice bath until use.
8. A 15 μl aliquot of 1.33× assembly master mix was thawed on ice for 5 min.
9. Equimolar amounts of DNA inserts and vector (total DNA concentration of up to 100 ng) were added to 15 μl of assembly master mix. Nuclease-free water were then added to a total reaction volume of 20 μl. 20 ng of linearized vector DNA was used during the assembly process. The amount of insert required could be empirically calculated by **Eqn. 3.1**.

Mass of insert(ng)

$$= \frac{\text{Length of insert (bp)}}{\text{Length of vector (bp)}} \times \text{Mass of vector (ng)} \quad \text{Eqn. (3.1)}$$

10. The reaction mixture was incubated at 50 °C in a thermocycler with heated lid for 1 h. The temperature inversion was necessary to avoid evaporation and the consequential loss of enzymatic activity. On completion, the reaction tube was held on ice for at least 15 min.
11. Next, 50 μl aliquot of chemically competent *E. coli* cells were thawed for 5 min on ice. 5 μl of the reaction mixture were then transformed into *E. coli* cells by 45s heat shock at 42 °C and 5 min incubation on ice. 400 μl of prewarmed SOC were added. Following, the entire transformation culture was transformed to a sterile round bottom capped tube and incubated in a shaking incubator set at 225 rpm and 37 °C for 40 min.

12. After transformation, 1/5 of the culture (~100 µl) were spread on an antibiotic-selective plate. The remaining 4/5 of the culture were diluted in 100 µl of SOC and plated on another antibiotic-selective plate.
13. Plates were incubated in a 37 °C incubator for 15 h and screened the next day by colony PCR.

3.1.3 Characterisation of Quorum Sensing Device

To evaluate the sensitivity and range of the quorum sensing device in an *E. coli* host, we cloned a GFP reporter downstream of the sensing device (QS-GFP) and measure the fluorescence output after induction with synthetic 3OC₁₂HSL molecule. Consequently, the characterisation profile of this biosensor can be used to estimate the amount of 3OC₁₂HSL that is secreted by *P. aeruginosa* clinical isolates. M9 medium was supplemented with appropriate antibiotic throughout this experiment unless otherwise stated.

1. A single colony of *E. coli* with the plasmid QS-GFP was inoculated from a fresh plate into 5 ml of M9 characterisation medium. Following, the culture was incubated in a 37 °C shaking incubator set at 225 rpm for 15 h.
2. After 15 h of incubation, the culture was diluted 100× in 10 ml of prewarmed M9 characterisation medium. The re-inoculated culture was then allowed to grow to OD₆₀₀ of 1.0 under the same conditions. This corresponded to mid-log phase of the cells.
3. While waiting for the re-inoculated culture to grow to the desired cell density, a 96-well microplate was prefilled with 110 µl of fresh M9 characterisation medium. 3OC₁₂HSL test solutions (0.1 µM, 1 µM, 10 µM, 0.1 mM and 1 mM) were vortexed evenly. 1 – 2 µl of each 3OC₁₂HSL

solution were then added into the media so that the final 3OC₁₂HSL concentrations in the 96-well microplate were in the range of 0 – 10⁻⁵ M.

4. Empty wells were filled with 220 µl of M9 characterisation medium to serve as both sterility control and liquid reservoir. A column of wells was also allocated for zeroing with 220 µl of M9 characterisation medium. The microplate was then prewarmed in a 37°C incubator for 30 min just before use. Of note, the microplate was covered with lid and sealed tight with parafilm when outside a biological hood or Bunsen flame.
5. The Synergy HT Multi-Mode Microplate Reader was prepared for end-point measurement of absorbance (OD₆₀₀) and fluorescence (485 nm excitation / 528 nm emission) at regular intervals of 5 min, with 30 s of rapid shaking before sampling. Temperature was set to 37 °C. Advance users could further program Biotek's Gen5 Data Analysis software to obtain triplicate average of background subtracted fluorescence per OD₆₀₀. Otherwise, data analysis could be performed in an exported excel sheet.
6. On reaching the desired cell density, the reinoculated culture was poured into a sterile petri dish. 110 µl of the re-inoculated culture was transferred into the prefilled microplate with a multi-channel pipette, effectively diluting the culture to OD of 1. The culture was evenly mixed by up and down pipetting actions, before being sealed with a transparent sealing film.
7. The microplate was transferred into the microplate reader and analysed for both fluorescence and absorbance for 3 h.
8. Raw experimental data were then into MS Excel. The fluorescence (RFU) and absorbance (OD₆₀₀) values of each well were normalised by zeroing with the average of pure M9 characterisation medium as shown in **Eqn. 3.2**

and **3.3**. The relative quantities of GFP molecules in single cells were derived as a ratio of background subtracted fluorescence to OD₆₀₀ values (RFU/OD₆₀₀).

Normalised Fluorescence of sample x at time t,

$$RFU_t^{x'} = RFU_t^x - RFU_t^{M9} \quad \text{Eqn. (3.2)}$$

Normalised OD₆₀₀ of sample x at time t,

$$OD_t^{x'} = OD_t^x - OD_t^{M9} \quad \text{Eqn. (3.3)}$$

9. Resultant values of RFU/OD₆₀₀ were averaged from technical triplicates. At least 3 independent experiments were performed in this study. Of note, only analysed data from independent experiments (biological replicates) were used to determine the standard deviations of statistical means.

10. The GFP production rate (RFU.OD₆₀₀⁻¹min⁻¹) was derived by taking the difference of RFU/OD₆₀₀ values from two time-points and dividing the result by the time interval δt as shown in **Eqn. 3.4**.

GFP Production Rate of sample x at time t,

$$\frac{RFU}{OD \cdot t} = \frac{RFU_{t+\delta t}^{x'}/OD_{t+\delta t}^{x'} - RFU_t^{x'}/OD_t^{x'}}{\delta t} \quad \text{Eqn. (3.4)}$$

11. Analysed data was plotted on a 3D plot using Matlab, setting 3OC₁₂HSL concentration, time and GFP production rate as the x-y-z axis respectively.

12. The period to which steady state GFP production rate was observed was determined with the 3D graphical plot obtained in **Step 10**. The mean GFP production rate were then calculated by averaging the numerical values of all GFP production rates within this identified period (see **Note 6**). The experimental results were then fitted to an empirical mathematical model

as shown in **Eqn. 3.5** using Matlab Curve Fitting Toolbox where A, B, C and n were curve-fitted empirical parameters.

GFP Production Rate,

$$Y = A + \frac{B [3OC_{12}HSL]^n}{C^n + [3OC_{12}HSL]^n} \quad \text{Eqn. (3.5)}$$

3.1.4 Estimation of Native Autoinducer Produced by *P. aeruginosa*

This protocol describes a method to measure the extracellular homoserine lactone produced by *P. aeruginosa* clinical isolates using the engineered biosensor QS-GFP¹⁵¹.

1. A single colony of *E. coli* carrying QS-GFP was inoculated into 5 ml of M9 characterisation medium separately. The culture was then incubated in a 37 °C shaking incubator set at 225 rpm for 15 h.
2. **Step 1** was repeated to grow all *P. aeruginosa* isolates separately.
3. After 15 h of incubation, the *E. coli* culture was diluted 100× in 20 ml of prewarmed M9 characterisation medium. Re-inoculated cultures of *E. coli* (QS-GFP) were allowed to grow to OD₆₀₀ of 0.5. Cultures were then held on ice until use.
4. Each *P. aeruginosa* culture from **Step 2** was diluted 100× in 5 ml of prewarmed M9 characterisation medium. Re-inoculated *P. aeruginosa* cultures were grown to OD₆₀₀ of 1.0, under the same conditions. Cultures were then held on ice until use.
5. The Synergy HT Multi-Mode Microplate Reader was prepared for end-point measurement of absorbance (OD₆₀₀) and fluorescence (485nm excitation / 528nm emission) at regular intervals of 5 min, with 30 s of rapid shaking

before sampling. Temperature was set to 37°C. Advance users could further program Biotek's Gen5 Data Analysis software to obtain triplicate average of background subtracted fluorescence per OD₆₀₀. Otherwise, data analysis could be performed in an exported excel sheet.

6. 600 µl of each *P. aeruginosa* culture was filter sterilised with 0.22 µm filter. The filtrates were then transferred to a 96-well microplate in aliquots of 100 µl to generate 1× stocks of *P. aeruginosa* 3OC₁₂HSL. 0.1× stocks of *P. aeruginosa* 3OC₁₂HSL were obtained by diluting 10 µl of aliquot cultures in 90 µl of M9 characterisation medium.
7. 200 µl of re-inoculated *E. coli* (QS-GFP) culture was transferred into a new 96-well microplate with a multi-channel pipette. Then, *E. coli* culture in each well was induced with 20 µl of 1× and 0.1× filtrates of *P. aeruginosa* cultures from **Step 4**. This effectively induced QS-GFP with 10× and 100× diluted *P. aeruginosa* filtrate.
8. **Steps 7 – 10 in Section 3.2.2** were repeated.
9. The concentration of 3OC₁₂HSL in each *P. aeruginosa* filtrate was determined by using the empirical transfer function equation in **Eqn. 5**.

3.1.5 Characterisation of Lysis Device

This protocol describes a method to characterise E7 lysis release device using high throughput absorbance assay and Bradford protein quantification. The protocol can also be applied to study the dynamics of other lytic systems including, bacteriocin release proteins of cloacin DF13, colicin E1, E3, A and D phage holin and endolysin proteins.

1. Single colonies of *E. coli* carrying the plasmid QS-E7 and QS-E7-S5 were separately inoculated into 5ml of M9 characterisation medium. Each culture was incubated in a 37 °C shaking incubator set at 225 rpm for 15 h.
2. After 15 h of incubation, each culture was diluted 100× in 10 ml of prewarmed M9 characterisation medium. The re-inoculated culture was allowed to grow to OD₆₀₀ of 1.0 under the same conditions. This corresponded to mid-log phase of the cells.
3. While waiting for the re-inoculated culture to grow to the desired cell density, a 96-well microplate was prefilled with 110 µl of fresh M9 characterisation medium. 3OC₁₂HSL test solutions (0 µM, 1 µM, 0.1 mM and 10 mM) were evenly vortexed. Then, 2 µl of each 3OC₁₂HSL solution were added in triplicates so that the final 3OC₁₂HSL concentrations in the 96-well microplate were in the range of 0 – 10⁻⁴ M.
4. Empty wells were filled with 220 µl of M9 characterisation medium to serve as both sterility control and liquid reservoir. A column of wells was allocated for zeroing with 220 µl of M9 characterisation medium. The microplate was then prewarmed in a 37 °C incubator for 30 min just before use. Of note, the microplate was covered with lid and sealed tight with parafilm when outside a biological hood or Bunsen flame.
5. The Synergy HT Multi-Mode Microplate Reader was prepared for end-point measurement of absorbance (OD₆₀₀) at regular intervals of 10 min, with 30 s of rapid shaking before sampling. Temperature was set to 37 °C. Advance users could further program Biotek's Gen5 Data Analysis software to obtain triplicate average of background subtracted fluorescence

per OD₆₀₀. Otherwise, data analysis could be performed in an exported excel sheet.

6. On reaching the desired cell density, the reinoculated culture was poured into a sterile petri dish. Next, 110 µl of re-inoculated culture was transferred into the prefilled microplate with a multi-channel pipette, effectively diluting the culture to OD of 0.5. The culture was evenly mixed by up and down pipetting actions, before being sealed with a transparent sealing film.
7. The microplate was transferred into the microplate reader and analysed for absorbance for 6 h.
8. Raw experimental data were exported into MS Excel. The absorbance (OD₆₀₀) value of each well was normalised by zeroing with the average of pure M9 characterisation medium as shown in **Eqn. 3**.
9. To characterise the efficiency of the lysis device in mediating pyocin release, QS-S5-E7 and QS-S5 plasmids were first labeled with hexahistidine tags on the 3' terminus of S5 gene with Phusion *DNA* polymerase and transformed into *E. coli* Top10. Overnight cultures of the His-tag version of QS-S5-E7 and QS-S5 were then diluted in LB and harvested at an OD₆₀₀ of 0.7. The collected cultures were induced with 1.0E-6 M 3OC₁₂HSL and incubated for 6 hours in a shaking flask culture set at 37 °C and 170 rpm. At regular intervals of 2 hours, cell cultures were drawn and filter sterilized. The filtered cultures were mixed with 1/10 volume of 100% trichloroacetic acid and incubated on ice for an hour to allow protein precipitation, before being washed with an equal volume of acetone. Precipitated proteins were reconstituted in 1 ml of reconstitution solvent and purified by immobilized metal affinity chromatography using Vivapure

miniprep MC according to the manufacturer's instruction. Finally, purified pyocin proteins were analysed by SDS PAGE¹⁵² and Bradford assay¹⁵³.

3.1.6 Electron Microscopy Analysis of *E. coli* Cytoplasmic Release

This protocol describes a method to visually inspect the extent of lysis and cytoplasmic release of *E. coli* with scanning electron microscopy. The fixation method discussed here can also be used for surface morphology studies of other bacteria species.

1. Single colonies of engineered *E. coli* (QS-S5-E7) and control *E. coli* (QS-S5) were inoculated into cell culture tubes each with 10ml of LB plus ampicillin. The cell cultures were incubated in a 37 °C shaking incubator set at 225 rpm for 15 hours.
2. Each culture was then diluted to OD₆₀₀ ~0.01 in 10 ml of LB plus ampicillin and grown to a final cell density of OD₆₀₀ ~0.5 in the same conditions as described above.
3. Optional. The *E. coli* cultures were further diluted 10× in 10 ml of LB plus ampicillin and grown to a final cell density of OD₆₀₀ ~0.5 (see **Note 7**).
3. Next, both the engineered and control *E. coli* were induced by adding 2 µl of 0.1 mM 3OC₁₂HSL to 2 ml of each culture. The induced *E. coli* cultures were incubated in a 37 °C shaking incubator set at 225 rpm for 2 h. This experiment was performed in duplicates.
4. After 2 h of induction, the optical density of each culture was determined. The optical density of control *E. coli* culture should be significantly larger than the engineered *E. coli*. Next, each sample was transferred to sterile 2

ml microcentrifuge tubes and centrifuged at $3000 \times g$ and 4°C for 10 min.

The supernatant from each sample was discarded.

5. The cell pellet from each sample was washed with 1 ml of 0.1 M sodium cacodylate and resuspended gently by pipetting. The washed samples were centrifuged at $3000 \times g$ and 4°C for 10min, with the supernatant discarded. This washing procedure was repeated thrice.
6. Primary fixation was performed by gently resuspending the cell pellet obtain from **Step 7** with 1 ml of 2.5% w/v glutaraldehyde in 0.1 M sodium cacodylate. Fixed samples were incubate at 4°C for 2 h or overnight.
7. The washing procedures in Step 7 were repeated with 1 ml of 0.1 M sodium cacodylate thrice.
8. The cell pellet obtain from **Step 9** was gently resuspended in 0.1 M sodium cacodylate (10 – 50 μl) (see **Note 8**).
9. PEI-coated silicon slide was then placed on a sterile petri dish with a tweezer.
10. 2 μl of engineered cell culture (QS-S5-E7) from **Step 10** was spotted on a predefined edge of a PEI-coated silicon slide. Similarly, 2 μl of control cell culture without the lysis device (QS-S5) was spotted on the edge diagonally across the same slide. The petri dish was covered with lid and incubated at 25°C for 30min.
11. Optional (secondary fixation). The loaded slide was immersed into a well on 24-well microplate that contained 1 ml of 1% w/v osmium tetroxide in 0.1 M sodium cacodylate and incubated at 25°C for 90 min (see **Note 9**).
12. Other wells on the 24-well microplate were filled with 1 ml of ethanol solution at various concentration v/v (37%, 67%, 95%, 100%, 100% and

100%). The loaded silicon slide was then dehydrated by immersing the slide in serial concentration of absolute ethanol. Dehydration was implemented at 25 °C for 15 min (see **Note 10, 11**).

13. The slide was subsequently dried in a vacuum dryer overnight set at 25 °C.

14. The biological samples were coated with 20 nm of gold-palladium alloy with an osmium plasma coater and examined using a field emission scanning electron microscope at 10 kV.

3.1.7 Overlay Inhibition Assay

The overlay inhibition assay described here provides a method for the *in vitro* evaluation of antimicrobial efficacy of pyocin produced and released by an engineered *E. coli*. The protocol may be modified accordingly to identify the minimum inhibitory concentration (MIC) of other antimicrobial compounds⁶⁹.

1. Single colonies of *P. aeruginosa* isolate In7, engineered *E. coli* (QS-S5-E7) and control *E. coli* (QS-S5) were inoculated into cell culture tubes each with 10 ml of LB plus ampicillin. The cell cultures were incubated in a 37 °C shaking incubator set at 225 rpm for 15 h.
2. Each culture was then diluted to OD₆₀₀ ~0.1 in 10 ml of LB plus ampicillin and grown to a final cell density of OD₆₀₀~1.0 in the same conditions as described above.
3. 10 ml of In7 culture was separated into 2 ml and 8 ml cultures, respectively. Re-inoculated *E. coli* cultures (QS-S5-E7 and QS-S5) and 2ml culture of In7 culture were then held on ice until use. This procedure prevented growth and preserved a constant cell density temporarily.

4. 8 ml of the re-inoculated In7 culture was centrifuged at $3000 \times g$ for 10 min. The supernatant was sterilized with a $0.2 \mu m$ membrane filter, and the filtrate was then transferred into a labelled round bottom capped tube. Filtered supernatants containing 3OC₁₂HSL from *P. aeruginosa* could be stored at $-20^{\circ}C$ for up to a week.
5. Engineered and control *E. coli* cells were harvested by centrifuging each culture at $3000 \times g$ for 5 min and discarding the supernatants. Both the engineered and control *E. coli* were then induced by adding the same volume of filtered In7 supernatant as the discarded supernatant of *E. coli* in new culture tubes. The induced *E. coli* cultures were incubated in a $37^{\circ}C$ shaking incubator set at 225 rpm for 5 h. This experiment was performed in duplicates.
6. A TSA plate was prewarmed in a $37^{\circ}C$ incubator for 15 min.
7. Soft agar in a bottle was melted in a microwave for 2 min. The bottle was cooled under running tap water while the agar was maintained in a liquid state. 2.7 ml of soft agar was further to a round bottom capped tube and held in a water bath set at $55^{\circ}C$.
8. 100 μl of In7 culture at OD₆₀₀ ~ 0.2 was added to 2.4 ml of soft agar and uniformly mixed by vigorous shaking.
9. 2.5 ml of In7 soft agar culture was transferred onto a prewarmed TSA plate and uniformly spread by tilting the plate. The plate was covered with lid and the agar overlay was allowed to solidify at room temperature for 1 h. During this process, the agar plate was left on a horizontal surface that was verified with a water leveller.

10. 10 μ l of filtered supernatants from induced engineered *E. coli* and control *E. coli* cultures were spotted onto the In7 agar overlay and left to dry in a biological hood for 1 h.
11. The plate was incubated in a 37 °C incubator for 6 h or more, and then analysed with ChemiDoc XRS bio-imager on epiwhite mode or with a high resolution digital camera.

3.1.8 Live and Dead Fluorescent Microscopy

This protocol describes a qualitative method to assess the effectiveness of engineered *E. coli* in killing *P. aeruginosa*.

1. Overnight cultures of In7 (*P. aeruginosa*) and QS-S5-E7 (*E. coli* Top10) were diluted in LB and harvested at an OD₆₀₀ of 0.5 and 1.0 respectively.
2. 3OC₁₂HSL from In 7 were obtained after passing In7 culture through a filter membrane (0.22 μ m) and the sterile filtrate was used to induce expression of engineered system by mixing it with QS-S5-E7 *E. coli* in 1:1 mixing ratio to a total volume of 2 ml.
3. Resultant culture was grown for 3 hours and filtered with a similar membrane to obtain sterile S5 filtrate.
4. The filtrate was mixed with In7 at OD₆₀₀ of 1.0 in 1:1 mixing ratio to a total volume of 2 ml and incubated for 3 hours.
5. 1 ml of the final culture was stained with bacterial viability kit according to the manufacturer's instruction and analysed with a fluorescent microscope.

3.1.9 Co-culture Inhibition Assay

The co-culture inhibition assay discussed here provides a method to determine the ratio of engineered *E. coli* (EC) to *P. aeruginosa* (PA) cells that was required to completely inhibit the proliferation of *P. aeruginosa*. The protocol may be modified accordingly for the co-culture of other cell lines.

1. Single colonies of *P. aeruginosa* isolate In7 (with plasmid pMC-PA_{GFP/CM}) and engineered *E. coli* (QS-S5-E7) were inoculated into cell culture tubes each with 10 ml of LB plus ampicillin. The cell cultures were incubated in a 37 °C shaking incubator set at 225 rpm for 15 h.
2. Each culture was diluted to OD₆₀₀ ~0.1 in 10 ml of LB plus ampicillin and grown to a final cell density of OD₆₀₀ ~1.0 in the same conditions as described above.
3. In7 and engineered *E. coli* cultures were mixed in round bottom capped tubes as described in **Table 3-3** on ice (see **Note 12**). The mixed cultures were topped up to a total volume of 2 ml with LB plus ampicillin.
4. The mixed cultures were incubated in a 37 °C shaking incubator set at 225 rpm for 12 h.
5. The Synergy HT Multi-Mode Microplate Reader was prepared for end-point measurement of fluorescence (485 nm excitation / 528 nm emission).
6. At regular intervals of 3 h, 100 µl aliquots of the mixed cultures were transferred into a 96-well microplate for a total of two technical replicates. The 96-well microplate was then transferred to the microplate reader and analysed for background subtracted green fluorescence with pure LB plus ampicillin as the zeroing medium.

Table 3-3. Mixing ratio of *P. aeruginosa* and engineered *E. coli* in co-culture inhibition assay

<i>P. aeruginosa</i> (ml)	<i>E. coli</i> (ml)	LB plus ampicillin (ml)	EC/PA Ratio
0.2	0	1.8	0
0.2	0.2	1.6	1
0.2	0.4	1.4	2
0.2	0.6	1.2	3
0.2	0.8	1	4

7. Optional. The mixed cultures were serially diluted in the 96-well plate 10× with pure LB plus ampicillin. CFU count was then performed on a chloramphenicol selective LB agar plate. Only *P. aeruginosa* cells which carried the chloramphenicol resistance marker would develop into viable colonies.
8. The mixed cultures were topped up with 200 µl of fresh LB plus ampicillin so that total volume of each co-culture was maintained at ~2.0 ml. The mixed cultures were incubated in the 37 °C shaking incubator with the same settings as described above.
9. Normalised results were expored into MS Excel and a graph of relative green fluorescence (in RFU) was plotted against time (h). The optimal EC/PA ratio in preventing the growth of *P. aeruginosa* could be identified with a relatively constant GFP expression profile in the co-culture (see **Note 13**).

3.1.10 Biofilm Inhibition Assay

This protocol provides a method to assess the effectiveness of engineered *E. coli* with Sense-Kill function (QS-S5-E7) in preventing the formation of *Pseudomonas* biofilm. The protocol may be modified accordingly for other co-culture systems.

1. **Steps 1 – 2** of **Section 3.2.8** were repeated with MHB ampicillin instead of LB.
2. Engineered *E. coli* and *P. aeruginosa* isolate In7/PAO1 from **Step 1** were mixed in the ratio 4:1 with a total cell density of 1.0×10^{-8} cfu/ml. The mixed culture was gently mixed with a vortex.
3. The co-culture mixture from **Step 2** was transferred into a sterile 12-well microplate in 4 technical aliquots of 1 ml each. A transparent glass coupon was immersed at 45° into the well, allowing only half of the coupon to be in contact with the co-culture. The microplate was sealed and incubated at 37 °C at 150 rpm for 18 h.
4. Glass coupons with intact biofilm were removed from the microplate with a tweezer. Planktonic cells were washed off by gently dipping the coupons in sterile water completely twice.
5. Spent media in the 12-well microplate were disposed, leaving behind only biofilm cells (almost). Each well was further washed with 2 ml of sterile water twice to remove planktonic cells. The washing step was performed with extra care to minimise mechanical disruption to biofilm structures.
6. Each well was filled with 1 ml of fresh MHB chloramphenicol. Biofilm cells were recovered in the fresh media by sonication or vigorous pipetting.

7. Biofilm cells form in each well were quantified by counting the number of viable cells on LB agar plates with chloramphenicol. The effectiveness of biofilm inhibition was determined using **Eqn. 3.6**,

% Biofilm Survival =

$$\frac{\text{CFU of } P.aeruginosa \text{ biofilm in treated sample} \times 100}{\text{CFU of } P.aeruginosa \text{ biofilm treated with WT } E.coli}$$

Eqn. (3.6)

8. Biofilm structures on the glass slides from **Step 4** could be visually inspected using a confocal microscope with GFP detection channel.

3.2 Engineering Digital Logic Operations in Microbe

3.2.1 Design of AND, OR, NOT, XOR and Half Adder Genetic Circuits

To equip commensal microbes with the ability to “think” and “process” logical decisions, the cells could be rewired to exhibit half adder digital operations, which is the basic unit of microprocessor chips in electronics. **Figure 3-3** shows the design of the biological half adder in a single *E. coli* cell. The half adder consisted of 3 independent biologically-derived AND, OR and NOT logic gate - a fourth AND logic function that was not a physical device, but a result of programmable decision making as a result of interconnecting logic functions.

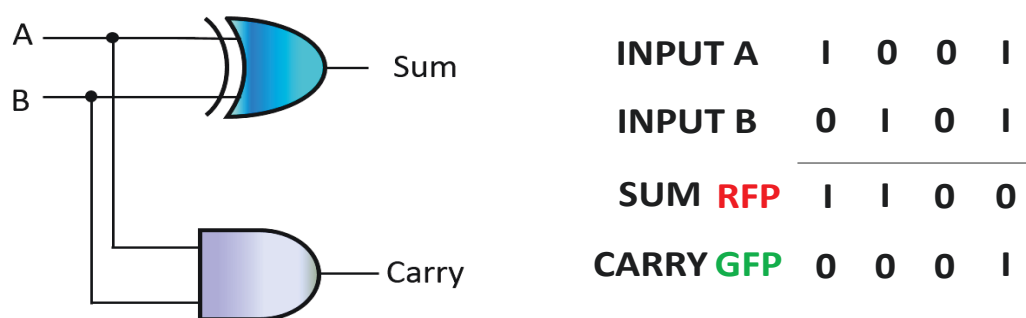


Figure 3-3: Logic output and design of half adder comprising of independent modules of AND, OR and NOT gates layered in series and in parallel.

The σ^{54} -dependent HrpRS regulation motif of *Pseudomonas syringae* T3SS secretion system was refactored for the design of the AND gate, as demonstrated in an earlier study ¹²⁷. The OR gate generated mRNA transcripts of the RFP gene upon induction with either arabinose or rhamnose. The NOT gate in the half adder was a hybrid promoter consisting of λ CI repressor binding sites downstream of the transcriptional start site (TSS) of the OR logic gate. Unlike conventional NOT gates which are designed to have transcriptional repressors competing for consensus RNAP binding sites, my NOT gate design functioned as orthogonal, molecular blockers to the RNA elongation process. This could potentially provide a more robust form of genetic inversion and be applied in different σ -dependent transcription systems. On induction with arabinose and rhamnose, the transcription factors

AraC and RhaS, both of which were constitutively expressed in a single transcript by promoter pCon, would associate with their corresponding inducers to activate expression of the enhancer-binding proteins HrpS and HrpR. This resulted in the activation of the AND logic and the concurrent synthesis of GFP reporter and lambda repressor (λ CI) by the pHrpL promoter. Consequently, genetic events of the OR gate, which ran in parallel with the HrpRS AND gate, would be turned off due to obstructive repression by λ CI molecules. In all, the half adder demonstrated both AND (SUM Output) and XOR (CARRY Output) logic operations, the latter operation was a processed outcome achieved by sequential and parallel layering of AND, OR and NOT logic (**Figure 3-4A**). By comparison, induction with either inducer singly would trigger only genetic operation of the OR gate, resulting in the synthesis of RFP reporter, but not GFP and λ CI molecules (**Figure 3-4B**).

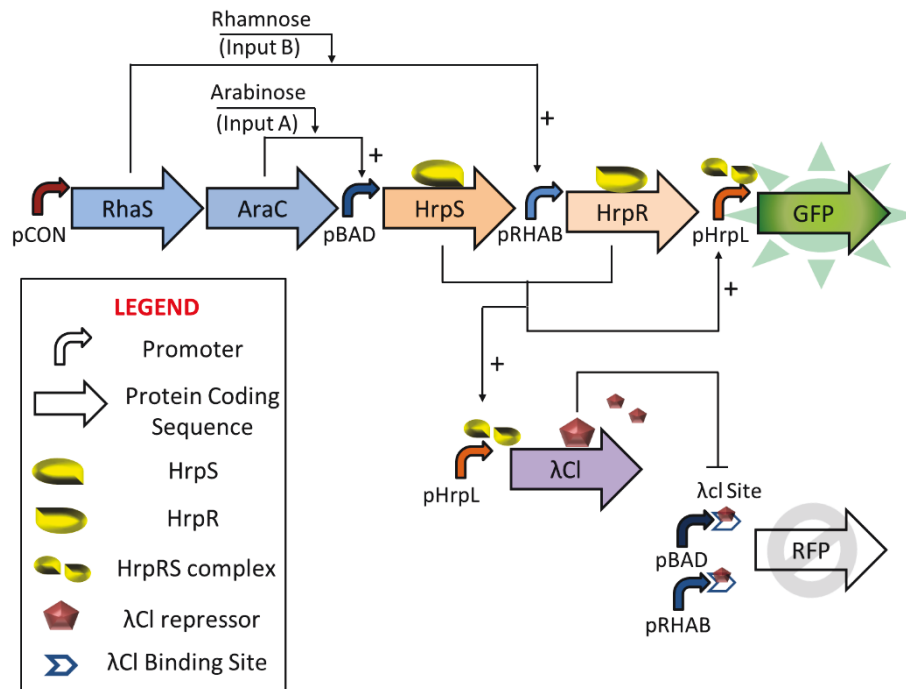
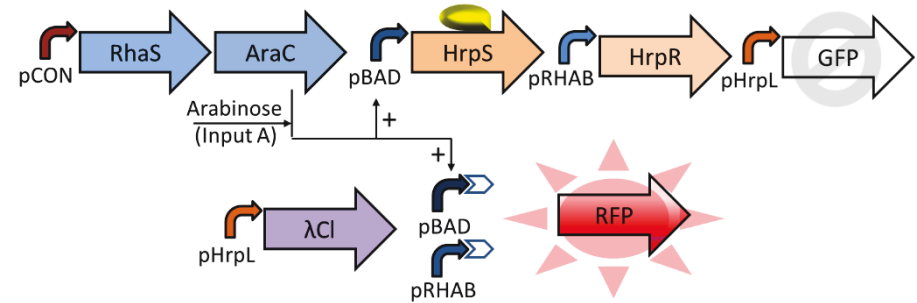
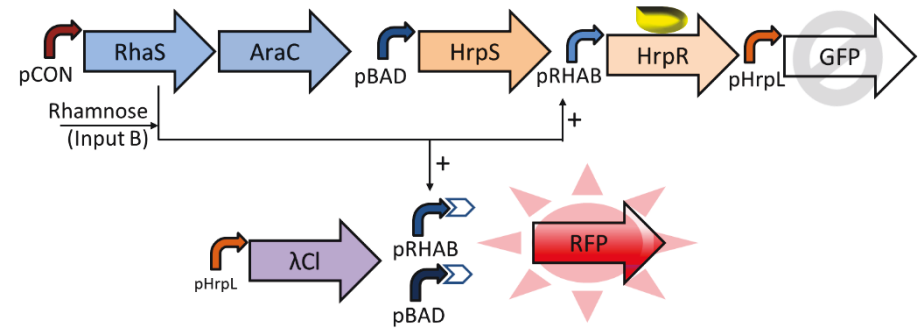
A**Induction with Arabinose and Rhamnose****B****Induction with Arabinose****Induction with Rhamnose**

Figure 3-4: Simplified schematics of biological half adder. (A) In the presence of two inputs, the AND gate was activated to produce GFP and lambda repressors, which further inactivated the OR gate to suppress RFP expression. (B) In the presence of either inputs singly, only the OR gate was activated to trigger RFP expression.

3.2.2 Biobrick Assembly

The biobrick assembly method can be used to construct synthetic genetic circuits using standardised biobrick vectors which possessed *EcoRI*, *XbaI*, *SpeI* and *PstI* restriction sites. This method allows the same restriction sites to be repeatedly used even when the engineered system gets progressively larger.

1. For the front insertion, 500 ng of PCR amplified insert and 300ng of biobrick vector were digested with *EcoRI-HF* / *SpeI*, and *EcoRI-HF* / *XbaI* for 5 h at 37 °C, respectively. For back insertion, 500 ng of PCR amplified insert and 300ng of biobrick vector were digested with *XbaI* / *PstI*, and *SpeI* / *PstI* for 5 h at 37 °C, respectively. In both cases, total reaction volume was adjusted to 25 µl (**Table 3-4**).
2. Alternatively, to transfer PCR amplified insert into an empty biobrick vector without upstream or downstream constructs, 500 ng of insert and 300 ng of vector were independently digested with *XbaI* / *PstI* for 5 h at 37 °C.
3. Digested insert and vector fragments were purified with Minelute PCR purification columns according to the manufacturer's instructions.

Table 3-4. Digestion reaction composition

Initial Concentration	Volume per 25 µl reaction
Sterile ddH ₂ O	up to 25 µl
NEB Buffer 2.1 (10×)	2.5 µl
Restriction Enzyme 1	1 µl
Restriction Enzyme 2	1 µl
DNA (300 ng Vector / 500 ng Insert)	variable

4. The amount of purified DNA was measured using Nanodrop.
5. 100 ng of purified vector was dephosphorylated with 1 μ l rAPid alkaline phosphatase in a total reaction volume of 10 μ l using the thermocycler settings as shown in **Table 3-5**.
6. T4 DNA ligase buffer was thawed on ice. Sterile water, digested purified and insert, and dephosphorylated vector were also held on ice until use.
7. 20-50 ng of phosphorylated vector was ligated with up to 70 ng of purified insert using 1 μ l of T4 DNA ligase in a total reaction volume not exceeding 20 μ l. The mixture was gently mixed by pipetting up and down and incubated overnight at 10 °C.
8. 10 μ l of ligation mixture was then transformed into competent *E. coli* cells and successful construct was screened by colony PCR.

Table 3-5. Dephosphorylation cycling conditions

Cycle No.	Temperature	Function
1	37 °C, 60 min	Dephosphorylation
2	75 °C, 5 min	Denaturation
3	4 °C, ∞	Cooling

3.2.3 Characterisation and Orthogonal Testing of Input Switches

1. A single colony of *E. coli* expressing relevant input switch construct was inoculated into 5 ml of LB medium with appropriate antibiotic. The culture was incubated in a 37 °C shaking incubator set at 225 rpm for 15 h.

2. After 15 h of incubation, the culture was diluted 100× in 10ml of prewarmed LB antibiotic medium and grown to an OD₆₀₀ of 0.5 under the same conditions. This corresponded to mid-log phase of the cells.
3. 100× stock solution of arabinose and rhamnose inducer 2.8 M were diluted in serial 2-fold dilution with sterile water to obtain 16 different concentrations of inducers ranging from 10⁻⁶ – 2.8 M.
4. While waiting for the re-inoculated cultures to grow to the desired cell density, 1.5 µl of each inducer concentration was added to the microplate in triplicates.
5. 150 µl of re-inoculated culture was then transferred into each well with inducers to activate gene expression. The cultures were incubated at 37 °C for 3 h in the microplate shaking incubator with 750 rpm shaking.
6. The cultures were analysed for background subtracted absorbance and RFP fluorescence using a microplate reader with excitation and emission wavelength of 590 nm and 640 nm respectively. The data were then to MS Excel. RFP fluorescence was normalised with cell density estimated from A₆₀₀ to obtain RFP/cell in arbitrary units.
7. Experimental results were fitted to an empirical mathematical model as shown in **Eqn. 3.7** using Matlab Curve Fitting Toolbox where A, B, C and n are curve-fitted empirical parameters.

$$\text{Output Y, RFP} = A + \frac{B(X^n)}{C^n + X^n} \quad \text{Eqn. (3.7)}$$

8. The experimental data and empirical transfer function of each input switch device were plotted on the same graph with MS Excel.

9. To evaluate the input switch devices for pair-wise compatibility, **Steps 1 – 8** were repeated with *E. coli* transformed with pRHAB-RFP-pBAD-GFP and induced with both arabinose and rhamnose inducers. A_{600} absorbance, GFP (480 nm / 530 nm) and RFP (590 nm / 640 nm) fluorescence were measured using appropriate excitation and emission filters.

3.2.4 Characterisation of AND, OR, IMPLY Logic Gates

1. **Steps 1 – 3** of **Section 3.2.3** were repeated with *E. coli* carrying “AND”, “OR” or “IMPLY” constructs.
2. While waiting for the re-inoculated cultures to grow to the desired cell density, 1.5 μ l of each inducer was added to the microplate in a 2D array as shown in **Figure 3-5**.

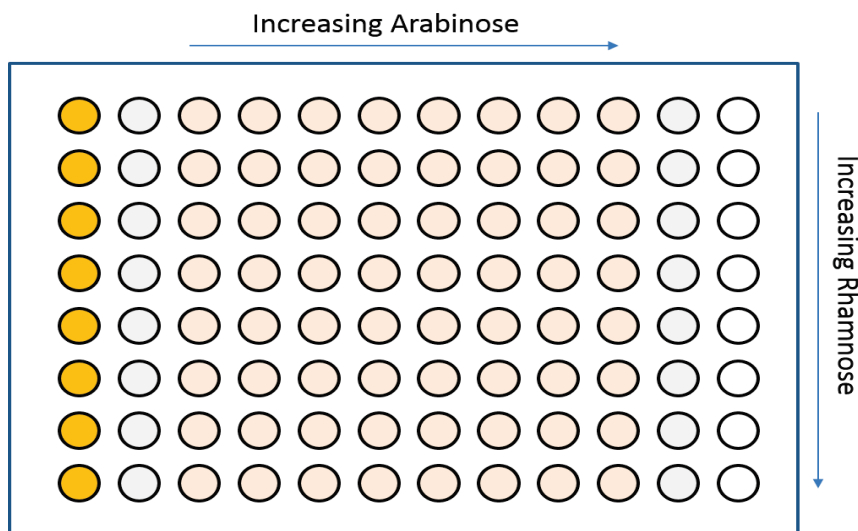


Figure 3-5: 2D array of sugar inducer on 96-well microplate. Row 1: LB blank, Row 2 & 11: Water.

3. 150 μ l of re-inoculated culture was transferred into each well with inducers to activate gene expression. The cultures were then incubated at 37 °C for 3 h in the microplate shaking incubator with 750rpm shaking.
4. Background subtracted GFP (480 nm / 530 nm) or RFP (590 nm / 640 nm) fluorescence and A_{600} absorbance were measured using microplate reader with suitable excitation and emission wavelength. The fluorescent values obtained were normalised with A_{600} estimated cell density to obtain fluorescence output per cell in arbitrary units.
5. Experimental results were plotted on a 3D surface plot with Input A (arabinose), Input B (rhamnose) and fluorescence output as X-Y-Z axis respectively using Matlab plotting tools.

3.2.5 Parts Mutagenesis of λ CI repressor Binding Sites

1. PCR library fragments of pBAD_{CI2}-RFP were generated using pBAD_{CI2A}-RFP as the template, and forward and reverse primers as listed in **Appendix A**. The PCR products were then ligated downstream of pHrpL- λ CI (pSB1C3, Cm^R) as described in **Section 3.2.2**.
2. The library of genetic circuits were then chemically transformed into *E. coli* cells with AND gate construct.
3. 24 single colonies of the transformed cells were picked with sterile toothpicks into 96-well plate prefilled with 200 μ l of growth LBAC media. The microplate was then sealed with air-permeable foil.
4. The microplate culture was incubated at 37 °C with 750 rpm shaking in a microplate incubator with heated lid for 6 h.

5. 200 μ l of fresh LBAC media was added to each well of a new 96-well microplate. Then, 2 μ l of 0.35 M arabinose inducer was added to each well in rows 2 and 6. Similarly, 2 μ l of 0.35 M arabinose and 2 μ l of 2.8 M rhamnose were added to wells in rows 3 and 7 (**Figure 3-6**), respectively.
6. 20 μ l of culture was transferred from each well into the new 96-well plate with fresh LBAC media in triplicates i.e., rows 2 – 4 and rows 6 – 8.
7. Background subtracted RFP (590 nm / 640 nm) fluorescence and A_{600} absorbance were measured using microplate reader with suitable excitation and emission wavelength.
8. Data were exported to MS Excel for analysis.
9. The desired clone was screened by reduction in red fluorescence and validated by DNA sequencing.

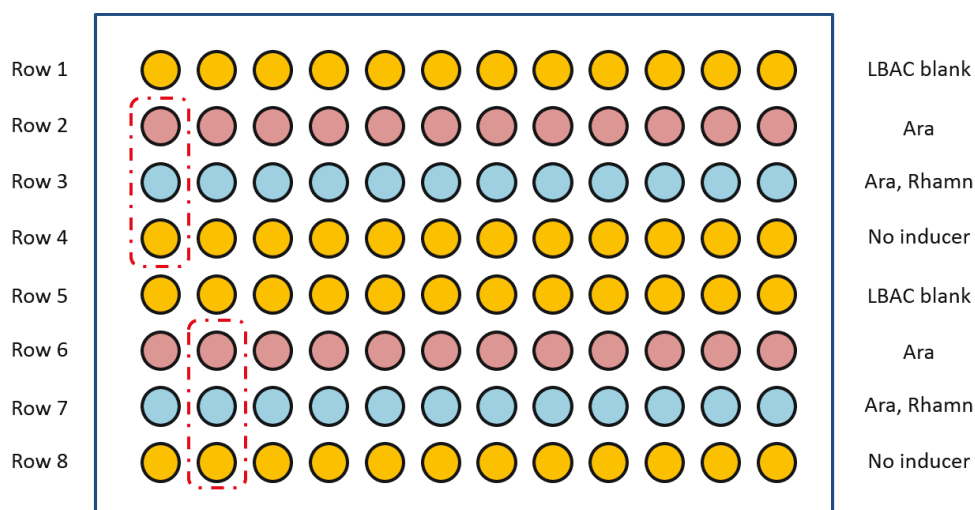


Figure 3-6: Layout of 96-well microplate for the screening of mutated λ CI binding sites. All wells were prefilled with LBAC media. Wells that were without inducers or serving as blank controls are in orange. Wells that were added with arabinose, or both arabinose and rhamnose inducers are in red and blue respectively. Each triplicate aliquot from a single re-inoculated colony is demarcated within red dotted lines. A successful screen expresses RFP in single induction of arabinose but not when both inducers are present, as represented by red and blue wells respectively.

3.2.6 Characterisation of XOR, Half Adder and Half Subtractor

DI H ₂ O	3.5µl	1.75µl	1.75µl	-
0.35M Arabinose	-	1.75µl	-	1.75µl
2.8M Rhamnose	-	-	1.75µl	1.75µl

Input Logic

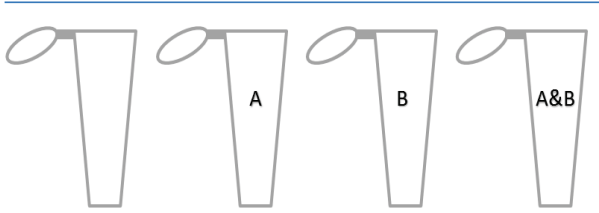


Figure 3-7: Inducer setup for XOR, half adder and half subtractor characterisation in 1.5 ml microcentrifuge tubes.

1. **Steps 1 – 3** of **Section 3.2.3** were repeated with *E. coli* carrying “XOR”, “Half Adder” or “Half Subtractor” constructs.
2. 1.5 µl of 2.8 M rhamnose and 0.35 M arabinose were added to 1.5 ml microcentrifuge tubes in 4 different logic combinations as shown in **Figure 3-7**. Simultaneously, the thermomixer was pre-heated to 37 °C.
3. 175 µl of re-inoculated culture was then transferred to each of the microcentrifuge tubes prefilled with input A and B. The sealed tubes were incubated at 37 °C on the thermomixer set to 1000rpm shaking for 4 h.
4. Background subtracted GFP (480 nm / 530 nm) or RFP (590 nm / 640 nm) fluorescence and A_{600} absorbance were measured using Biotek HTm microplate reader with monochromatic function. This prevented GFP spill over effect to RFP readings due to overlapping excitation and emission spectrum. The fluorescent values obtained were normalised with A_{600}

estimated cell density to obtain fluorescence output per cell in arbitrary units.

5. The normalised outputs of half adder and half subtractor were plotted on MS Excel. In the case of XOR gate, the results were analysed on a 3D surface plot with Matlab as described in **Step 5 of Section 3.2.3**.

3.2.7 Flow Cytometry of Half Adder and Half Subtractor

1. Steps 1 – 3 of **Section 3.2.6** were repeated with *E. coli* carrying “Half Adder” or “Half Subtractor” constructs.
2. The culture tubes were then transferred to a 4 °C fridge and chilled for 15 h to allow GFP and RFP molecules to completely mature and stabilize.
3. 5 µl of each culture was subsequently diluted in 1.5 ml of filtered DI water in separate flow cytometry tubes and mixed by vortexing (**Note 14**).
4. BD LSRFortessa X-20 flow cytometer was set up according to the manufacturer’s instruction. *E. coli* cells were then gated using both forward (550 v, threshold 1500 v) and side scatter (310 v) with the neutral density filter removed (**Note 15**). FITC (530 nm ±30) and PE594 (610 nm ±20) were set to 466 v and 852 v respectively.
5. At least 10,000 events were recorded. Data was analysed using the histogram overlay feature of Flowjo.

3.3 Statistical Analysis

In all figures presented in this thesis, error bars represent the standard deviation of the mean of n independent experiments or biological replicates, where $n \geq 2$. The mean, $\bar{y}$ and standard deviation, σ were determined using **Eqn. 3.8** and **3.9**, respectively.

$$\text{Statistical Mean, } \bar{y} = \frac{\sum_{i=1}^{i=n} y_i}{n} \quad \text{Eqn. (3.8)}$$

$$\text{Standard Deviation, } \sigma = \sqrt{\frac{1}{n} \sum_{i=1}^{i=n} (y^i - \bar{y})^2} \quad \text{Eqn. (3.9)}$$

3.4 Notes

1. pMC-PA_{GFP/CM} carried both chloramphenicol and carbenicillin selection marker and drove constitutive expression of GFP in *P. aeruginosa* with a Lac promoter. It allowed for quantification of *P. aeruginosa* viability by both fluorescence measurement and CFU count and could be used to image *Pseudomonas* biofilm by confocal fluorescence microscopy.
2. Inserts below 300 bp are recommended to be spliced together into longer fragments by overlap extension PCR. Splicing overlap extension (SOE) could be performed with equimolar ratio of DNA fragments, setting the largest fragment as the basis of comparison (100 ng). Of note, the primers designed for SOE PCR should have at least 20 – 25 bp of overlapping sequences, and designed without secondary structures that may otherwise interfere with DNA recombination.

3. Primer design is a balance between economics and having sufficient overlapping sequence such that the T_m is greater than 50 °C. Most gene synthesis companies offer standard desalted primers at affordable rates for oligos of up to 50 bp.
4. Spacer sequences were incorporated when repeated gene sequences were present in the construct, such as the repetitive use of similar promoters and terminators. Essentially, spacer sequences should be designed with ~50% GC content and distinctly different from other spacer sequences to prevent cross interference. Also, it is imperative that the spacer sequences (1) do not form hairpin structure in the 5'UTR or introduce unwanted transcription and translation termination (TAA, TGA & TAG), (2) are free from possible Shine-Dalgarno sequences, start codons (ATG, GTG & TTG) and methylation sites (GATC) of *E. coli*, (3) are free from unwanted restriction sites.
5. The concentration of amplified DNA recovered with QIAquick Gel Extraction Kit could be enhanced by recovering the two 40 µl volumes of PCR reaction in a single column. The final elution volume with nuclease-free water could be reduced to 20 µl instead of 30 µl as stated by the kit's instructional manual. Alternatively, 30 µl of eluted DNA were concentrated with a vacuum evaporator. Higher yield of DNA could be recovered during elution step by leaving the spin columns to stand in a 55 °C heat block for 2 min after nuclease-free water is added before collection.
6. In the previous work using pTet-LasR-pLux-GFP as the reporting device, we observed relatively uniform GFP production rate between 20 – 80 min after AHL induction¹⁴⁴. Note that the period in which steady state GFP

production rate was observed could vary in disparate designs of quorum sensing devices. Thus, characterisation on a case-by-case basis may be necessary.

7. This additional dilution step reduced the amount of aging cells with unhealthy surface morphology and enhances surface contrast between lysed and unlysed cells.
8. The precise volumes to resuspend the cell pellets depend on the amount of cells and some trial and error optimisation may be necessary. Generally after 2 h of induction, we observed that the engineered *E. coli* with lysis device could be resuspended in 10 μ l while the control *E. coli* with much more viable cells could be resuspended in 40 μ l for good comparison.
9. While primary fixation with glutaraldehyde serves to cross-linked proteins, secondary fixation with osmium tetroxide enables the fixation of lipids and is recommended for surface morphology studies. Note that osmium tetroxide is rapidly converted to osmium dioxide which is no longer able to fix cells on exposure to heat or light.
10. Prolonged dehydration may lead to cell shrinkage, especially at low ethanol concentration.
11. A secondary dehydration step with acetone/HMDS (hexamethyl disilazane) solvent could be performed to improve contrast by serial immersion of the loaded silicon slides in 2:1 acetone:HMDS, 1:2 acetone:HMDS and 100% HMDS (see Ref.¹⁵⁴).
12. Here, we have established that an OD₆₀₀ ~1.0 corresponds to 1.0E8 cfu/ml of *E. coli* cells with a spectrophotometer. Due to optical variability in different spectrophotometers, we advised that OD₆₀₀-CFU calibration

curves be generated to determine the actual cell densities before mixing different cell cultures together.

13. GFP molecules are stable for more than 24 h and therefore a constant level of GFP fluorescence still exist for growth-inhibited *P. aeruginosa* cells. Alternatively, a less stable variant of GFP reporter with shortened half-life may be used to assess the number of viable directly cells by flow cytometry.
14. In this experiment, 3 pulses of 3 s on vortex set at medium intensity was sufficient for good mixing and prevented bacteria from clogging up as a single particle.
15. Removing the neutral density filter allowed the flow cytometer to detect bacterial cells with greater sensitivity. Care should be noted to avoid gating at high voltage as background noise was also amplified.

Chapter Four

4. ENGINEERING COMMENSAL MICROBE TO SENSE AND ERADICATE PSEUDOMONAS AERUGINOSA

Communications between *P. aeruginosa* cells are mediated by diffusible chemical signals through a process known as quorum sensing. Quorum sensing is a cell density dependent property; the synthesis of homoserine lactones are regulated by a positive feedback mechanism that is proportional to the population density of the pathogen⁸⁵. In synthetic biology, genes that encode for biological functions are considered as fundamental building blocks for engineering biology. That is, by transferring the genes encoding for quorum sensing function from *Pseudomonas* into target chassis, the engineered microbe could also be functionalised with the ability to sense *Pseudomonas* pathogen. In this chapter, the development of a synthetic genetic system, which comprises quorum sensing, killing, and lysing devices, that enabled *Escherichia coli* to sense and kill a pathogenic *Pseudomonas aeruginosa* strain through the production and release of pyocin is discussed. The sensing, killing, and lysing devices were characterised to elucidate their detection, antimicrobial and pyocin release functionalities, which subsequently aided in the construction of the final system and the verification of its designed behavior. Constructs developed in this chapter are shown in **Figure 4-1**. The work was published in Molecular Systems Biology, EMBO Press¹⁴⁴.

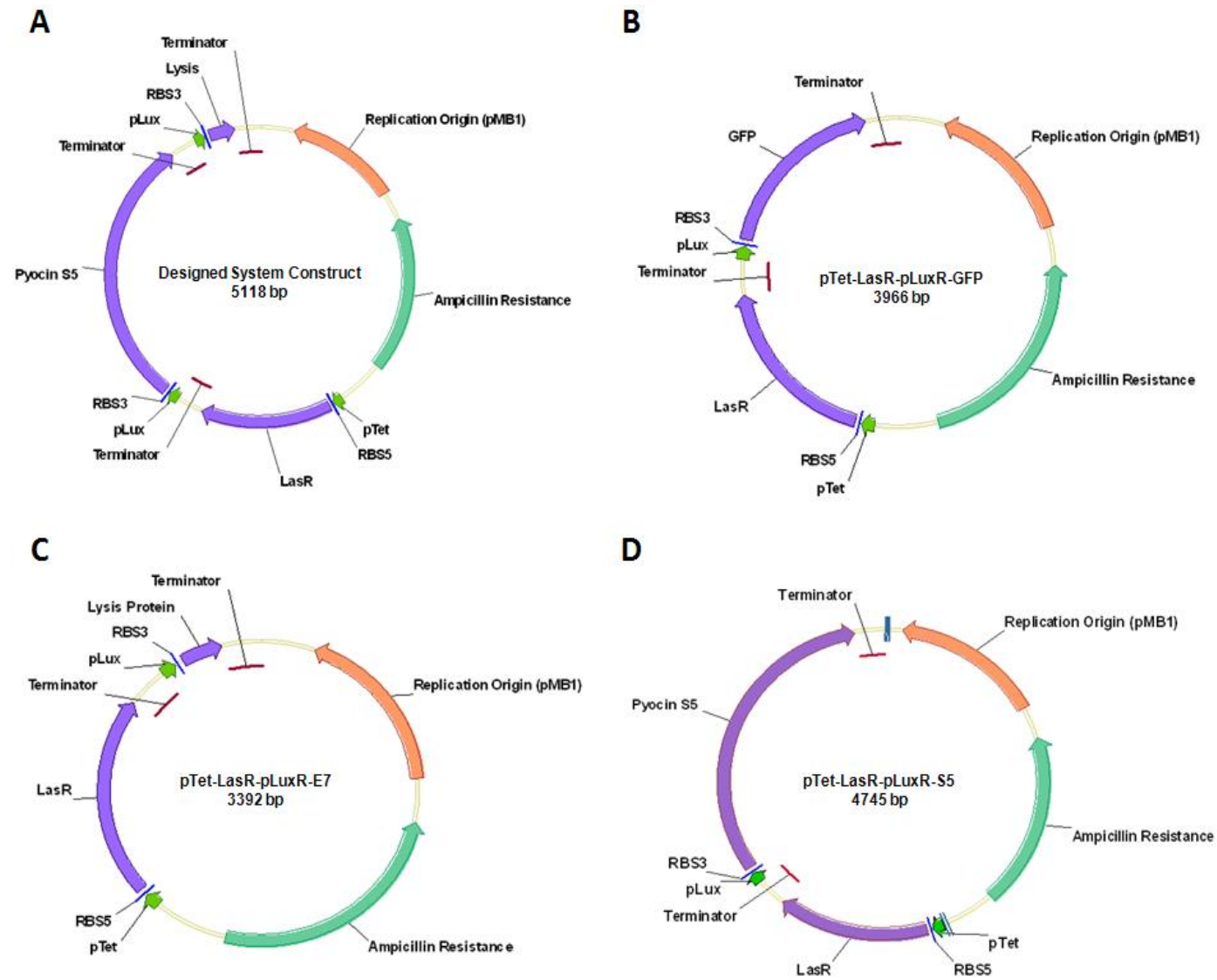


Figure 4-1: Plasmid map of engineered system and devices. (A) The final engineered system, control constructs include: (B) Sensing device coupled to GFP, (C) sensing device with E7 lysis, and (D) sensing device with pyocin S5. The figure is reproduced from ¹⁴⁴ with permission.

4.1 Characterisation of Quorum Sensing Device

To evaluate and characterise the sensing device, the gene encoding the green fluorescent protein (GFP) was fused to the sensing device (i.e. pTetR-LasR-pLuxR-GFP; the plasmid map is shown in **Figure 4-1B**) and GFP expressions were monitored over a range of concentrations of 3OC₁₂HSL. GFP content in bacterial culture and cell density were quantified in terms of the relative fluorescence unit (RFU) and optical density (OD). Assuming a linear dependence of GFP concentration with RFU and cell density with OD, the quotient RFU/OD provided a reasonable quantification of the amount of GFP per cell. Further, the rate of GFP synthesis per cell could be estimated by taking the ratio of $\Delta(\text{RFU/OD})$ over a small time interval Δt . From the measured GFP synthesis rates (**Figure 4-2**), we observed a basal expression level of 0.1 RFU OD⁻¹min⁻¹ without induction, followed by a sharp increase in GFP production rate as the concentration of 3OC₁₂HSL were increased beyond 1.0E-7 M. This transition peaked at 1.0E-6 M of 3OC₁₂HSL and exhibited a sharp decline afterward. These results suggest that the optimal detection range of the sensing device was between 1.0E-7 M and 1.0E-6M 3OC₁₂HSL. At 3OC₁₂HSL concentration above 1.0E-6 M, diffusion of quorum signals into cell was not rate-limiting, leading to rapid gene expression within the first 20 min. Significant metabolic burden was exerted on the cell and this resulted in inadvertent failure of the sensing device after about 60 min.

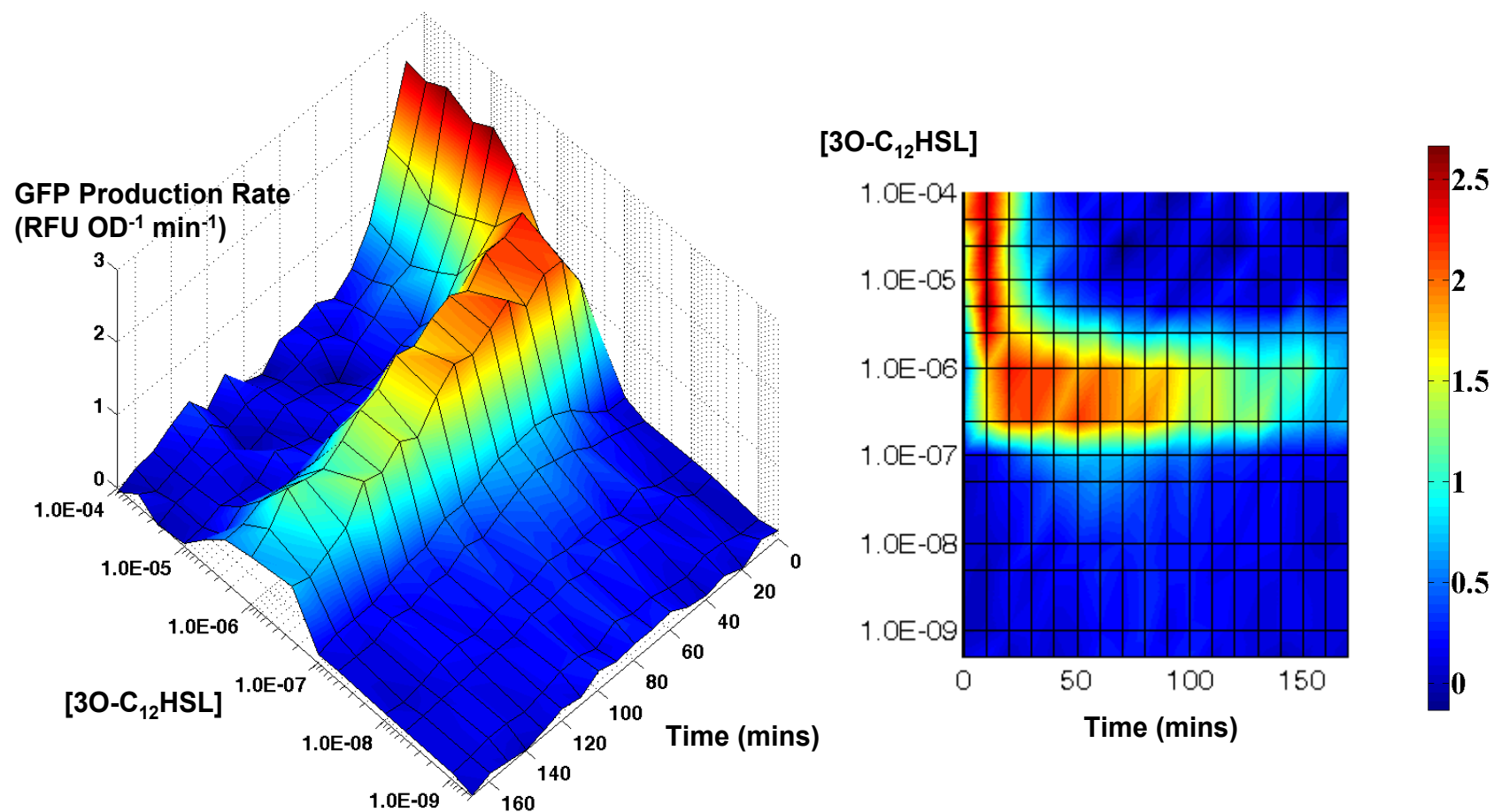


Figure 4-2: Characterisation of quorum sensing device with GFP reporter. GFP production rate is reported over a range of autoinducer (3OC₁₂HSL) concentration and time. The figure is reproduced from ¹⁴⁴ with permission.

A goal of synthetic biology is to ensure that the compositional property of an engineered device is preserved when used jointly with other biological parts. However, complex biochemical kinetics usually prevents a thorough understanding of novel devices and hinders any efforts in process optimisation. Herein, quorum sensing dynamics of the sensing device was accurately described by fitting an empirical mathematical model (Hill equation) to the experimental data where the input 3OC₁₂HSL concentration was smaller than 1.0E-6 M. The performance of quorum sensing receiver was modelled by determining the average GFP synthesis rate under two conditions: (1) at inducer concentration below 1.0E-6 M 3OC₁₂HSL and (2) at time between 20 to 100 minutes after induction when GFP synthesis rate was assumed to be at pseudo steady state with fairly constant expression throughout for a particular inducer concentration. Nonlinear curve fitting of the experimental results into a transfer function model was implemented with Matlab Curve Fitting toolbox to derive the characteristic hill coefficient and constant. The resulting best fit model demonstrated that the static performance of the sensing device follows a Hill equation below the input concentration of 1.0E-6 M 3OC₁₂HSL (**Figure 4-3**).

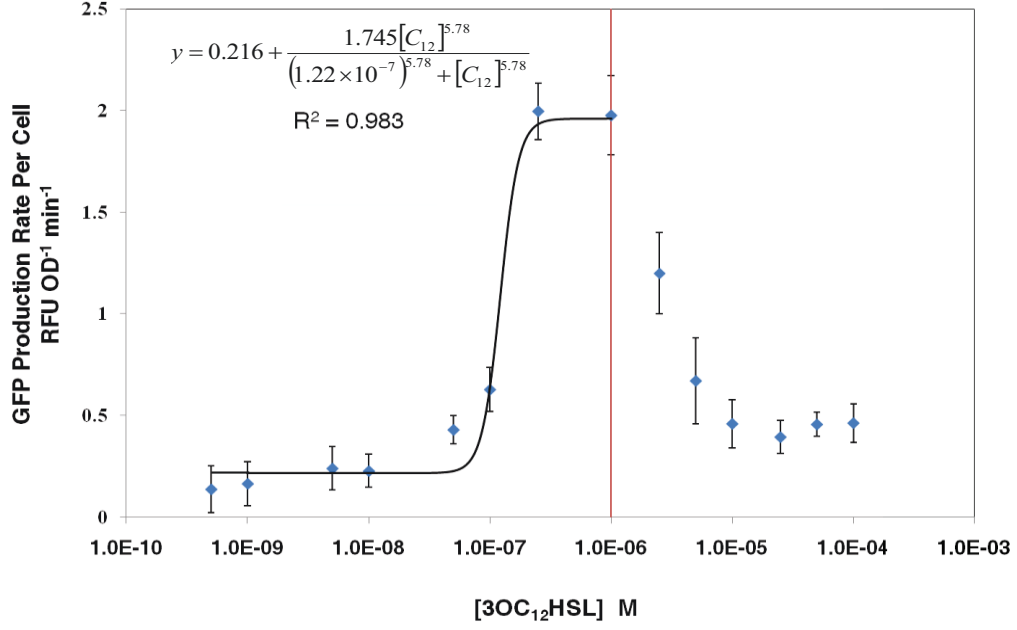


Figure 4-3: Time-averaged GFP production rate per cell at different input 3OC₁₂HSL concentrations, showing that the optimal operating concentrations for the sensing device range from 1.0E-7 to 1.0E-6M 3OC₁₂HSL. Error bars represent the standard deviation of statistical means between 20 and 80 minutes after induction. The figure is reproduced from ¹⁴⁴ with permission.

$$y = 0.216 + \frac{1.745 [C_{12}]^{5.78}}{(1.22 \times 10^{-7})^{5.78} + [C_{12}]^{5.78}} \quad \text{Eqn (4.1)}$$

The model showed that the sensing device saturated at a maximum output of 1.96 RFU OD⁻¹ min⁻¹ at input concentration greater than 3.3E-7 M but smaller than 1.0E-6 M 3OC₁₂HSL, and the switch point for the sensing device was 1.2E-7 M 3OC₁₂HSL, the input concentration at which output was at half-maximal. Since the switch point concentration was smaller than the concentration of 3OC₁₂HSL present (1.0E-6 M to 1.0E-4 M) near the site of *P*.

aeruginosa infection as described in the previously literature^{146, 147}, the sensing device would be responsive to detect the amount of 3OC₁₂HSL natively produced by *P. aeruginosa*.

4.2 Estimation of Native Autoinducer Produced by *P. aeruginosa*

As stated above, the characterisation of the sensing device indicated that it produced an optimal output at 1.0E-7 M to 1.0E-6 M 3OC₁₂HSL. To verify that the sensing device would be able to sense the amount of 3OC₁₂HSL natively produced by *P. aeruginosa*, the sensing device coupled with a GFP reporter (i.e. pTetR-LasR-pLuxR-GFP) was induced using filtered cultures of different *P. aeruginosa* clinical isolates. Filtered supernatant of the *Pseudomonas* isolates were mixed with *E. coli* carrying the sensing device with GFP reporter at ratios of 1:100 and 1:10. This was necessary as the earlier characterisation results with synthetic 3OC₁₂HSL implied that a certain GFP production rate per cell could correspond to inducer concentration either below or above 1.0E-6 M. To estimate the concentration of 3OC₁₂HSL produced by various *P. aeruginosa* isolates, time-averaged GFP production rates (**Figure 4-4**) due to induction by AHL from *P. aeruginosa* were substituted in the transfer function model. **Figure 4-5** shows the estimated concentration of exogenous 3OC₁₂HSL produced and released by *P. aeruginosa* clinical isolates. The average concentration of 3OC₁₂HSL in liquid cultures of the *P. aeruginosa* strains were estimated to be approximately 1.0E-6 M 3OC₁₂HSL. This result was coherent with previous studies that had estimated that the extracellular concentration of 3OC₁₂HSL was between 1.0E-6 and 1.0E-4 M^{146, 147}. Results from this experiment also suggests that certain strains of *P. aeruginosa* such as In4 may

not synthesize autoinducer under the same conditions as other strains due to slight variation in regulatory pathway. In7 was identified as the strain with the highest secretion of type I autoinducer and would be further tested for co-culturing with engineered treatment system.

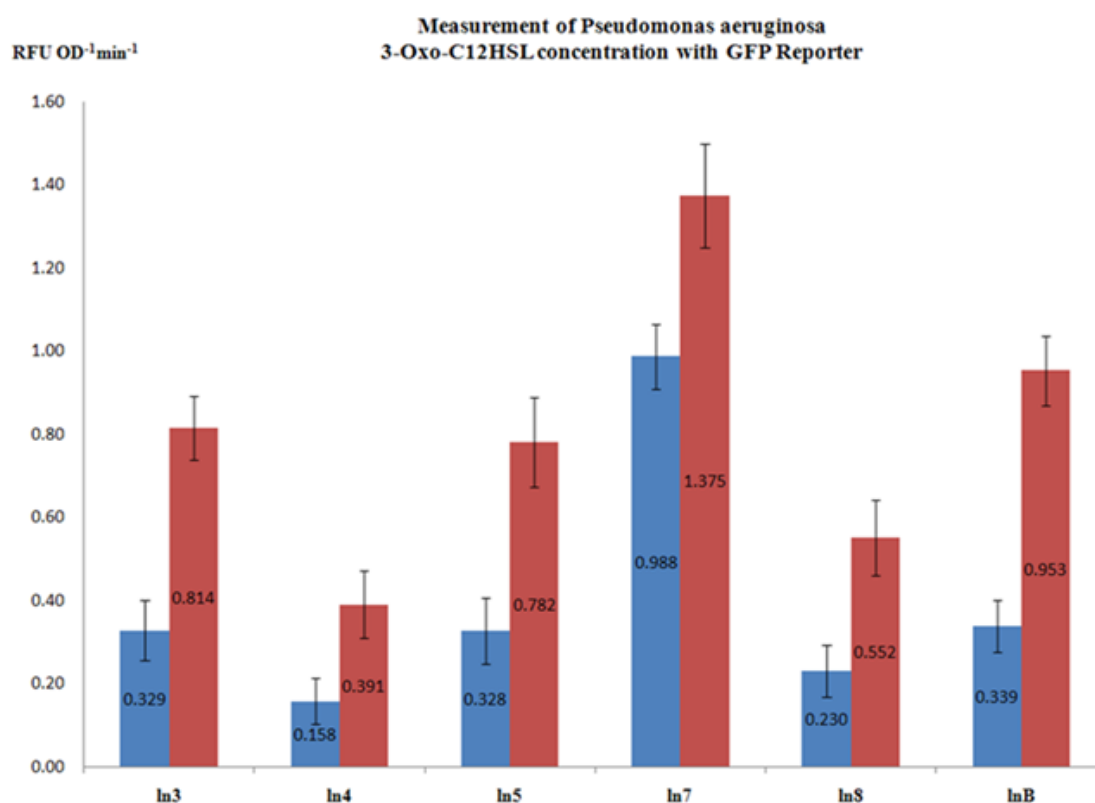


Figure 4-4: GFP production rate of different *P. aeruginosa* isolates. Time-averaged GFP production rate between 20 ~ 80 minute after induction with 1:100 and 1:10 filtered supernatant. Error bars represent the standard deviation of three biological replicates.

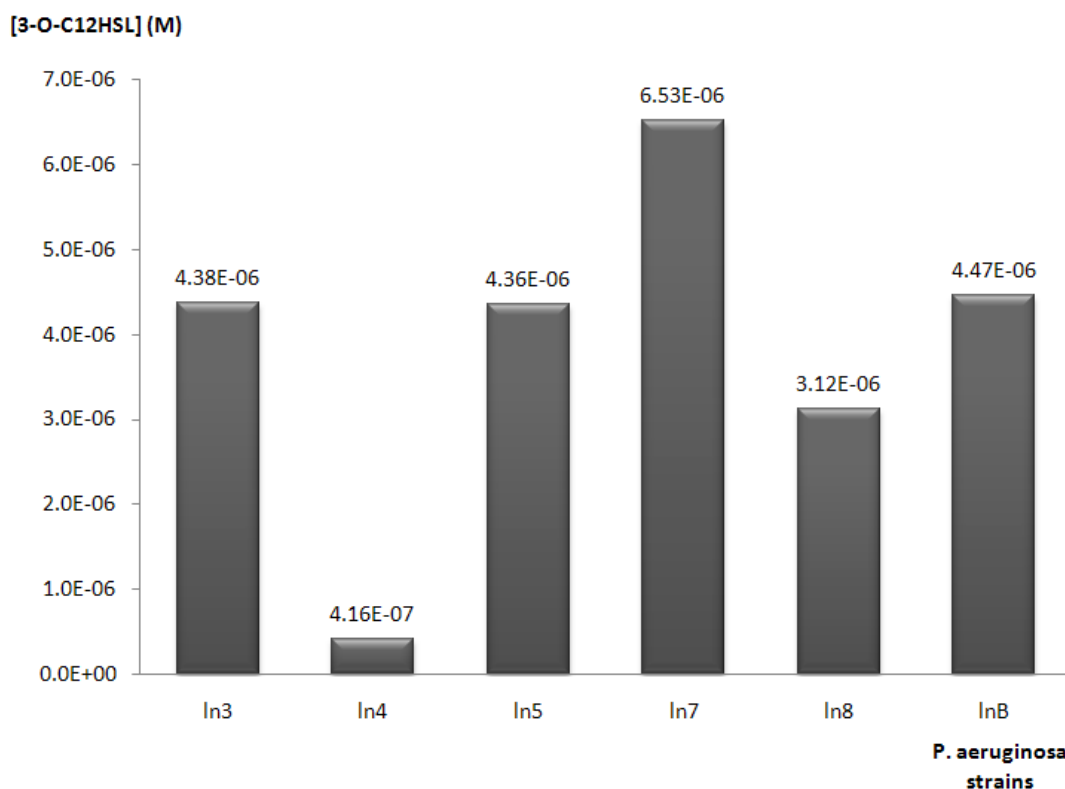


Figure 4-5: Concentration of 3OC12HSL autoinducer secreted by different *P. aeruginosa* clinical isolates. The average concentration of 3OC12HSL in liquid cultures of *P. aeruginosa* was estimated to be 1.0E-6 M. The figure is reproduced from ¹⁴⁴ with permission.

4.3 Characterisation of Lysis Device

In the conceptual design of the engineered commensal bacteria, E7 lysis device was introduced to mediate the release of accumulated S5 pyocins on quorum sensing detection of extracellular 3OC₁₂HSL released by *P. aeruginosa*. Henceforth, to characterise the activity of the lysis device, the E7 lysis gene was ligated downstream to the sensing device (i.e. pTetR-LasR-pLuxR-E7; the plasmid map is shown in **Figure 4-1C**) and its performance was assessed in an *E. coli* chassis by measuring absorbance at OD₆₀₀ over a range of concentrations of 3OC₁₂HSL and time. **Figure 4-6A** shows that at 0 and 1.0E-8 M 3OC₁₂HSL, the growth rates of *E. coli* underwent no noticeable transition

into a lysis state. However, at higher concentrations of 3OC₁₂HSL (i.e., 1.0E-6M and 1.0E-4 M), the cells exhibited a significant reduction in optical density, likely due to the lysis activity.

The results imply that 1.0E-6M or higher concentrations of 3OC₁₂HSL cause observable cell lysis with a delay of approximately 120 minutes. To verify the effect of the lysis, cell integrity was examined with and without 1.0E-6M 3OC₁₂HSL using Field Emission Scanning Electron Microscopy (FESEM). **Figure 4-6B** illustrates that *E. coli* containing pTetR-LasR-pLuxR-E7 and induced with 3OC₁₂HSL appeared shrivelled with corrugated surface morphology as opposed to the distinct 'rod-like' features of healthy *E. coli* cells that were not induced with 3OC₁₂HSL. The result suggests that lysed cells tend to implode from its initial shrivelled state, possibly due to the release of cellular content as similar to air escaping from a balloon. Under the collapsed state, light from spectrophotometer would less likely be absorbed by opaque cellular material, resulting in lower absorbance measurements. To further confirm that the lysis activity can be sustained in the final system including pyocin S5, the morphology of *E. coli* containing the final system (i.e. pTetR-LasR-pLuxR-S5-pLuxR-E7; the plasmid map is shown in Figure 9A) were monitored using FESEM.

Figure 4-6C shows that *E. coli* cells having the final system and induced with 3OC₁₂HSL also appeared shrivelled with corrugated surface morphology, while *E. coli* cells having the final system (without induction) remained 'rod-like' in shape. These observations were comparable to those obtained in the earlier

experiment with *E. coli* containing only the lysis device (i.e. pTetR-LasR-pLuxR-E7) and suggestively implied the engineered *E. coli* containing the final system were able to lysed themselves on sensing exogenous 3OC₁₂HSL.

In line with the overall objective of the E7 lysis device in mediating the export of pyocin, we assessed the efficiency of the lysis device in the final system by measuring the amount of the pyocin released. Histidine-tagged S5 protein was purified by immobilized metal affinity chromatography from the filtered supernatant and analysed by SDS PAGE (**Figure 4-7A**) and Bradford assay after induction with 1.0E-6 M 3OC₁₂HSL (**Figure 4-7B**). Whereas no bands were seen on the SDS PAGE of an incomplete system without the lysis device (i.e. pTetR-LasR-pLuxR-S5; the plasmid map is shown in **Figure 4-1D**), distinct bands that are indicative of pyocin S5 were observed on the SDS PAGE of the final system (i.e. pTetR-LasR-pLuxR-S5-pLuxR-E7).

Additionally, we validated the results by estimating the protein concentrations in the supernatant with Bradford assay and showed that the amount of pyocin released by the final system was eight fold higher than a system without the lysis device. Activity of the lysis device in the final system was characterised by an impulse release of protein two hours after induction, which was eventually succeeded by a steady discharge of pyocin S5.

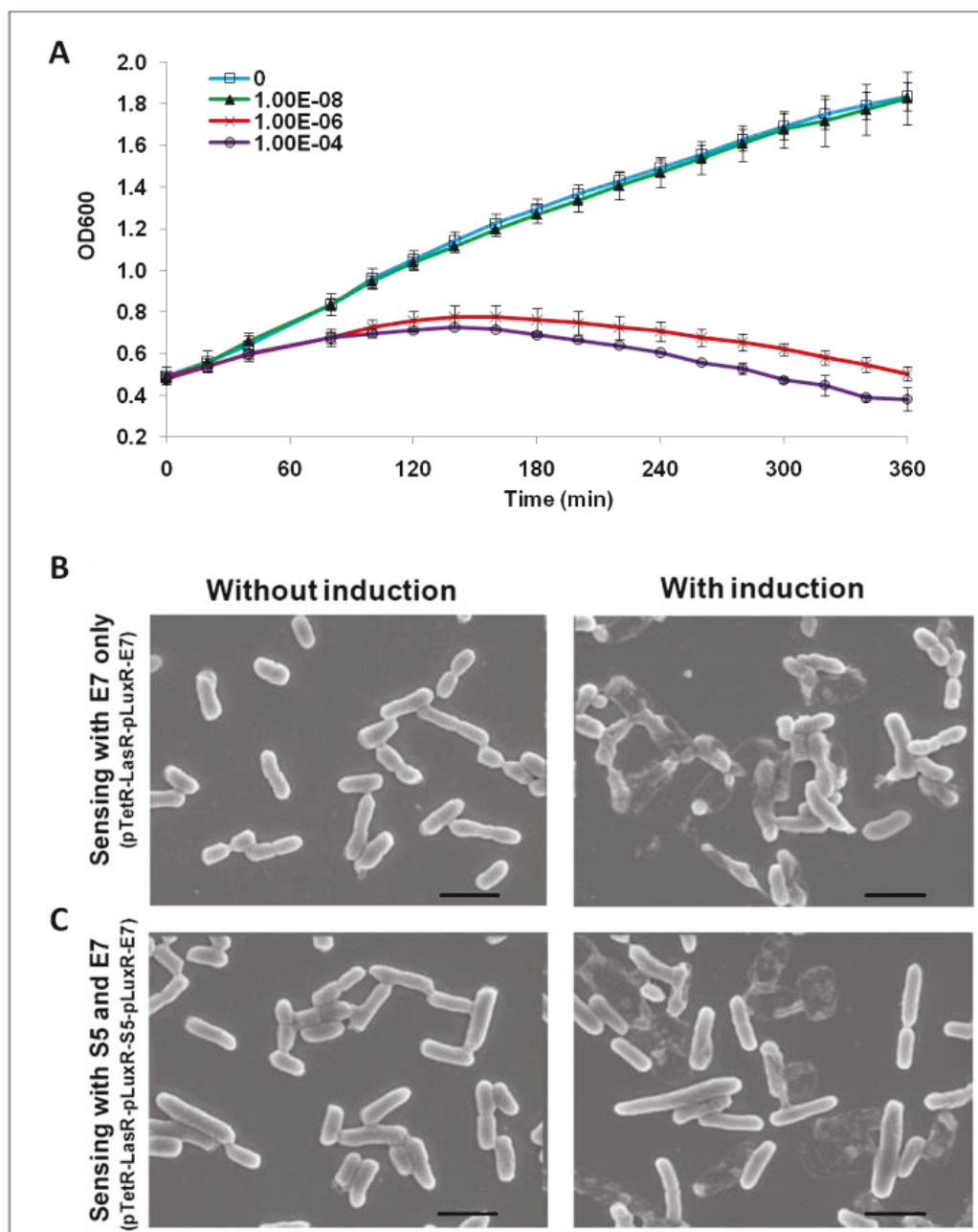


Figure 4-6: Characterisation results of lysis device using 3OC₁₂HSL. (A) Growth curve of *E. coli* expressing E7 lysis protein after induction with different concentrations of 3OC₁₂HSL. (B and C) Effects of lysis protein on *E. coli* surface morphology as observed using FESEM. The results show that the surface of the *E. coli* was damaged when *E. coli* carrying pTetR-LasR-pLuxR-E7 and *E. coli* carrying pTetR-LasR-pLuxR-S5-pLuxR-E7 (the final system) were induced with 3OC₁₂HSL. Scale bar: 1 μ m. Error bars represent the standard deviation of 4 replicates. The figure is reproduced from ¹⁴⁴ with permission.

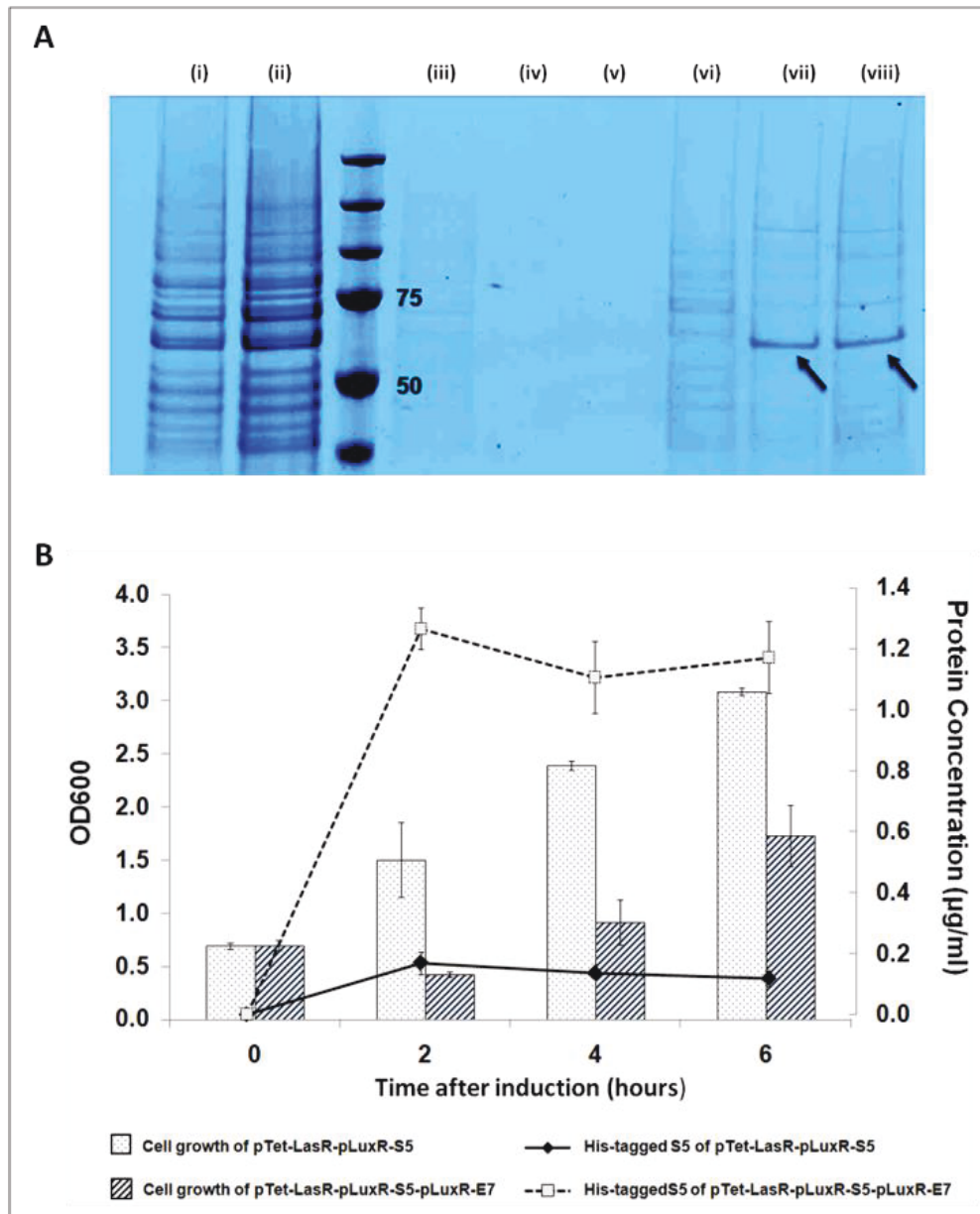


Figure 4-7: Characterisation results of the lysis device in the final system using 3OC₁₂HSL. **(A)** SDS PAGE of (i - ii) total extracellular proteins and (iii - viii) IMAC purified His-tagged S5 protein sampled from the extracellular supernatant. Total extracellular proteins exported from (i) *E. coli* without lysis device was significantly lesser than that exported from (ii) *E. coli* carrying the final system. *E. coli* without lysis device (iii - v) and *E. coli* carrying the final system (vi - viii) at 0, 2 and 4 hours after induction. The results show that pyocin S5 (57 kDa; arrowed) was only detected in lanes of *E. coli* with the final system and not in lanes of *E. coli* without the lysis device. Ladder: Bio-Rad's Precision Plus Protein standards. **(B)** Characterisation of lysis device in the final system by optical density (bar graphs) and concentration of pyocin released (lines) after induction. Error bars represent the standard deviation of 2 replicates. The figure is reproduced from ¹⁴⁴ with permission.

4.4 Overlay Inhibition Assay with AHL and the Final System

Thus far, commensal *E. coli* were engineered to sense 3OC₁₂HSL signals and lysed itself to mediate the export of intracellular proteins such as pyocin S5. To study whether the sensing of 3OC₁₂HSL also triggers the killing of *P. aeruginosa* as designed, the growth of *P. aeruginosa* were monitored in the presence of the engineered *E. coli* containing the final system.

To determine if the concentration of AHL required to elicit the expression and production of *P. aeruginosa* bacteriocidal peptides were within the range of concentrations of 3OC₁₂HSL naturally produced by the pathogen, the engineered *E. coli* were induced with synthetic 3OC₁₂HSL at 0, 1.0E-8 M, 1.0E-6 M, and 1.0E-4 M, after which their filtered supernatants were spotted onto *P. aeruginosa*-grown agars. Clearly, the growth of *P. aeruginosa* were suppressed by filtered supernatants of the *E. coli* cultures exposed to 1.0E-6M and 1.0E-4 M 3OC₁₂HSL, as distinguished by the halo clearings in the overlay inhibition assay, in contrast to very pale inhibition zones from possible basal expression of pyocin S5 and E7 lysis proteins observed at 0 and 1.0E-8 M 3OC₁₂HSL (**Figure 4-8A**).

Consequently, to confirm the killing activity by the native 3OC₁₂HSL produced by *P. aeruginosa*, the filtered supernatant of *P. aeruginosa* cultures were mixed with the *E. coli* cultures, whose supernatants were then added to *P. aeruginosa*-grown agars. **Figure 4-8B** shows that *P. aeruginosa* growth were significantly suppressed by the engineered *E. coli* cultures exposed to the supernatant of *P. aeruginosa* cultures, while neither the wild-type *E. coli* cells

with nor without the *P. aeruginosa* supernatant led to growth inhibition. This result indicates that the final system produces pyocin S5 and E7 lysis proteins on sensing native 3OC₁₂HSL from *P. aeruginosa*, subsequently leading to the killing of the pathogen.

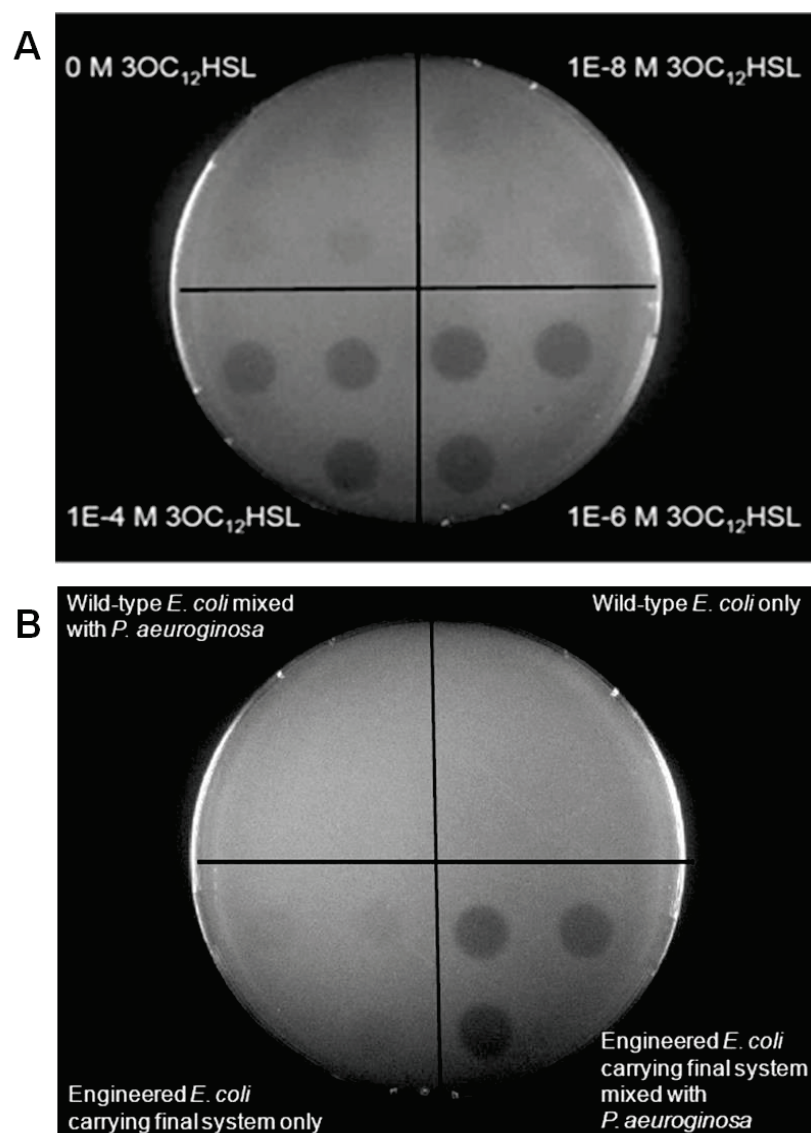


Figure 4-8: Overlay inhibition assay on *P. aeruginosa*-grown agar. (A) Inhibition assay of *P. aeruginosa* after exposure to supernatant of the *E. coli* carrying the final system at different 3OC₁₂HSL concentrations. Faint inhibition areas were observed with 0 M and 1E-8 M 3OC₁₂HSL. The results show that supernatant of engineered *E. coli* culture induced by 1E-6 M 3OC₁₂HSL produced wider and clearer inhibition zones relative to other inducer concentrations. (B and C) Inhibition assay of *P. aeruginosa* after exposure to supernatant of 4 different cultures. First, *P. aeruginosa* exposed to supernatant of wild-type *E. coli* showed no bactericidal activity. Second, *P. aeruginosa* exposed to supernatant of wild-type *E. coli* mixed with *P. aeruginosa* produced no inhibition zones. Third, exposure to supernatant of *E. coli* carrying final system did not produce any inhibition as well. Fourth, only *P. aeruginosa* exposed to supernatant of *E. coli* carrying final system with *P. aeruginosa* displayed clear inhibition zones, which suggested that the system produced sufficient pyocin S5 to exhibit bactericidal activity. The figure is reproduced from ¹⁴⁴ with permission.

4.5 Live and Dead Fluorescent Microscopy with Final System

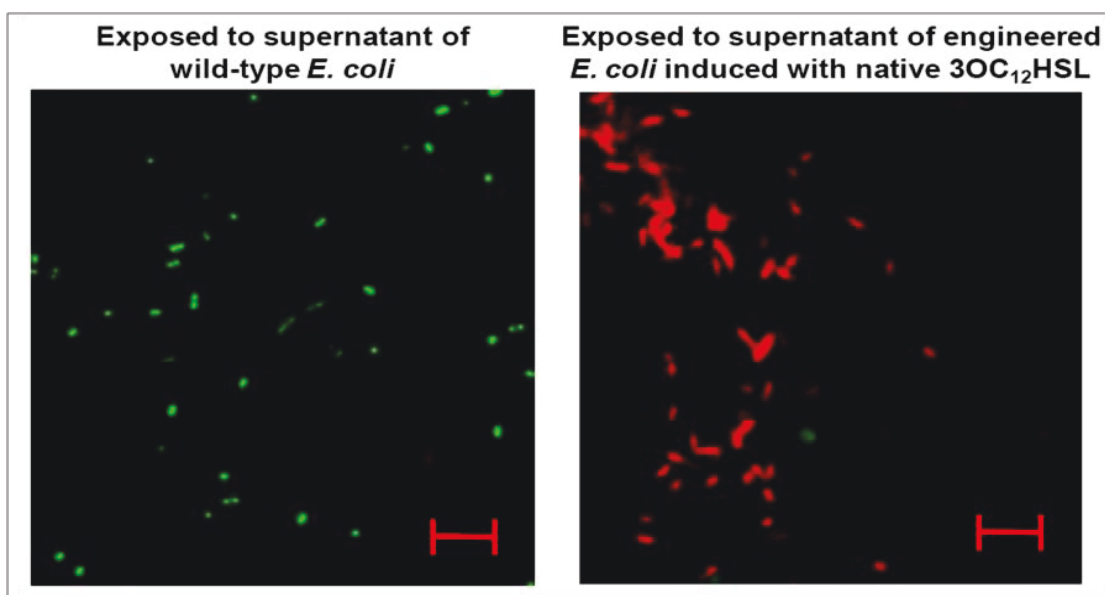


Figure 4-9: *P. aeruginosa* cells stained using the LIVE/DEAD cell viability assay. Many *P. aeruginosa* cells were stained with PI dye, which indicate dead cells, when exposed to supernatant of engineered *E. coli* carrying the final system that was induced by native 3OC₁₂HSL produced by *P. aeruginosa*. Scale bar: 5 μm. The figure is reproduced from ¹⁴⁴ with permission.

To visualise the extent of inhibition by the engineered *E. coli*, *P. aeruginosa* were examined after exposure to the supernatant of the *E. coli* cultures induced with native 3OC₁₂HSL from *P. aeruginosa* using the LIVE/DEAD cell viability assay. Results from fluorescent microscopy shows that *P. aeruginosa* cells that were exposed to the supernatant of the engineered *E. coli* were stained with the red PI dye, which stains a dead cell, whereas those that were incubated with the wild-type *E. coli* were mostly stained with the green SYTO 9 dye, which stains a live cell (**Figure 4-9**). This result suggests that the engineered *E. coli* carrying the final system can kill *P. aeruginosa* in response to 3OC₁₂HSL natively produced by *P. aeruginosa*.

4.6 Coculturing of engineered *E. coli* and *P. aeruginosa*

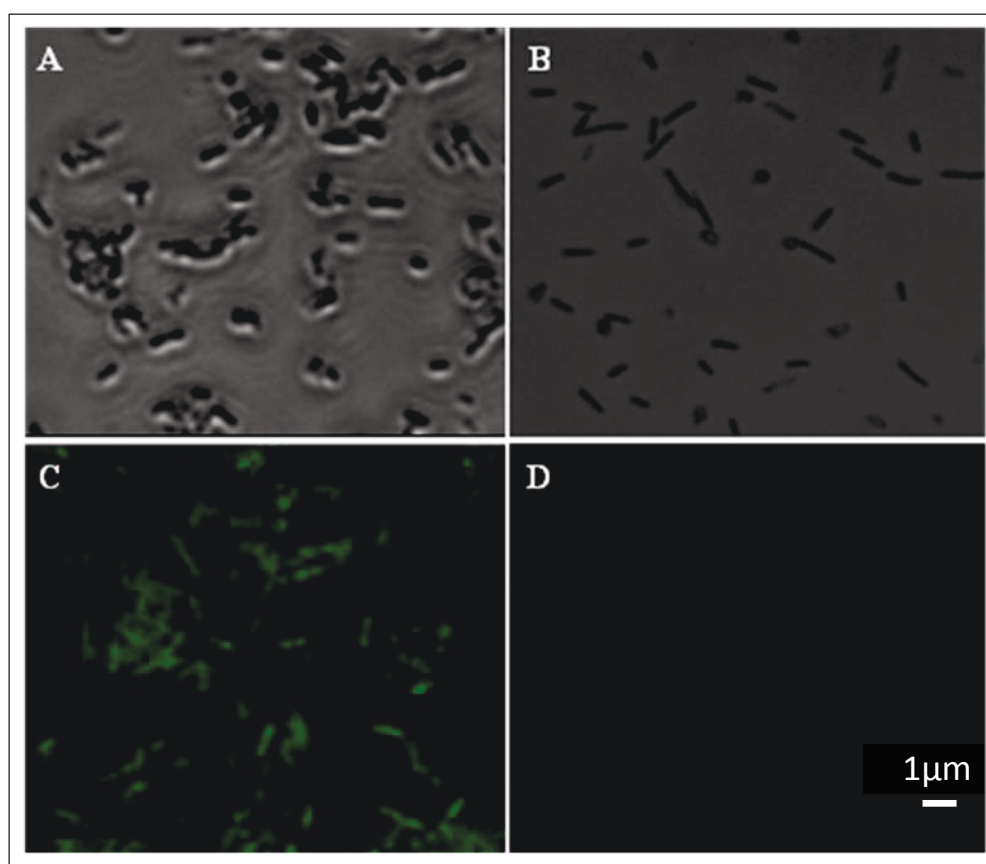


Figure 4-10: Microscopy images of *P. aeruginosa* In7 constitutively expressing GFP and wild type In7.

To verify that the engineered *E. coli* which contained the final system was able to inhibit *P. aeruginosa* in a mixed culture, the growth of *P. aeruginosa* co-cultured with the engineered *E. coli* in the ratio 1:4 was evaluated. Since both strains of bacteria display rod-like morphology, *P. aeruginosa* was transformed with pMRP9-1 to differentiate the pathogen from *E. coli* by green fluorescence. **Figure 4-10** shows normal light and fluorescent microscopy images of *P. aeruginosa* clinical isolate In7 that were transformed with (A and C) and without GFP reporter (B and D). Further, to demonstrate that only the engineered *E. coli* equipped with all three biological device – sensing, lysing

and killing, were able to suppress the pathogen, *E. coli* systems without either the pyocin S5 or E7 lysis devices were used as negative controls in this study.

Figure 4-11 shows that the GFP expression level of the *P. aeruginosa* co-cultured with the *E. coli* that carried the final system remained low and almost constant, whereas the GFP level of *P. aeruginosa* that was cultured together with the negative control *E. coli* systems increased continuously. To verify the efficiency in growth inhibition, I also performed CFU count on mixed cultures using *P. aeruginosa* that was transformed with chloramphenicol resistant plasmid. **Figure 4-12A** shows that the engineered *E. coli* effectively inhibited the growth of *P. aeruginosa* when the pathogen produced sufficient autoinducer molecules in the late exponential and early stationary phase. The results also implied that only the engineered *E. coli*, were able to suppress *P. aeruginosa* by 99% while growth of the pathogen persisted when treated with incomplete *E. coli* systems without either killing or lysis devices (**Figure 4-12B**).

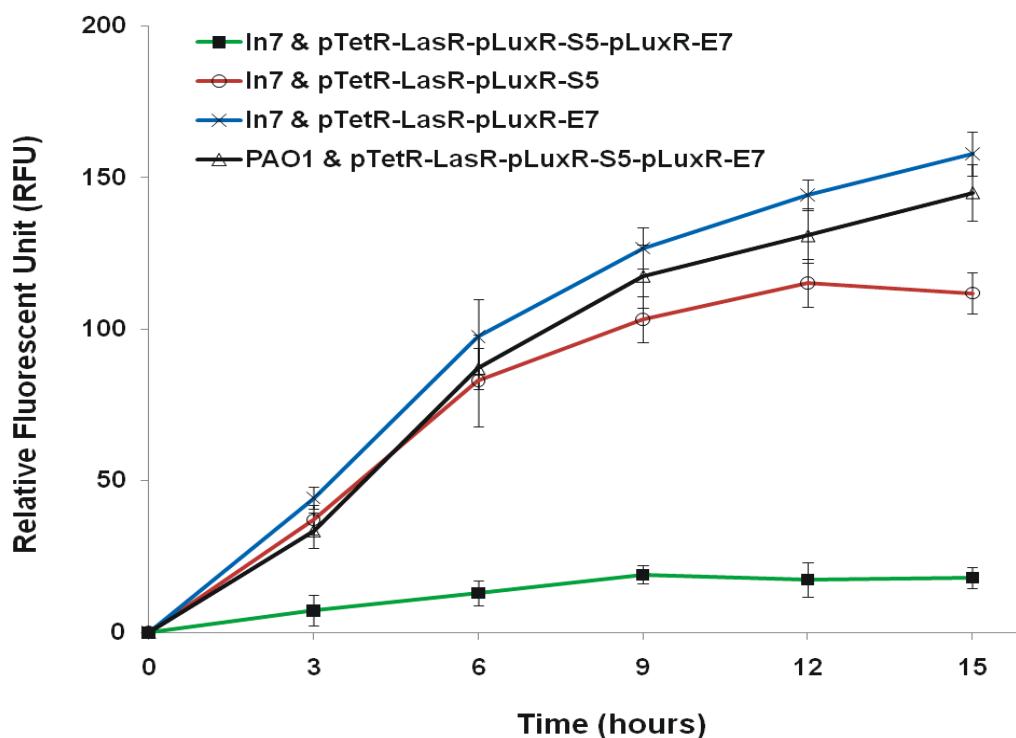


Figure 4-11: Fluorescence measurement of *P. aeruginosa* that constitutively expresses GFP in mixed culture with engineered *E. coli*. The result from the mixed culture with the engineered *E. coli* carrying pTetR-LasR-pLuxR-E7 (without killing device) and pTet-LasR-pLuxR-S5 (without lysis device) shows continuous increase in fluorescence readings, whereas the mixed culture with *E. coli* carrying pTetR-LasR-pLuxR-S5-pLuxR-E7 (the final system) exhibited no increase in the readings. This implies that the growth of *P. aeruginosa* was suppressed in the mixed culture with engineered *E. coli* carrying the final system. PAO1, which pyocin S5 was derived from, was included as a negative control. Error bars represent the standard deviation of 6 replicates. The figure is reproduced from ¹⁴⁴ with permission.

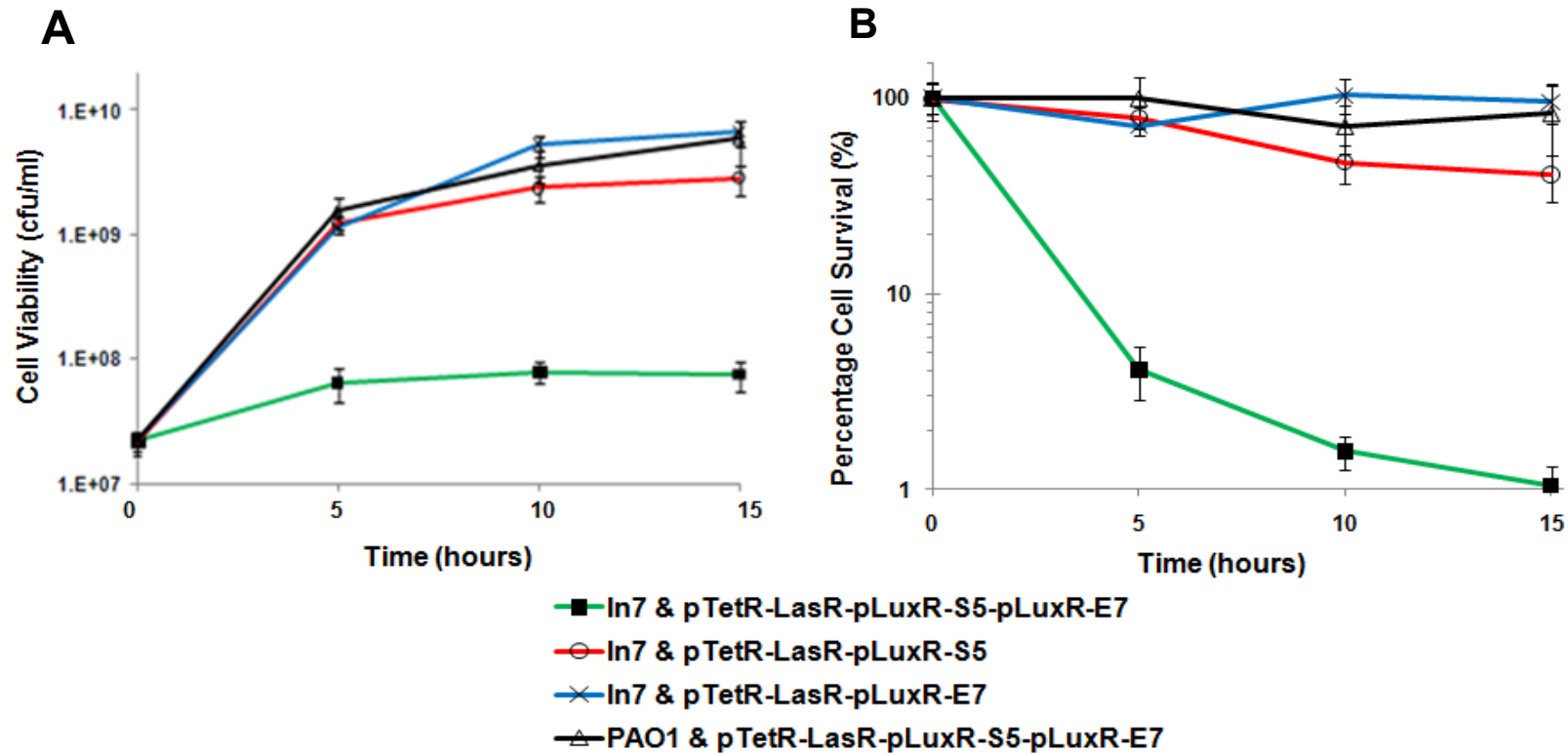


Figure 4-12: (A) Cell viability and (B) Percentage survival of *P. aeruginosa* in mixed culture with the engineered *E. coli*. *Pseudomonas* in the mixed culture was quantified by viable cell count using chloramphenicol selection. The result shows that the engineered *E. coli* suppressed the growth of *P. aeruginosa* by 99%. In contrast, inhibition was less observed in *P. aeruginosa* co-cultured with incomplete *E. coli* systems without either the killing device or lysis device. Error bars represent the standard deviation of 3 replicates. The figure is reproduced from ¹⁴⁴ with permission.

4.7 Biofilm Inhibition Assay

To examine the potential application of the engineered system against a *pseudo* disease state of *Pseudomonas*, a static biofilm inhibition assay was performed by culturing *P. aeruginosa* carrying a chloramphenicol resistance plasmid with the engineered *E. coli*. **Figure 4-13** shows that the engineered *E. coli* effectively inhibited the formation of *P. aeruginosa* biofilm by 90%. To visualise the extent of biofilm inhibition, biofilm cells with green fluorescence were grown in the presence of engineered *E. coli* on glass slide substrate and examined with confocal laser scanning microscopy (CLSM).

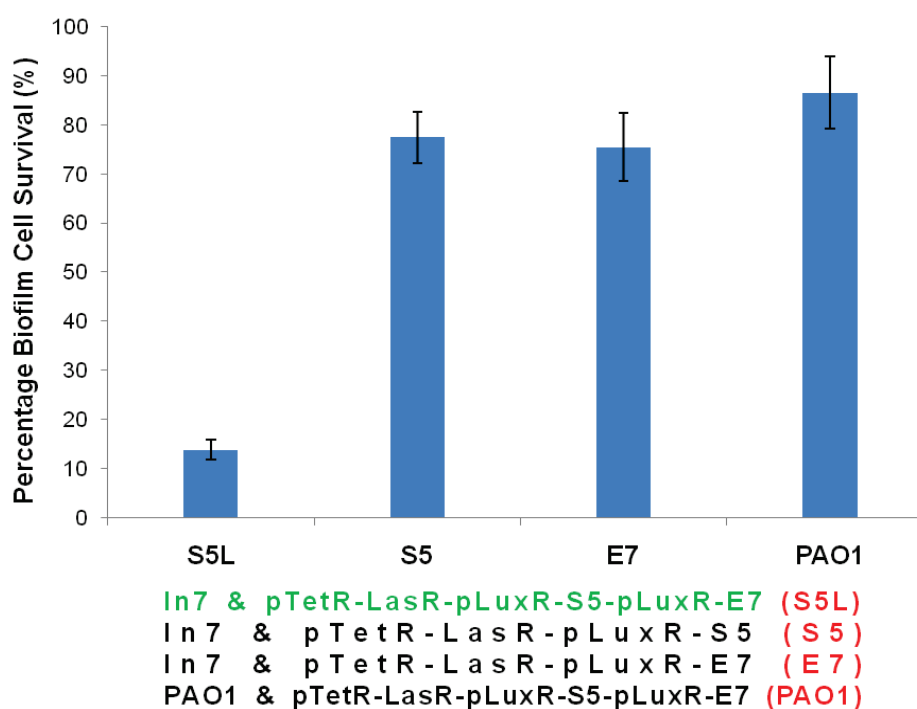


Figure 4-13: Biofilm inhibition assay with engineered *E. coli*. *Pseudomonas* biofilm was grown in a polystyrene 24-well plate in the presence of the engineered *E. coli* for 18 hours and quantified by CFU count. The results imply that the formation of *Pseudomonas* biofilm was inhibited by close to 90% with the engineered *E. coli* carrying the final system as compared to biofilm grown with wild type *E. coli* or incomplete *E. coli* systems. *P. aeruginosa* PAO1, which pyocin S5 was derived from, was included as a negative control. Error bars represent the standard deviation of 6 replicates. The figure is reproduced from ¹⁴⁴ with permission.

Figure 4-14 shows that the morphology of *Pseudomonas* biofilm treated with the engineered *E. coli* appeared thinner and sparser while elaborated water channels and honey-combed features were seen in the control experiments. This observation implies that the engineered *E. coli* was able to inhibit biofilm formation during the initial attachment phase and prevented subsequent progression into mature micro-colonies. Despite the preliminary success, it is apparent that further extensive experimental studies in direct disease-relevant models are necessary. In particular, future studies should examine the *in vivo* efficacy of the engineered *E. coli* in controlling the growth of *P. aeruginosa* in infected murine models.

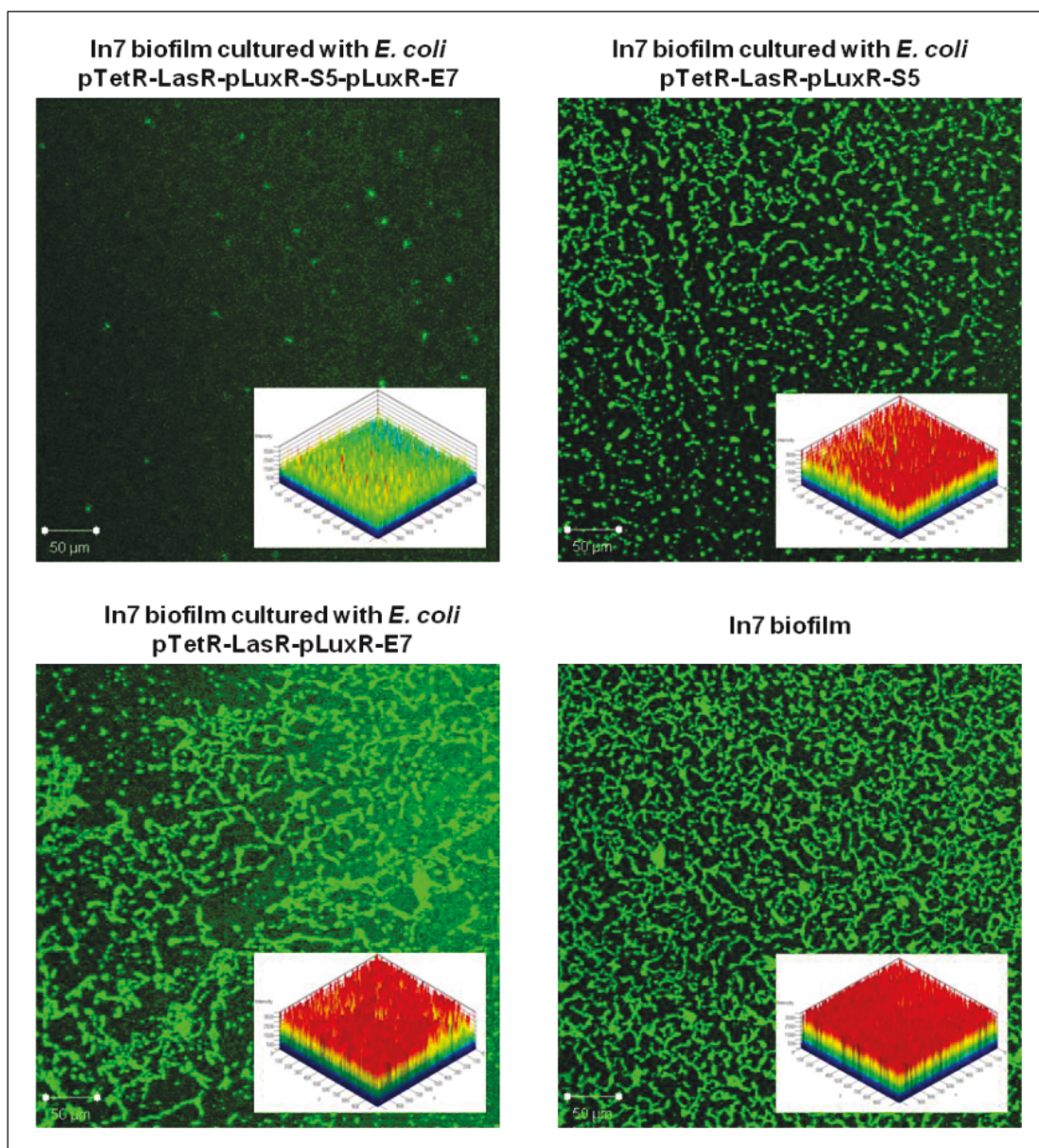


Figure 4-14: Biofilm inhibition observed under CLSM microscopy. *Pseudomonas* biofilm with green fluorescence was grown on glass slide in the presence of the engineered *E. coli* for 18 hours and visualised by CLSM. Images reconstructed from biofilm Z-stacks using Zeiss 2.5D software implied that the initialization and progression of biofilm cells into multi-layers were inhibited for *Pseudomonas* grown with *E. coli* carrying the final system as opposed to lush and elaborated biofilm formation observed in *Pseudomonas* grown alone or with incomplete *E. coli* system missing either pyocin S5 or E7 lysis genes. Scale bar: 50 µm. Z stack: 40 µm. The figure is reproduced from ¹⁴⁴ with permission.

4.8 Conclusion

One of the major health concerns of today is the emergence of superbug infections that cannot be controlled with antibiotics. Probiotics are commensal bacteria that promote human health benefits and have been used for prophylactic and therapeutic control of microbial infections. Despite the effectiveness of probiotics, their applications are confined to a narrow spectrum of infection types. Drawing inspiration from nature, we developed a prototype of designer probiotic to combat superbug infection by reprogramming human commensal *E. coli* with genetic devices that enabled *E. coli* to sense biomarkers from microbial pathogen and produce corresponding killing molecules to eradicate the pathogen. The sensing, killing, and lysing devices were characterised to elucidate their functionalities, which subsequently aided in the construction of the final system and the verification of its designed behavior. Based on the characterisation and modeling results, we verified that the sensing device had an optimal activity approximately in the range of $1.0\text{E-}7$ to $1.0\text{E-}6$ M 3OC₁₂HSL, which was in line with the concentration of 3OC₁₂HSL secreted by *P. aeruginosa*. We further confirmed the activities of the killing and lysing devices in response to this range of 3OC₁₂HSL concentration. Finally, we demonstrated that the engineered *E. coli* with the complete system effectively killed *P. aeruginosa* in both planktonic and biofilm states when those two microbes were grown together. The synthetic biology framework and genetic devices developed in this work could potentially be transferred into other microbial chassis such as probiotics and residential microbes of the upper respiratory tract^{39, 155}. Further, this study presents the possibility of engineering potentially beneficial microbiota into therapeutic bioagents to

arrest *Pseudomonas* infection. Given the stalled development of new antibiotics and the increasing emergence of multi-drug-resistant pathogens, this study provides the foundational basis for a novel synthetic biology-driven antimicrobial strategy that could be extended to include other pathogens such as *Vibrio cholera* and *Helicobacter pylori*.

Chapter Five

5. ENHANCING WHOLE CELL BIOSENSORS WITH AND & OR LOGIC OPERATIONS

In the previous chapter, a commensal microbe with sensing, lysing and killing functions was developed to mitigate the proliferation of the *Pseudomonas* superbug *in vitro*. While quorum sensing with N-3-oxododecanoyl homoserine lactone is by and large, a successful method to detect a growing population of *Pseudomonas*, there are reports that quorum regulatory mechanisms are quiescent in certain virulent isolates of *P. aeruginosa*, possibly to enhance survival under more stressful environmental conditions or to evade immune arrest^{156, 157}. The recent discovery that a second class of quorum sensing mechanism mediated by 4-hydroxy-2-alkylquinolines, exists in *P. aeruginosa* may be an additional tool to identify the pathogen¹⁵⁸. Alternatively, direct sensing of virulent determinants such as exotoxins may provide a more responsive and expansive detection. Despite the many biomarkers that are available for whole cell biosensing, an apparent shortage of genetic interface that could integrate and compute multiple input signals into the desired outputs has hindered the development of whole-cell biosensors with better specificity and sensitivity. In this chapter, a novel forward engineering approach to enable digital operations in synthetic biological systems will be discussed. In particular, an orthogonal set of AND and OR logic gates were developed and rigorously

characterised using a bottom-up synthetic biology approach. The design principles and scientific findings documented from this chapter will be valuable reference for any synthetic biologists who are keen in developing biological systems with advanced complexity. In this proof of concept study, prebiotic sugar arabinose and rhamnose will be used to represent input A and input B respectively¹⁵⁹.

5.1 Predicting the Effect of Ribosome Binding Sites on Transfer Function of Input Devices

A key outcome of synthetic biology is to demonstrate that reliable and robust engineering of synthetic biological systems can be achieved by applying classical engineering theorems (standardisation, modularisation, characterisation and abstraction) during the design process prior to system assembly. This could be achieved if the outcomes of genetic switches and logic devices, each composed of diverse biological parts, can be dependably predicted by forward computational approaches. Towards the design and optimisation of synthetic biological systems with more complicated circuitry and computational functions, we examined the effect of ribosome binding sites (RBS) on the steady state transfer function of input switch devices. By analysing reference data¹²⁷ that had previously characterise the input-output relationship of genetic switches in the form of **Eqn. 5.1**, where A, B, C and n are empirically derived parameters and X is input concentration,

$$\text{Output } Y = A + \frac{B(X^n)}{C^n + X^n} \quad \text{Eqn. (5.1)}$$

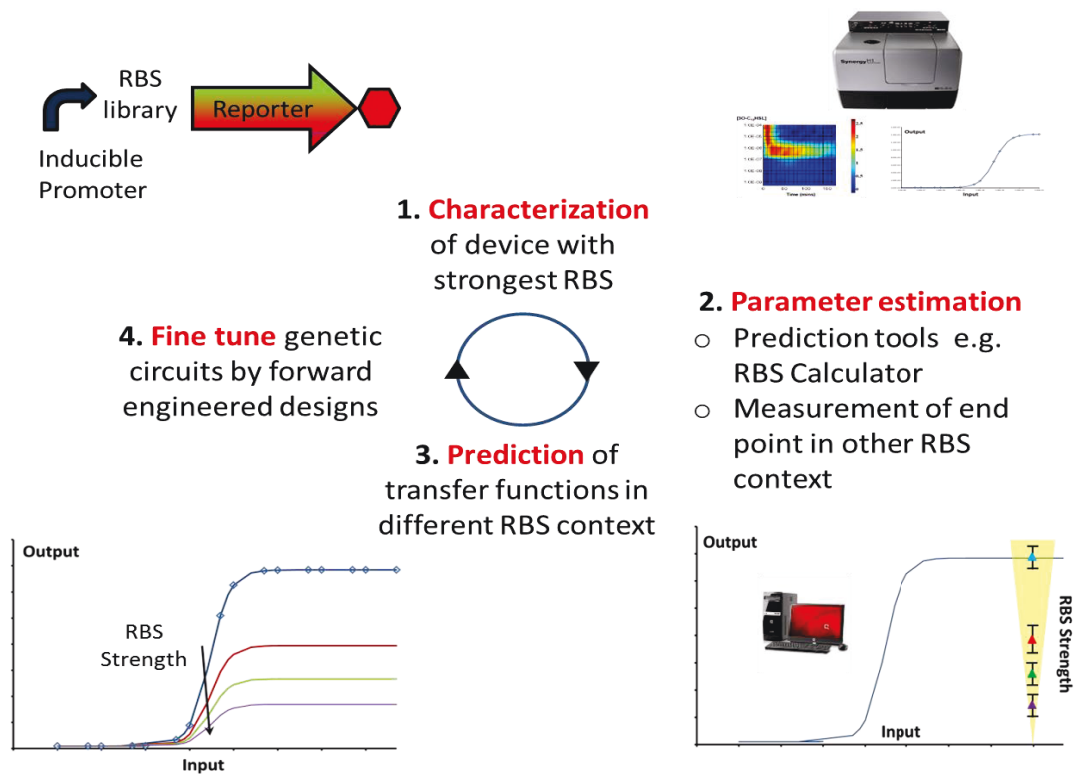


Figure 5-1: Proposed framework to predict transfer functions of input devices with different ribosome binding sites (RBS).

It was observed that parameters that are most sensitive to changes in RBS are parameters A and B. Accordingly, knowing the relative output of switch devices with weaker RBS by prediction from reliable software or single experimental measurement of device's output at input maximal, the parameters A and B can be scaled proportionally to obtain similar parameters that relate to switch devices which are engineered with weaker RBS (**Figure 5-1**). We validated the approach with previously obtained data sets (**Table 5-1**) and showed that the transfer function of input devices pLuxR (**Figure 5-2A**) and pBAD (**Figure 5-2B**) with different RBS can be reliably estimated without much experiments.

Table 5-1. Transfer functions of input switch devices derived from experimental results and model prediction. Input devices marked with asterix * were used to derive the values of parameter A and B for other devices with the same inducible promoter but with different RBS.

Input Device	A	B	C (mM)	n	R ²	Ref
pLuxR-Rbs34-GFP*	2010.01	1.349E+05	2.890E-09	1.898	0.994	¹²⁷
pLuxR-Rbs31-GFP	969.32	7.693E+04	2.955E-09	1.771	0.999	¹²⁷
pLuxR-Rbs32-GFP	581.16	5.143E+04	3.509E-09	1.655	0.999	¹²⁷
pLuxR-RbsH-GFP	364.12	3.194E+04	3.784E-09	1.616	0.998	¹²⁷
pLuxR-Rbs34-GFP	2010.01	1.349E+05	2.890E-09	1.898	0.994	This work
pLuxR-Rbs31-GFP	1198.15	7.693E+04	2.890E-09	1.898	0.986	This work
pLuxR-Rbs32-GFP	801.00	5.143E+04	2.890E-09	1.898	0.965	This work
pLuxR-RbsH-GFP	497.45	3.194E+04	2.890E-09	1.898	0.946	This work
pBAD-Rbs34-GFP*	705.50	1.411E+05	5.240E-04	1.173	0.999	¹²⁷
pBAD-RbsH-GFP	304.56	9.229E+05	4.800E-04	1.415	0.999	¹²⁷
pBAD-Rbs32-GFP	135.41	5.208E+04	5.160E-04	1.268	0.999	¹²⁷
pBAD-Rbs33-GFP	16.77	1.290E+04	5.130E-04	1.323	0.999	¹²⁷
pBAD-Rbs34-GFP	705.50	1.411E+05	5.240E-04	1.173	0.999	This work
pBAD-RbsH-GFP	461.45	9.229E+05	5.240E-04	1.173	0.947	This work
pBAD-Rbs32-GFP	260.40	5.208E+04	5.240E-04	1.173	0.979	This work
pBAD-Rbs33-GFP	64.50	1.290E+04	5.240E-04	1.173	0.983	This work

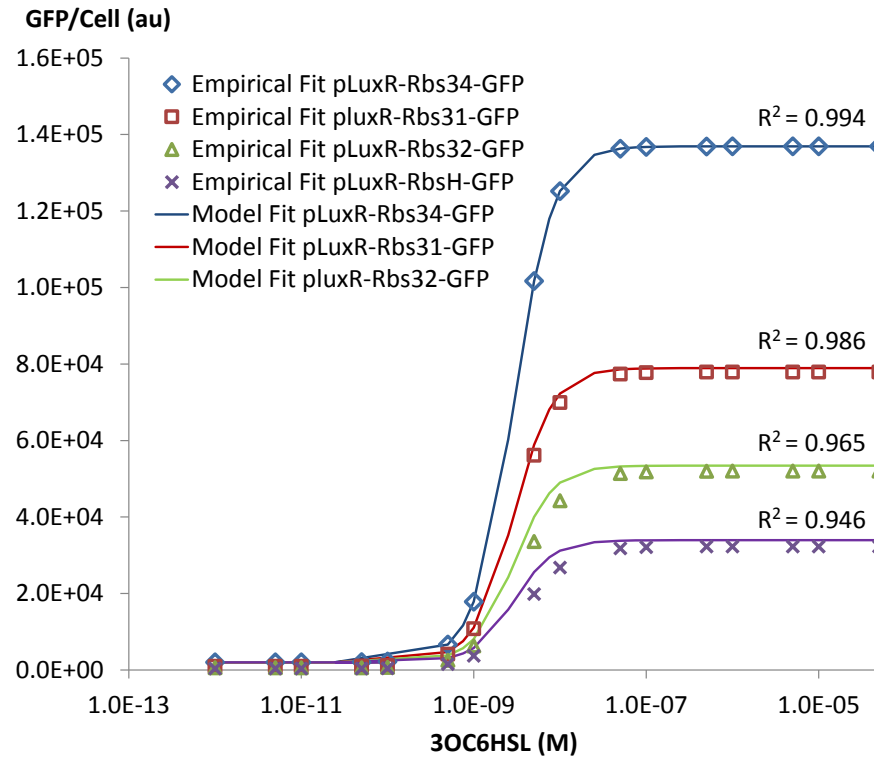
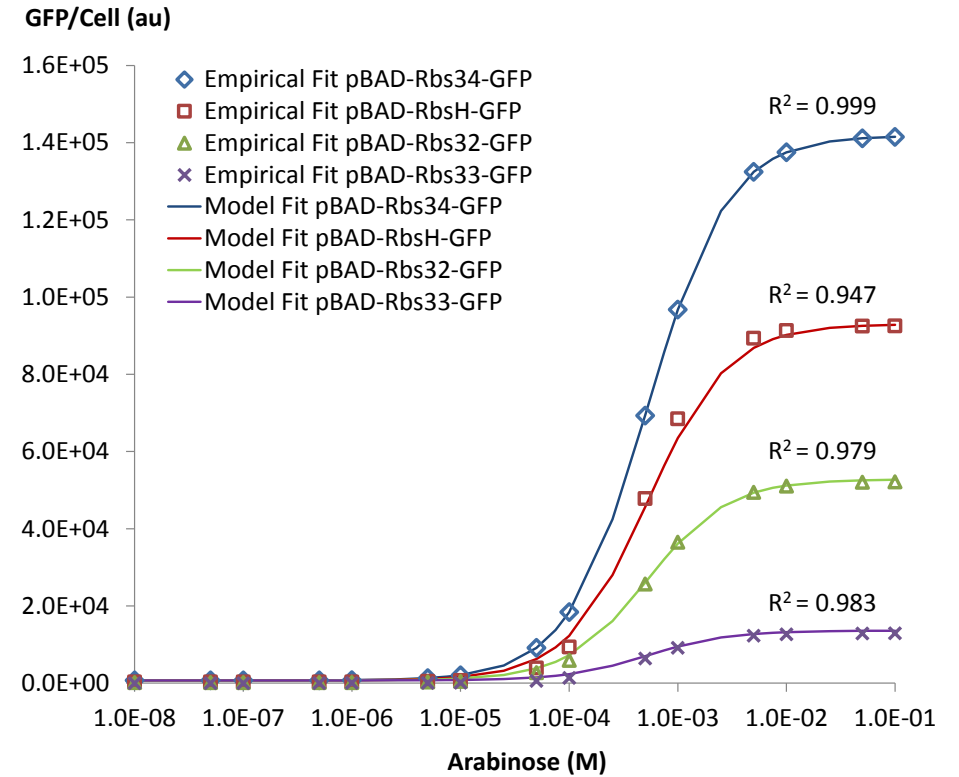
A**B**

Figure 5-2: Predicting the transfer functions of input devices with different ribosome binding sites (RBS). Validation of the suggested forward engineering approach with (A) pLuxR and (B) pBAD expression systems with different ribosome bind sites upstream of GFP reporter. Solid lines represent the forward predicted transfer functions, with corresponding R-squared values, while symbols represent empirically-derived transfer functions using total experimental fitting. All the empirically-derived transfer functions have R-squared values of 0.998 or greater and are obtained from an earlier study by Wang et al 2011.

5.2 Characterisation of Input Devices

The choice of input signals presents the first possible complication of parts modularity. For this reason, genetic circuits of higher complexity with multiple inputs often utilise promoter systems which are activated by inducers of vastly dissimilar chemical nature, namely IPTG, tetracycline, arabinose, 3OC12HSL and C4HSL. Previous studies have shown that a subset of quorum sensing promoters can be activated by homoserine lactone inducers of similar carbon chain length¹⁶⁰. Similarly, incidences of cross-phosphorylation have also been observed in two component signal transduction systems between otherwise distinct pathways¹⁶¹. Thus, it is important that inducible input devices are carefully characterised for their steady state transfer function and pairing compatibility before further assembly into higher ordered logic devices.

The arabinose inducible promoter pBAD is tightly repressed by its corresponding transcription factor AraC in the absence of arabinose. However, on induction with arabinose, pBAD undergoes conformational changes in its DNA structure that enables the assembly of RNAP holoenzyme and initiation of transcription. While previous studies with pRHAB promoter involved genetic circuits that include both RhaR and RhaS transcription factors¹⁶²⁻¹⁶⁴, in this section I demonstrate that the rhamnose inducible promoter pRHAB requires only RhaS for full activation and displays tight regulation even when RhaS is overexpressed. **Figures 5-3C and 5-4C** show the steady state transfer functions of input device A, pBAD (**Figure 5-3A**) and input device B, pRHAB (**Figure 5-4A**) expressing RFP under strong RBS by their corresponding inducers respectively.

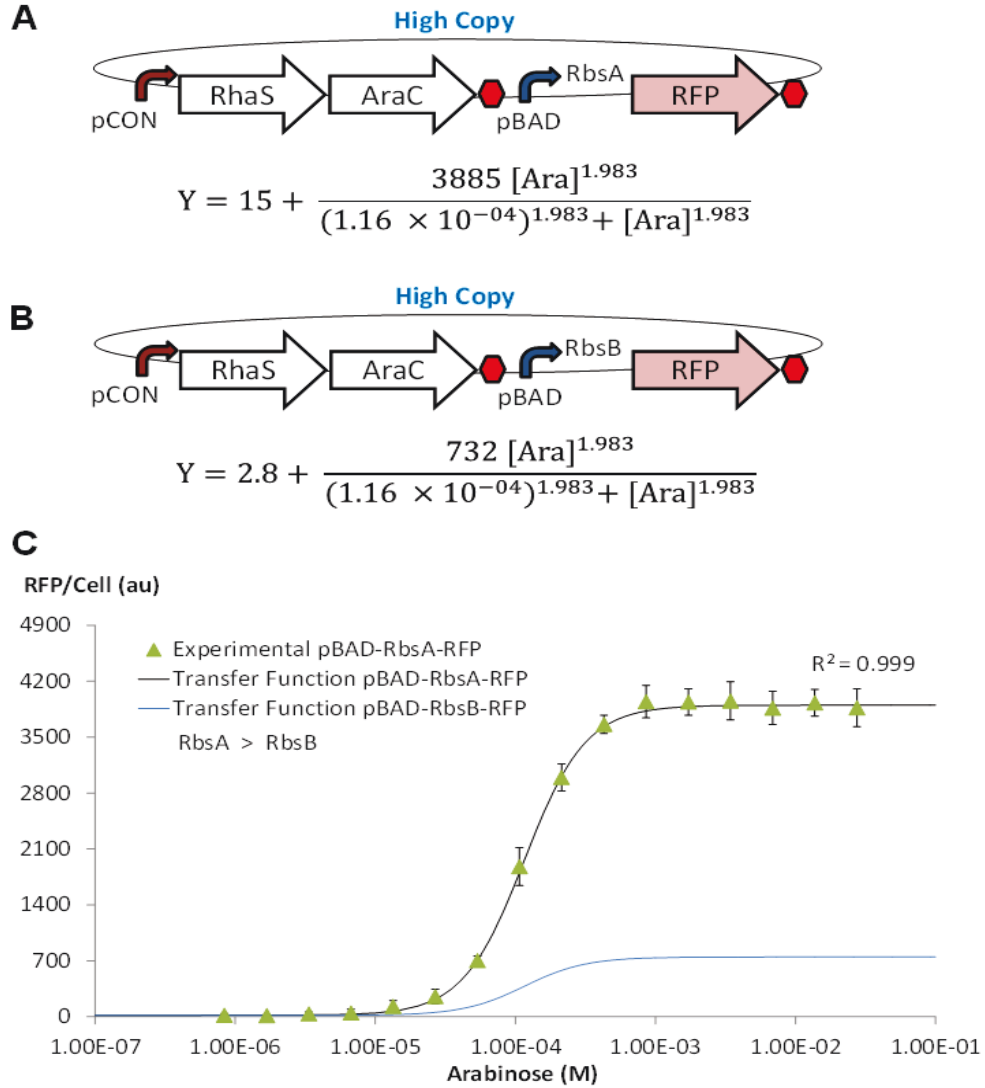


Figure 5-3: Design of input device A with (A) strong and (B) weak ribosome binding sites respectively. Input device A is modelled after the arabinose-induced expression system. It comprises of the cognate AraC transcription factor that is constitutively expressed by the pCON promoter and the inducible pBAD promoter. In the presence of arabinose, AraC binds and triggers conformational change in the pBAD promoter to activate RFP expression. The RhaS transcription factor (for use with rhamnose-inducible expression system) is included in the design to enable accurate characterisation of input device B in the context of the overall half adder genetic circuit. (C) Transfer function of pBAD promoter. Green triangles represent experimental data while black line represents empirically-derived transfer function for construct with strong ribosome binding site as denoted in the equation above. Blue line represents predicted transfer function for construct with weak ribosome site. Error bars represent the standard deviation of biological triplicates.

To examine the possibility of genetic cross-communication, I constructed genetic circuits that couple GFP production to pBAD activation and RFP production to pRHAB activation. The results showed that varying concentration of arabinose did not display any effect on pRHAB promoter activity (**Figure 5-5A**). A similar orthogonal trend was observed in pBAD promoter with rhamnose (**Figure 5-5B**). Interestingly, the simultaneous introduction of both sugars modified the transfer function of each promoter slightly, which may be a result of differential cell growth and sugar import rate. This effect however is insignificant as definite ON and OFF switch behaviours are apparent, thereby confirming the pairing compatibility of pBAD and pRHAB promoters.

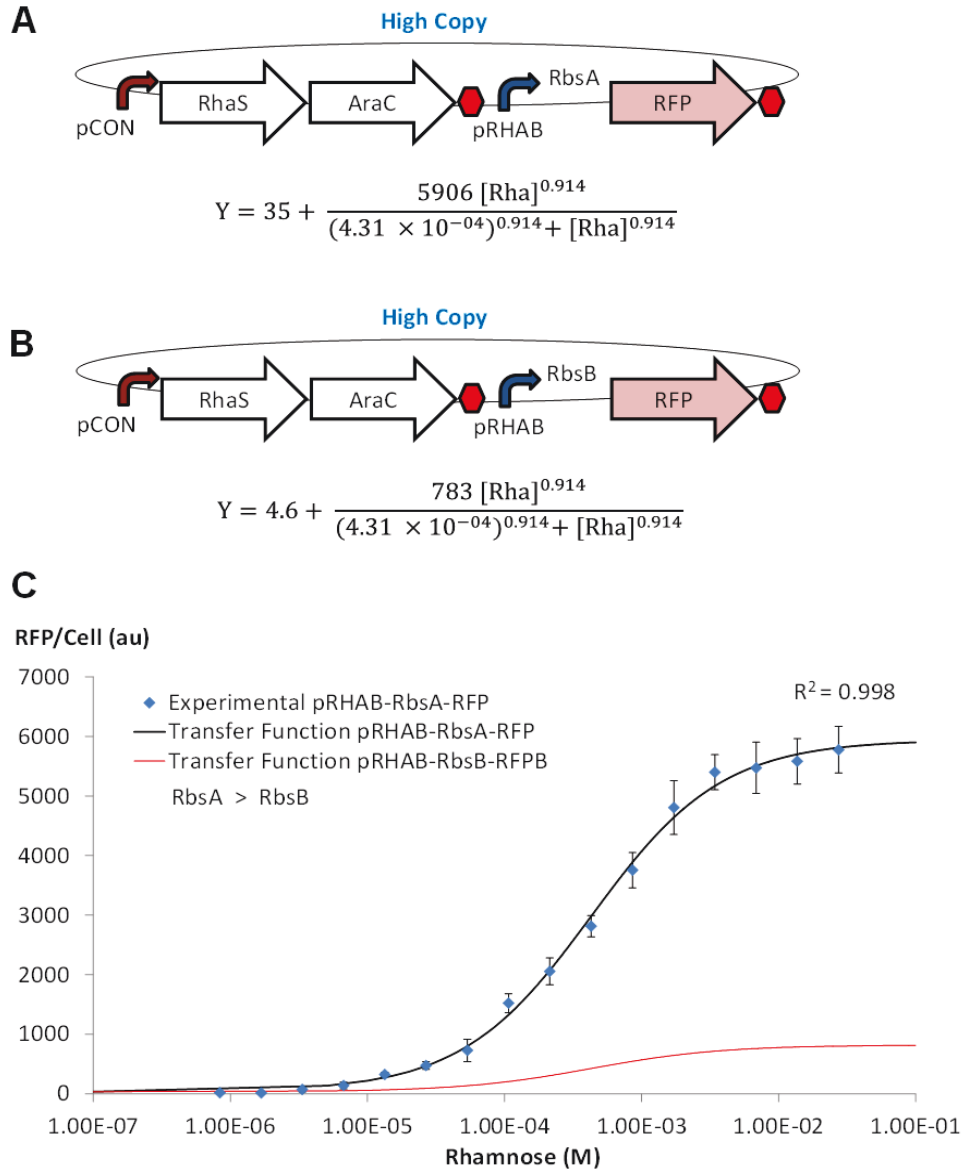


Figure 5-4: Design of input device B with (A) strong and (B) weak ribosome binding sites respectively. Input device B is modelled after the rhamnose-induced expression system. It comprises of the cognate RhaS transcription factor that is constitutively expressed by the pCON promoter and the inducible pRHAB promoter. In the presence of rhamnose, RhaS binds and triggers conformational change in the pRHAB promoter to activate RFP expression. The AraC transcription factor (for use with arabinose-inducible expression system) is included in the design to enable accurate characterisation of input device B in the context of the overall half adder genetic circuit. (C) Transfer function of pRHAB promoter. Blue diamond represent experimental data while black line represents empirically-derived transfer function for construct with strong ribosome binding site as denoted in the equation above. Red line represents predicted transfer function for construct with weak ribosome site. Error bars represent the standard deviation of biological triplicates.

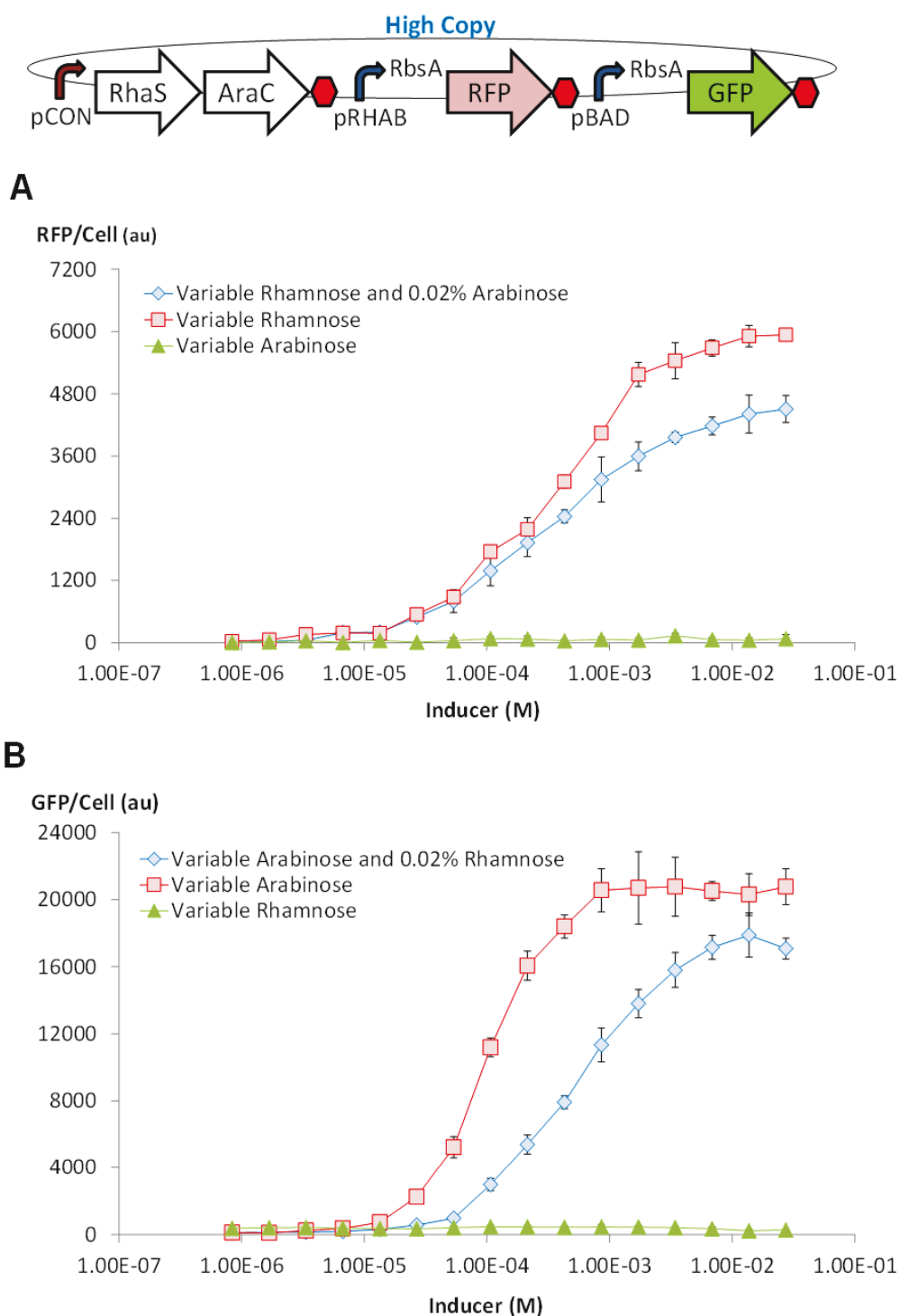


Figure 5-5: Pair-wise compatibility assessment of (A) pRHAB promoter with RFP expression and (B) pBAD promoter with GFP expression. Red square and green triangle show the characteristics of each promoter following induction with its natural and heterologous sugar respectively. Blue diamond represents the performance of each input device in the presence of both sugars. Error bars represent standard deviation of biological triplicates.

5.3 Model-aided Design of AND & OR Logic Gates

To model an AND logic gate, the transfer functions of individual switch devices are incorporated to the following equation similar to how the effective resistance of a parallel arrangement of resistors is calculated.

$$AND_{Abs} = \left[\frac{\alpha_{AND}}{\frac{1}{TF1} + \frac{1}{TF2}} \right] \quad \text{Eqn. (5.2)}$$

Where α_{AND} is an empirically-derived parameter, and TF1 and TF2 are transfer functions of input switch device 1 and 2 respectively.

From **Eqn. 5.1**, the transfer functions of input 1 and 2 are presented as,

$$TF1 = A_1 + \frac{B_1(X_1^{n1})}{C_1^{n1} + X_1^{n1}} \quad \text{Eqn. (5.3)}$$

$$TF2 = A_2 + \frac{B_2(X_2^{n2})}{C_2^{n2} + X_2^{n2}} \quad \text{Eqn. (5.4)}$$

Substituting, **Eqn. 5.3 and 5.4** into **Eqn. 5.2**, the normalised output of AND logic gate:

$$AND_{Norm} = \frac{\left[\frac{\alpha_{AND}}{\frac{1}{TF1} + \frac{1}{TF2}} \right]}{\lim_{X_1 X_2 \rightarrow \infty} \left[\frac{\alpha_{AND}}{\frac{1}{TF1} + \frac{1}{TF2}} \right]}$$

$$= \frac{\left[\frac{1}{\frac{1}{A_1 + \frac{B_1(X_1^{n1})}{C_1^{n1} + X_1^{n1}}} + \frac{1}{A_2 + \frac{B_2(X_2^{n2})}{C_2^{n2} + X_2^{n2}}}} \right]}{\lim_{X_1 X_2 \rightarrow \infty} \left[\frac{1}{\frac{1}{A_1 + \frac{B_1(X_1^{n1})}{C_1^{n1} + X_1^{n1}}} + \frac{1}{A_2 + \frac{B_2(X_2^{n2})}{C_2^{n2} + X_2^{n2}}}} \right]} \quad \text{Eqn. (5.5)}$$

From **Eqn. 5.3 and 5.4**, as X_1 and X_2 approaches infinity, TF_1 and TF_2 are B_1 and B_2 respectively. Thus, the normalised output of AND logic gate is derived as:

$$AND_{Norm} = \left[\frac{B_1 + B_2}{B_1 B_2 \left(\frac{1}{TF_1} + \frac{1}{TF_2} \right)} \right] \quad \text{Eqn. (5.6)}$$

Table 5-2. Transfer functions of input switch devices in the modelling of AND & OR gates.

Input Device (Rbs)	A	B	C	n	Abbrev.
pBAD-HrpS (strong)	15	3885	1.16E-4	1.983	TF1S
pBAD-HrpS (weak)	2.8	732	1.16E-4	1.983	TF1W
pRHAB-HrpR (strong)	45	5906	4.31E-4	0.914	TF2S
pRHAB-HrpR (weak)	4.6	783	4.31E-4	0.914	TF2W

Normalised outputs of four hypothetical AND gate designs were modelled with transfer functions from 4 input switch devices of differing expression strength as listed in **Table 5-2**. **Figure 5-6** shows the predicted output of four different AND gate designs. From the simulation, two possible AND gate designs were derived by matching input device A which generates HrpS, to the corresponding input device B which generates HrpR. The design in **Figure 5-6D** was further chosen for AND gate assembly as it generated less transcription factors, thus imposing less metabolic burden on the cell.

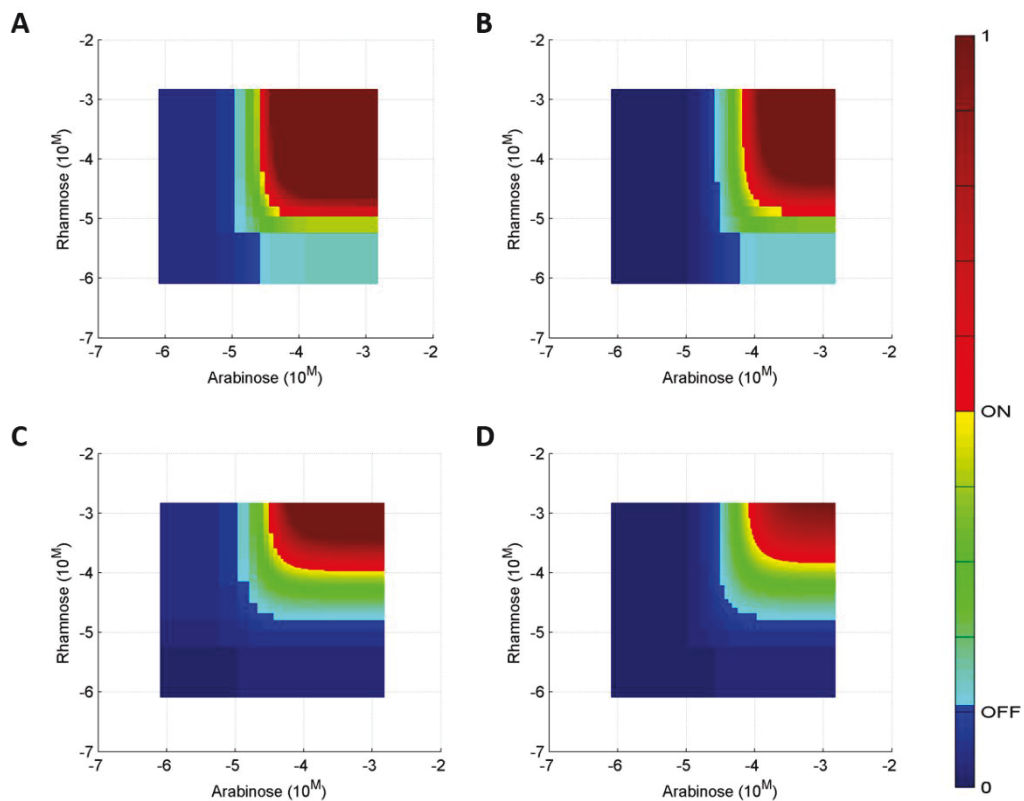


Figure 5-6: Predicted normalised outputs of 4 different AND gate designs with transfer functions corresponding to (A) TF1S-TF2S, (B) TF1W-TF2S, (C) TF1S-TF2W, (D) TF1W-TF2W. From the model, design C and D are functional AND gates, while design A and B may exhibit leaky expression when only arabinose is present.

To model an OR logic gate, the transfer function of individual switch devices are incorporated to the following equation similar to how the effective resistance of serial arrangement of resistors is calculated.

$$OR_{Abs} = [\alpha_{OR} (TF1 + TF2)] \quad \text{Eqn. (5.7)}$$

Substituting, **Eqn. 5.3 and 5.4** into **Eqn. 5.7**, the normalised output of OR logic gate:

$$\begin{aligned} OR_{Norm} &= \frac{[\alpha_{OR} (TF1 + TF2)]}{\lim_{X_1 X_2 \rightarrow \infty} [\alpha_{OR} (TF1 + TF2)]} \\ &= \frac{(TF1 + TF2)}{\lim_{X_1 X_2 \rightarrow \infty} \left[A_1 + \frac{B_1(X_1^{n1})}{C_1^{n1} + X_1^{n1}} + A_2 + \frac{B_2(X_2^{n2})}{C_2^{n2} + X_2^{n2}} \right]} \end{aligned} \quad \text{Eqn. (5.8)}$$

Thus, the normalised output of OR logic gate is derived as:

$$OR_{Norm} = \left[\frac{(TF1 + TF2)}{B_1 + B_2} \right] \quad \text{Eqn. (5.9)}$$

The normalised outputs of four hypothetical OR gate designs were modelled with transfer functions from 4 input switch devices of differing expression strength. **Figure 5-7** shows the predicted output of four different OR gate designs. From the simulation, two possible OR gate designs were derived by matching input device A to input device B with similar output expression strength. Designs in **Figure 5-7C and D** were constructed and evaluated in the later parts of Chapter 5 and 6.

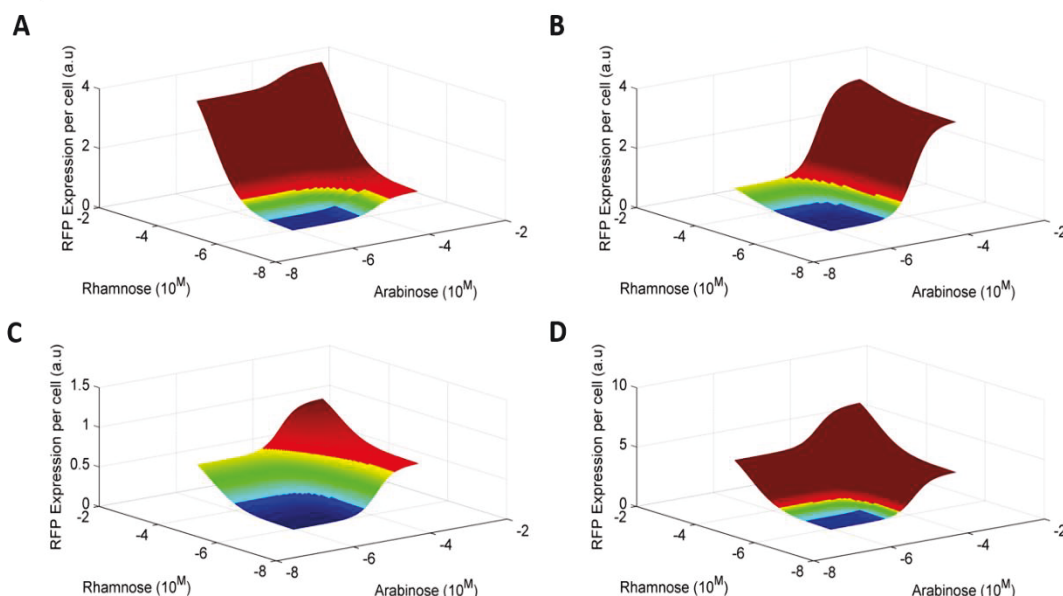


Figure 5-7: Predicted normalised outputs of 4 different OR gate designs with transfer functions corresponding to (A) TF1W-TF2S, (B) TF1S-TF2W, (C) TF1W-TF2W, and (D) TF1S-TF2S. From the model, only design C and D are functional OR gates, while design A and B are imbalanced in the overall expression profile.

5.4 Characterisation of AND Logic Gate

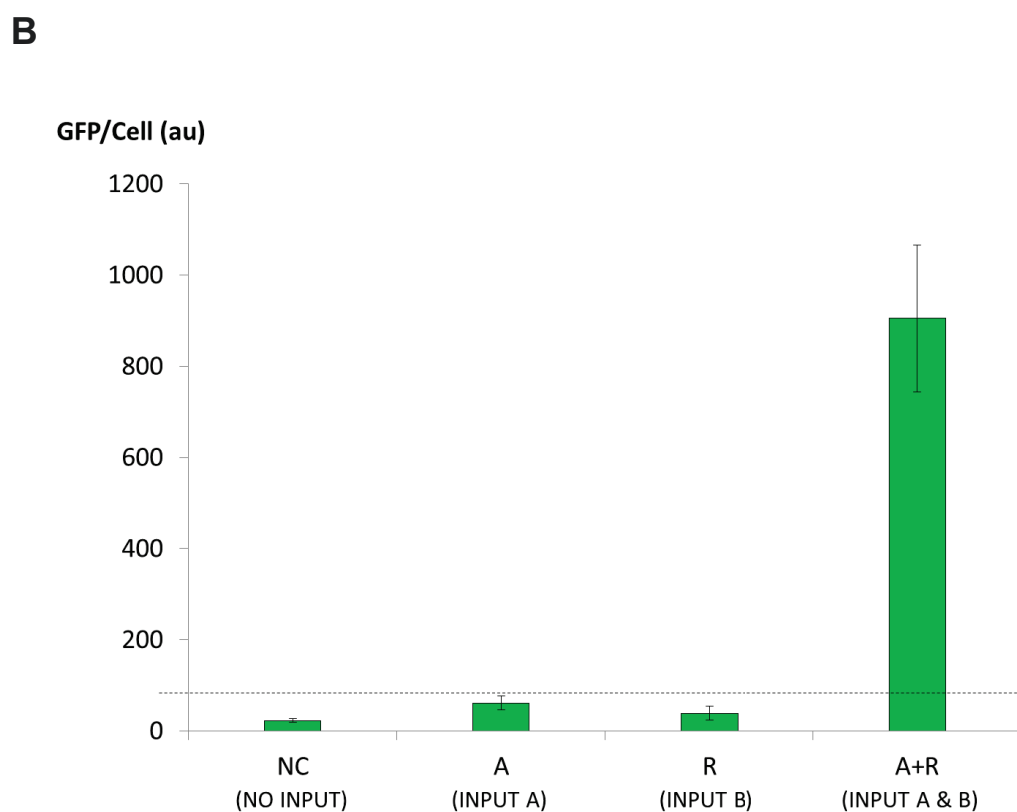
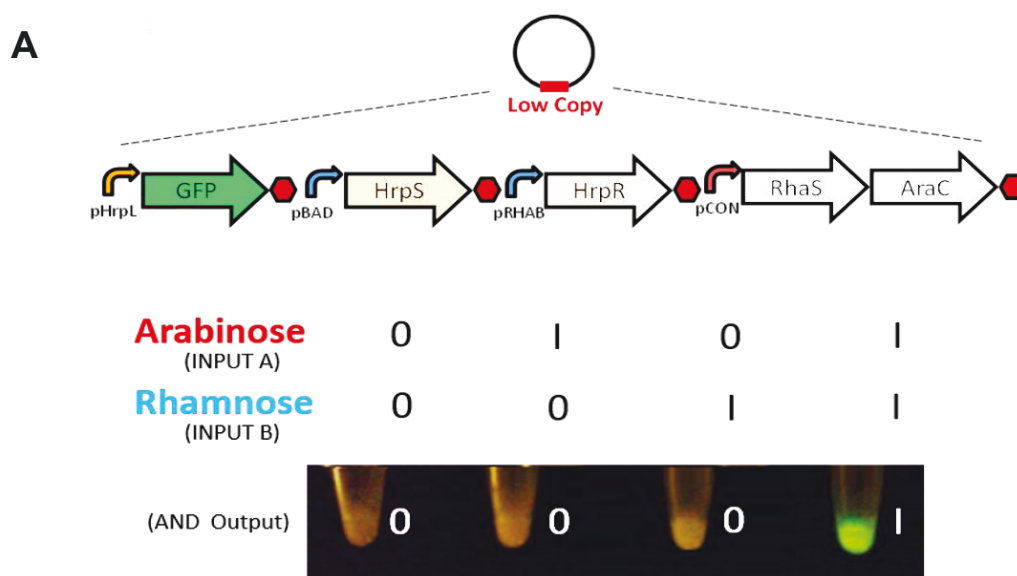
To develop a biological AND logic gate, the σ^{54} -dependent HrpRS regulation motif of *Pseudomonas syringae* T3SS secretion system was refactored by placing the expression of HrpRS transcription factors in two inducible expression systems¹²⁷. The advantage is that HrpRS AND gate offers dual layer of orthogonal control in *E. coli* host. This means that (a) the majority of transcription events occurs via σ^{70} -dependent transcription, and (b) HrpRS transcription factors are absent in wild type *E. coli*. Transcription occurs when enhancer-binding proteins HrpS and HrpR, which are regulated by arabinose (input A) and rhamnose (input B) induction respectively, are coexpressed and bound to the upstream activator sites of pHrpL promoter. This binding event then triggers an ATPase-dependent conformational change within the

promoter through a molecular interplay with the σ^{54} -RNAP holoenzyme, thereby allowing RNA synthesis and elongation after the transcription start site.

Designs of whole cell AND logic devices have hitherto involved the use of multiple plasmids^{101, 120, 127, 133}. Towards the progression of programmable biological systems with advance functionalities, it is beneficial to minimise the usage of expression vectors and contain genetic logic gates in single plasmid as far as possible. Such design procedure would effectively modularise logic devices in a “plug and play mode”, and facilitate system transfer between biological chassis for diverse applications. More importantly, modularising logic devices simplifies the integration and evaluation of layered genetic circuits with multiple components – allowing smoother process for troubleshooting and fine tuning of genetic circuits.

In this study, refactored modules of the HrpRS transcription machinery were systematically designed and assembled into a low copy plasmid (**Figure 5-8A**). The module which expressed GFP from pHrpL promoter was assembled upstream of pBAD-HrpS and pRHAB-HrpR modules to avoid any genetic context dependent effects that might arise from transcriptional overrun of the stronger pBAD and pRHAB input expression modules as a result of inefficient transcription termination. The “on” and “off” digital performance of the AND gate at steady state was qualitatively and quantitatively assessed by introducing inputs well above switch points in four different logic conditions (**Figure 5-8A and 5-8B**). The results showed that the AND gate was only activated in the presence of both inputs with approximately 900 fold

expression increase, as compared to conditions whereby only single input is present (or no inputs). Next, steady state profile of the functional AND gate was characterised by titrating with varying concentration of arabinose (input A) and rhamnose (input B) as shown in (**Figure 5-8C**).



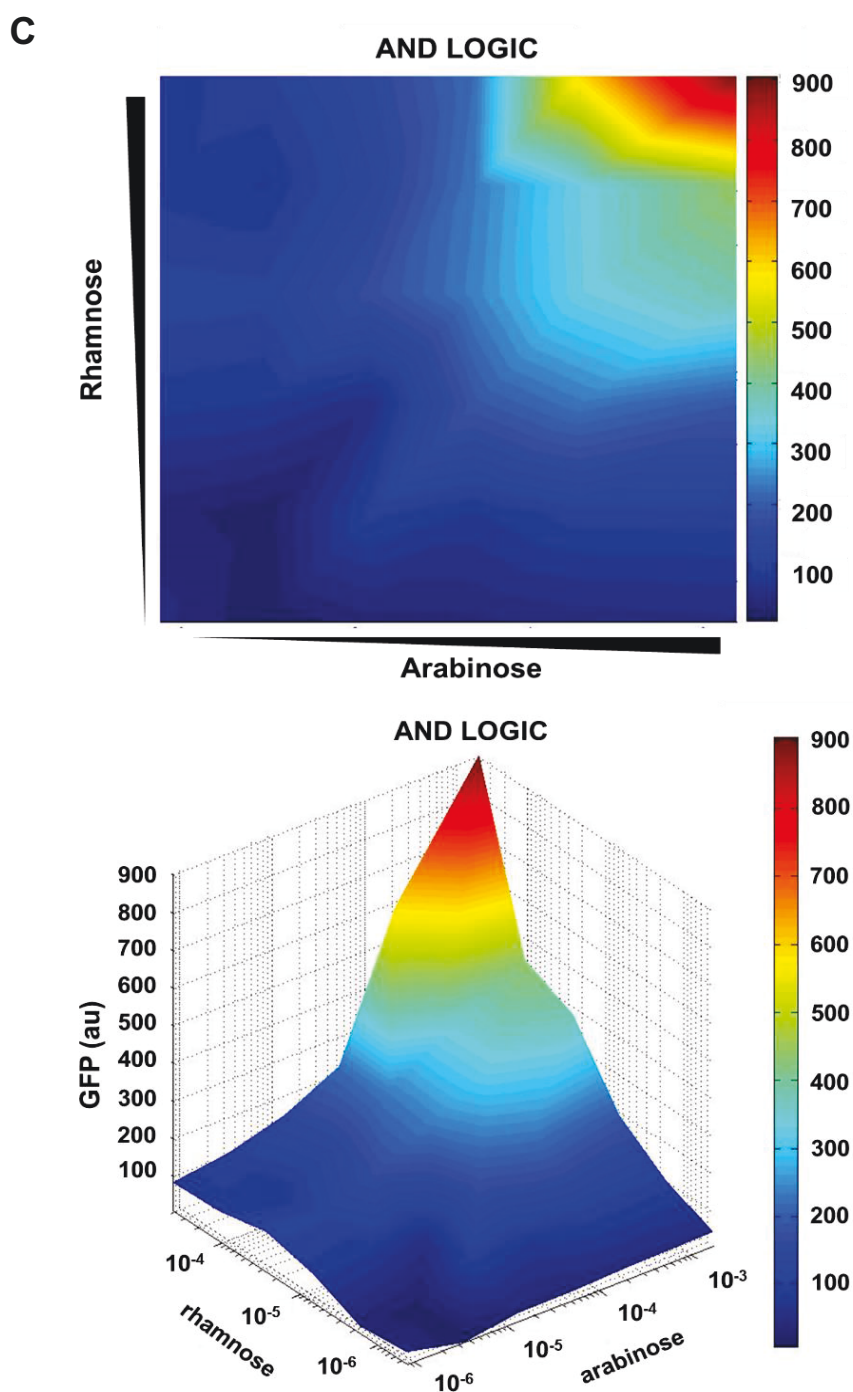


Figure 5-8: Design and characterisation of biological AND gate. (A) Design and logic output of Hrp-based AND gate. The AND gate comprises of HrpS and HrpR transcription factors that are unregulated under the control of pBAD and pRHAB promoters respectively. In the presence of both inputs, HrpRS jointly bind and induce conformational change in the pHrPL promoter, thereby enabling DNA transcription and the expression of GFP reporter. (B) Digital performance of AND gate at steady state. Error bars represent the standard deviation of biological triplicates. (C) Steady state profile of AND gate in varying concentration of arabinose (input A) and rhamnose (input B).

To assess the effect of plasmid copy number on the performance of the AND gate, modules which generated the HrpRS transcription activators (pBAD-HrpS-pRHAB-HrpR) were constructed. This produces GFP output (pHrpL-GFP) in separate low and high copy plasmids (co-transformed with HrpRS-generating plasmid *E. coli* cells). The relative GFP output of each system was measured (**Figure 5-9A**). The results showed that the AND gate system with GFP-producing module in high copy plasmid and HrpRS transcription activators in low copy plasmid produced a >4 fold higher GFP than AND gate systems with GFP-producing module in low copy plasmid and HrpRS (**Figure 5-9B**). The result indicates that a higher concentration of HrpRS transcription activators, above the saturation limit of the pHrpL promoter, do not produce greater GFP output. It is likely that the transcriptional output of the HrpRS AND gate is limited by the strength of the weak pHrpL promoter. Hence, the conclusion is that when pHrpL-GFP module was expressed in high copy plasmids, intracellular availability of pHrpL promoters were increased – resulting in the amplification of GFP output.

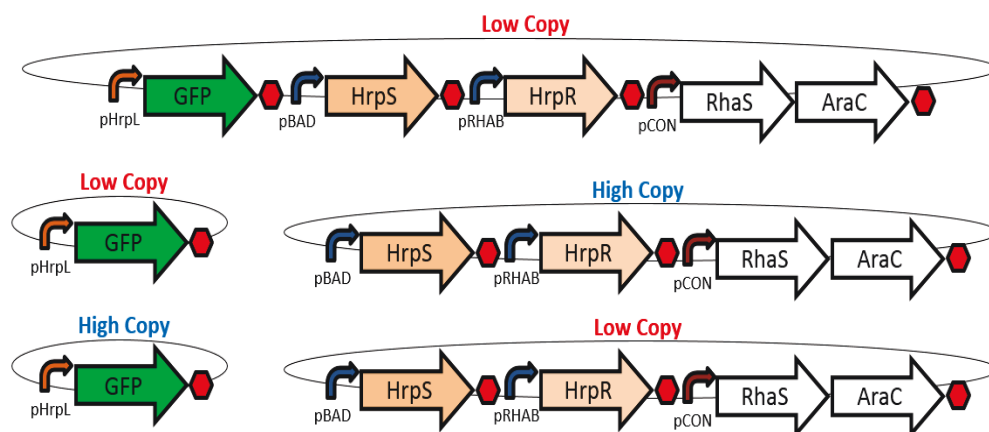
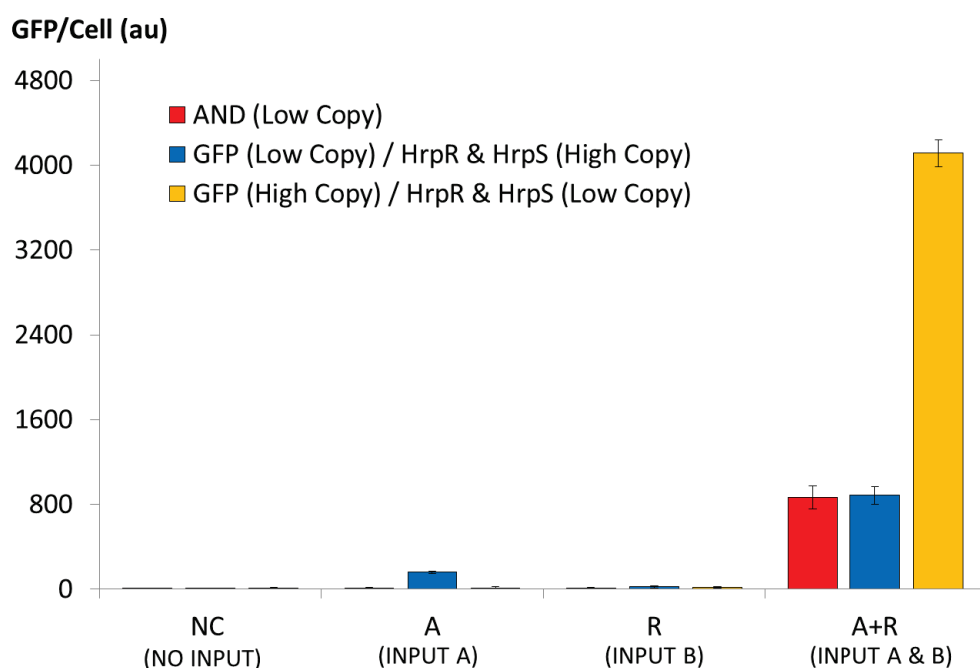
A**B**

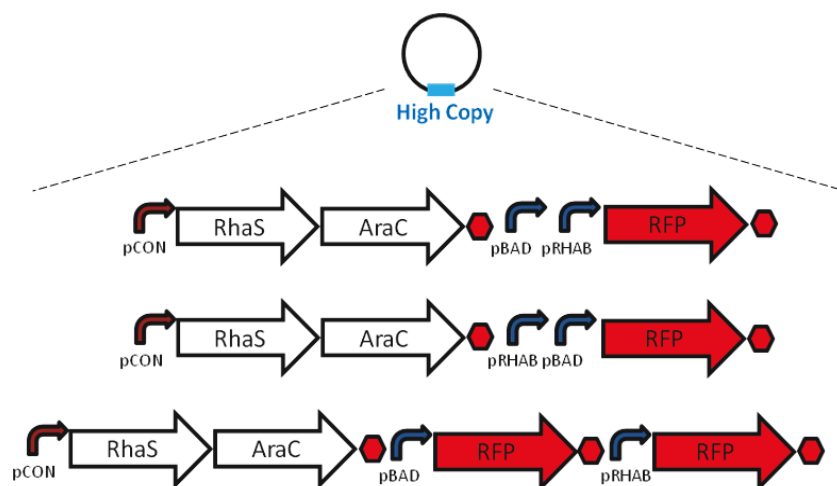
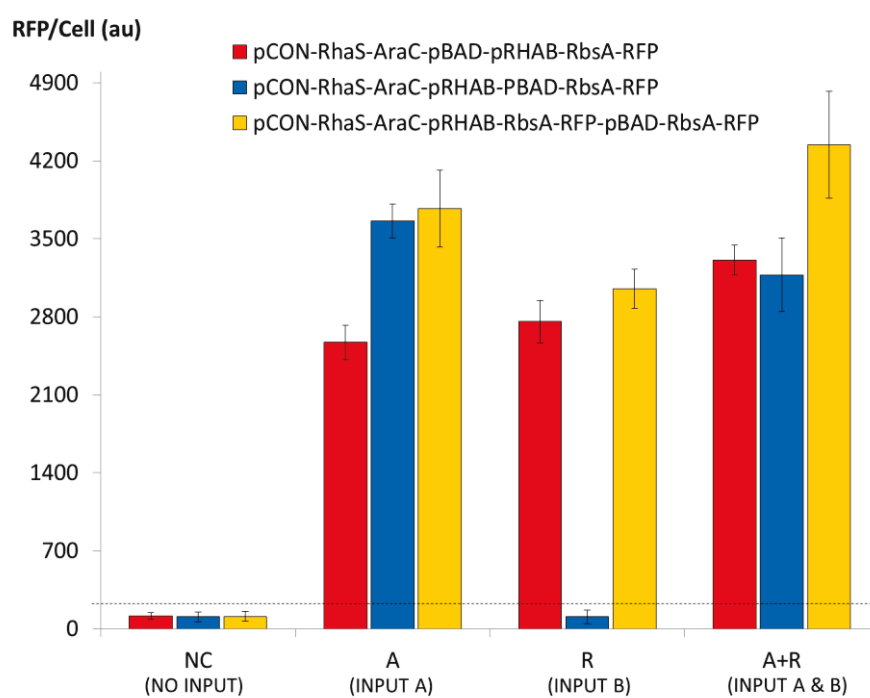
Figure 5-9: Effect of plasmid copy number on AND gate performance. (A) Genetic blueprint and (B) evaluation of HrpRS AND gate system in both high and low copy plasmids. Input devices generating HrpRS transcription factors and pHrL-GFP reporter module are placed in plasmids of different copy numbers to study the effect of plasmid copy on precision control and tuning of Hrp-based AND gate. Error bars represent the standard deviation of biological triplicates.

5.5 Characterisation of OR Logic Gate

Genetic OR gates can be achieved by designing tandem promoter genetic circuits or by expressing a target gene in two discrete expression cassettes. Nonetheless, tandem promoter OR gate circuits may fail when repression of downstream promoter prevents the proper functioning of the upstream promoter¹⁶⁵. To develop the OR logic gate of the half adder, three prototype designs were constructed; two of which comprised of pBAD and pRHAB promoters in different tandem arrangements upstream of an RFP reporter gene with strong RBS, and a third design that produces RFP in two distinct expression cassettes (**Figure 5-10A**). The three OR gate designs were then introduced with input A and B above their switch points and assessed for the respective RFP outputs (**Figure 5-10A and 5-10B**). The results showed that design I and III are functional OR gates with >2500 folds higher RFP expression when either or both inputs were present. The OR gate design II, which composed of pRHAB promoter upstream of pBAD promoter and RFP reporter, was activated only in the presence of rhamnose but not arabinose. These results agree with previous finding that no expression was detected when pBAD promoter was fused downstream of tetracycline-inducible pTET promoter and upstream of a YFP reporter¹⁶⁵.

The conclusion is that this observation is likely an effect of the AraC transcription factor which can function as both repressor and activator. In the absence of arabinose, AraC when over expressed, remains bound to operator sites that induce DNA looping of the pBAD promoter, thereby obstructing the elongation of mRNA by initiated RNA polymerase. The result also suggests

that genetic circuits which involved tandem promoters in serial arrangement can be used to elucidate the biochemistry of inducible promoters and their associated transcription factors – exemplifying a bottom-up approach to understanding biology. As will be shown in Chapter 6 of this thesis, in order to achieve other applications of the OR gate design I, the construct was characterised for its steady state profile by titrating with varying concentration of arabinose and rhamnose (**Figure 5-10C**).

A**B**

C

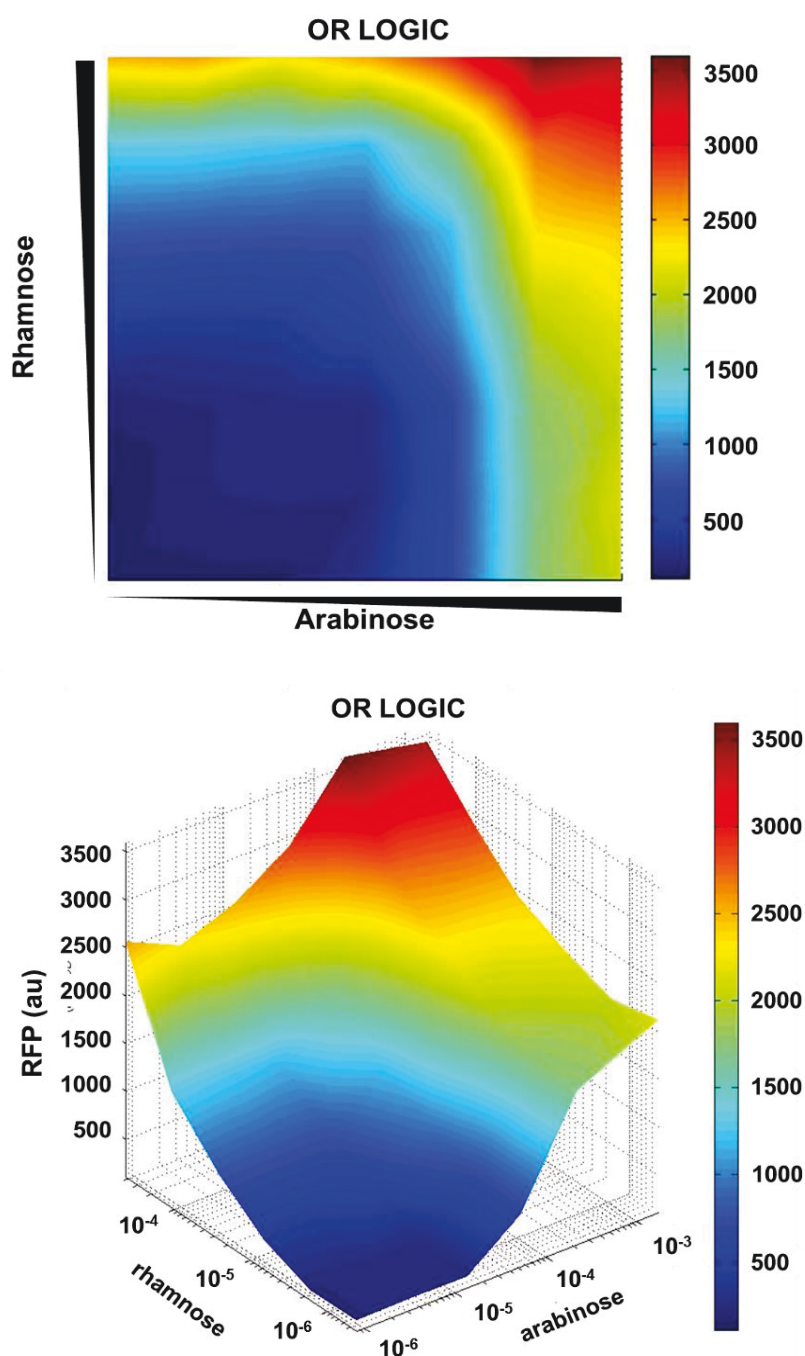


Figure 5-10: Design and characterisation of biological OR gates. (A) Genetic blueprint and logic output of three OR gate designs. Design I and II are tandem promoters in opposite arrangement while design III expresses RFP reporter in two distinct transcripts. Only design I and III are functional OR gates that generates RFP in the presence of either inputs. (B) Digital performance of OR gates at steady state. Error bars represent the standard deviation of biological triplicates. (C) Steady state profile of OR gate I in varying concentration of arabinose (input A) and rhamnose (input B).

5.6 Conclusion

To expand the biosensing capabilities of designer probiotics, we applied the synthetic biology engineering framework to develop orthogonal sets of AND and OR genetic logic gates that can be used as a processing interface to assimilate multiple input signals. As a proof of concept study, we created two orthogonal input devices to sense arabinose and rhamnose inputs from a library of standardised biological parts comprising of promoters, ribosome binding sites, transcription factors, gene reporters and transcription terminators. The input switch devices were rigorously characterised and modelled for different expression strength when coupled to different ribosome binding sites.

While it is generally known that simple genetic switches can be modelled in the form of Hill-like transfer function equations (**Eqn. 5.1**), it remains ambiguous as to how the choice of ribosome binding sites will affect the overall properties of these transfer functions. Herein, we verified that ribosome binding sites can function as direct amplifiers of reporter output in genetic biosensors, being most sensitive to parameters A and B when n is less than 2. Switch points of most inducible promoters assessed in this study were more or less coherent when tested with different ribosome binding sites.

Next, a novel forward engineering approach was implemented to design both the AND and OR logic gates by considering the transcriptional machinery of each sub-components as orthogonal, modular units that functioned like resistors in wired electrical circuits. For example, the normalised output of AND gate which required parallel layering of HrpS and HrpR expression

cassettes was modelled according to the net resistance of resistors connected in parallel arrangement. Likewise, the normalised output of OR gate was modelled according to the net resistance of resistors in serial arrangement. From the modelling prediction, optimal designs of AND and OR gates were then constructed and characterised accordingly. Of note, the predicted, normalised output of AND and OR gate were largely congruent with the normalised, experimental results, with goodness of fit, R^2 value > 0.9 .

To facilitate studies in the next chapter which required the layering of independent logic gates, AND and OR logic gates developed in this chapter were rigorously characterised in different context including genetic architectural designs or plasmid copy numbers. Importantly, OR gate which are designed from tandem promoter genetic circuits may fail when downstream promoters are naturally repressed by strong DNA bending elements. In such situation, characterisation in different genetic context is necessary to avoid bad designs. Finally, we showed that the expression output of HrpRS AND gate is limited by the strength of the weak pHrpL promoter. By increasing the total intracellular availability of pHrpL promoters on high copy plasmids, higher expression output can be generated from the HrpRS system.

Chapter Six

6. PROGRAMMING AUTONOMOUS DECISION MAKING IN COMMENSAL MICROBE WITH BIOLOGICAL HALF ADDER

Engineering digital operations in biological system is often far from trivial and requires considerable time and effort during functional testing and tuning of synthetic genetic circuits. Apart from the scarcity of reliable and well characterised biological parts, digital performances in biological system are further implicated by the cellular and genetic context dependent effects of the parts applied^{128, 166}. Cellular context effects are fairly well understood and occurred as a result of genetic cross-talk between engineered circuits with the endogenous networks of host cell. For this reason, molecular parts and devices that were orthogonal to the cell native machineries with roles in either genetic transcription or protein translation had been created to enable predictable engineering of genetic circuits^{120, 127, 167-169}. Demonstrations of layered genetic circuits in single cell, such as the execution of 4-input AND gate in bacteria¹²⁰ and biological half adder and half subtractor in mammalian cells¹⁷⁰ have revealed that orthogonal logic gates can be interlinked to perform digital operations of higher complexity and computational strength.

While the capability to program cells with memory^{101, 110} and decision making functions presents many opportunities in biotechnological applications, a lack

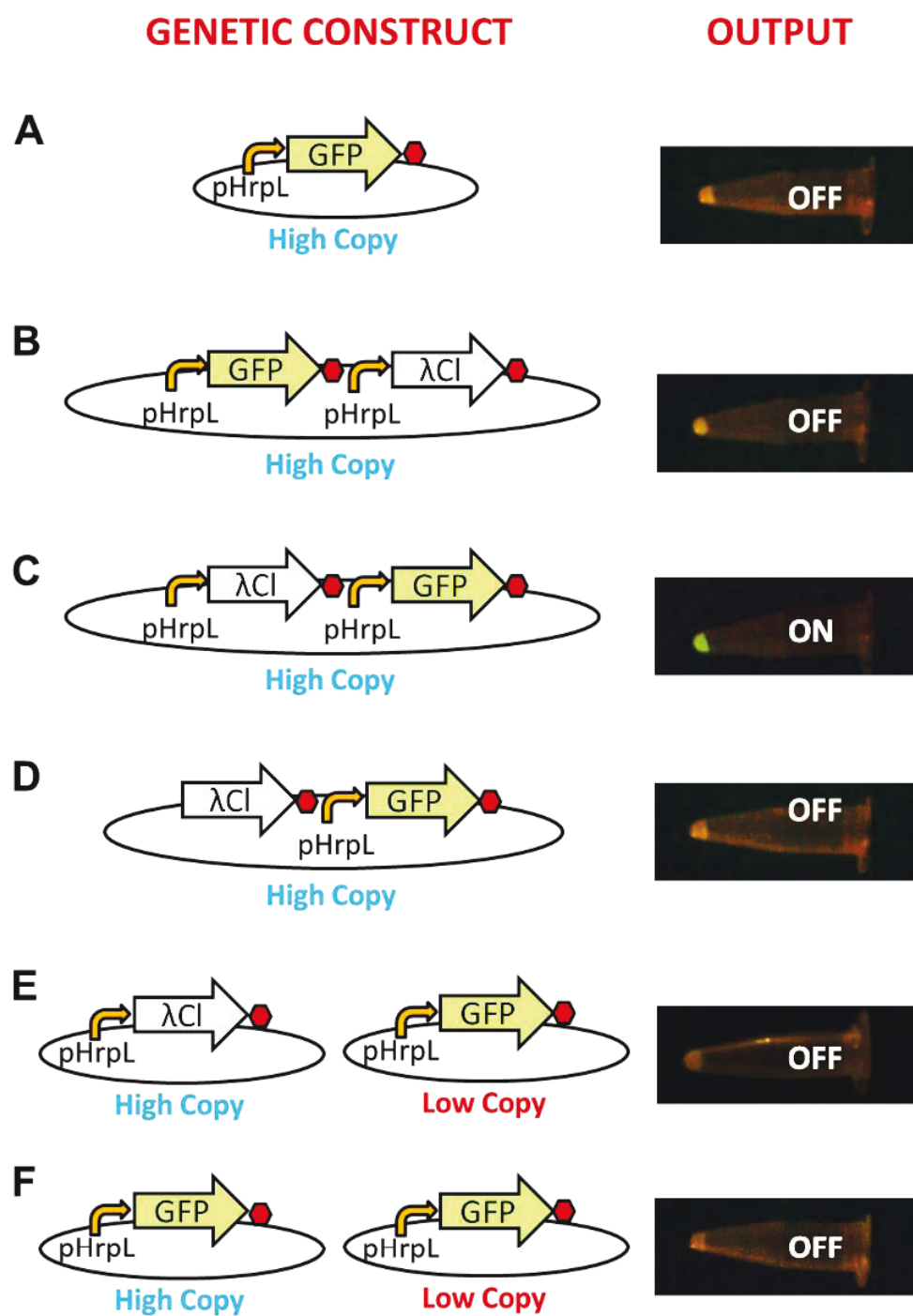
of formal understanding associated with genetic context dependent effects had undermined our progress in engineering biology. In this respect, two studies have shown that the 5' untranslated region (5'-UTR) of mRNA can affect the temporal control of multigene operons or inverter-based genetic circuits, and RNA processing using CRISPR RNA endonuclease or ribozyme can serve as effective genetic insulators to buffer such context dependent effects^{128, 129}. In this chapter, we have sought to elucidate the limitations of engineering biology from an architecture point of view, with the hope of creating a general set of design heuristics that will serve as fundamental guidelines for the design and implementation of synthetic genetic circuits in the future.

In this study we were interested to develop a biological half adder in prokaryotic systems, particularly in microbes which exhibit much faster cell division and cycle – so that they can be broadly applied in different biotechnological applications. In contrast to the mammalian half adder which was developed mainly for therapeutic and biosensing applications, a prokaryotic half adder can be used to enhance molecular process control and decision-making in drug and biofuel production, biosensing and bioremediation¹⁷¹, and probiotic engineering for the treatments of metabolic disorders¹⁸, cancer^{172, 173} and infectious diseases^{144, 174}. In digital processing, half adders form the key building blocks for shift registers, binary counters and serial parallel data converters. Half adder logic can also be further connected in tandem to display output signals of full adder and other advanced digital operations such as ripple-carry adders, which may eventually pave the way for development of true 'microprocessors' in biological platforms.

6.1 Genetic Context Effect of σ 54-dependent pHrpL promoter

To enable sufficient expression of λ CI repressor by an AND gate system, the gene encoding for λ CI repressor was assembled downstream of σ 54-dependent pHrpL promoter on a high copy plasmid. Fortuitously, we discovered that pHrpL promoter located downstream of another pHrpL expression cassette can be turned on even in the absence of its cognate HrpRS transcription factors (**Figure 6-3C**). The converse is not true for an upstream pHrpL promoter (**Figure 6-3B**). Negative controls with just GFP reporter or RBS- λ CI gene upstream of pHrpL-GFP module confirmed that pHrpL promoter alone is not leaky and that cryptic promoter is absent in the λ CI gene (**Figure 6-3A and 6-3D**).

To buffer against this genetic context dependent effect of the pHrpL promoters, we assembled pHrpL-GFP and pHrpL- λ CI modules on separate plasmids. This successfully prevented the genetic interference of both pHrpL expression modules on one another (**Figure 6-3E and 6-3F**). **Figure 6-3G** shows a quantitative assessment of pHrpL promoter activation due to the presence of another upstream pHrpL promoter and the use of plasmids as genetic insulators.



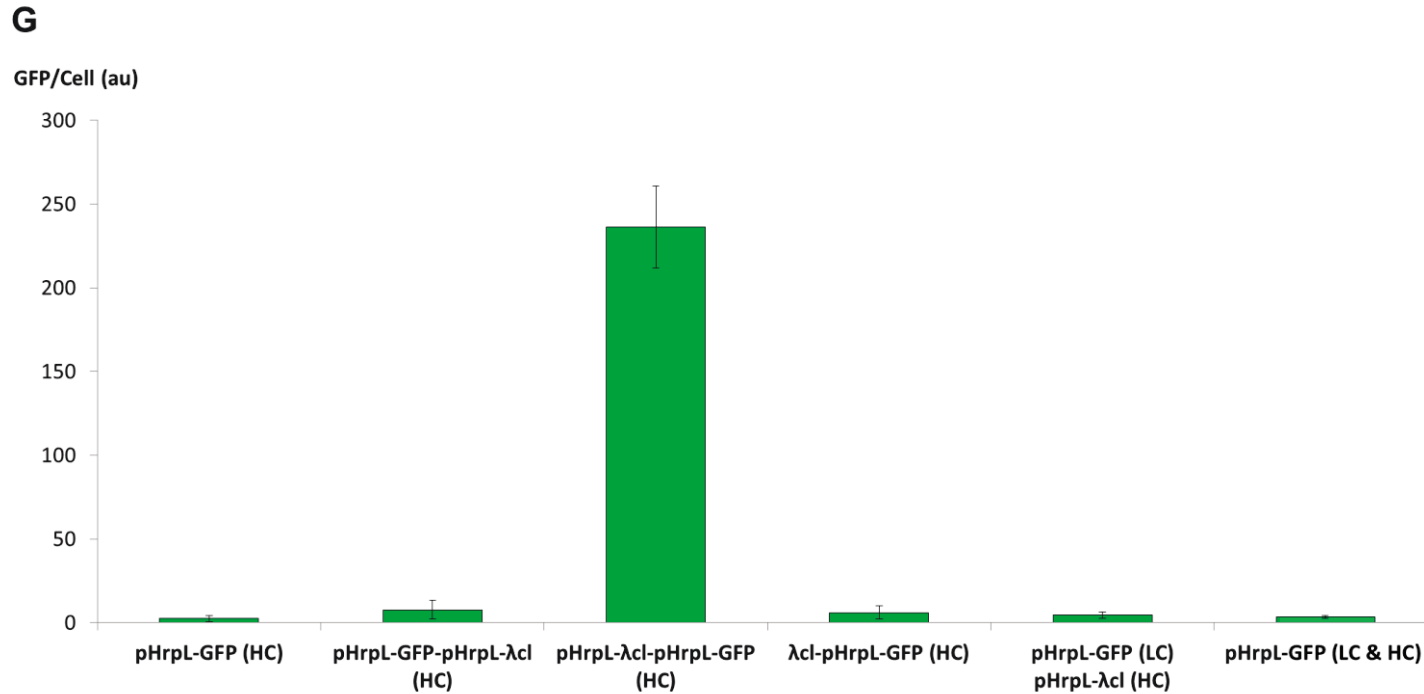
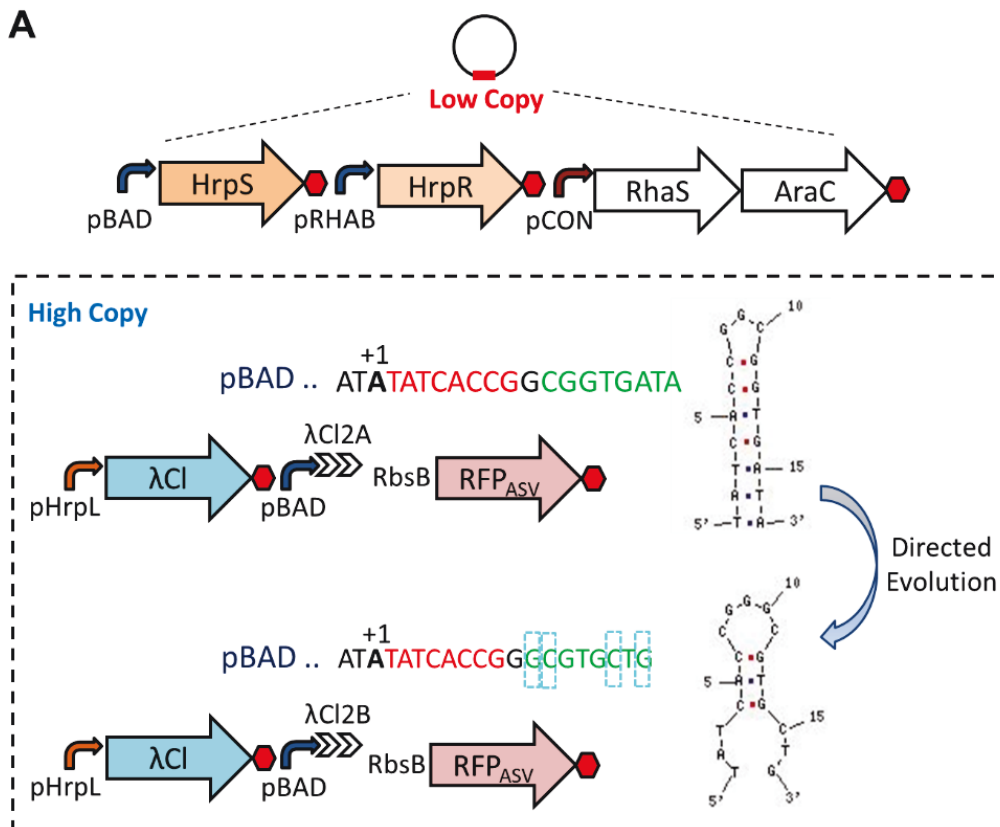


Figure 6-3: Effects of genetic architecture on the switching behaviour of σ_{54} -dependent pHrpL promoter. (A) pHrpL-GFP reporter in high copy plasmid, (B) pHrpL-GFP reporter assembled upstream of pHrpL- λ Cl in high copy plasmid, (C) pHrpL-GFP reporter assembled downstream of pHrpL- λ Cl in high copy plasmid, (D) pHrpL-GFP reporter assembled downstream of λ Cl in high copy plasmid, (E) pHrpL-GFP reporter and pHrpL- λ Cl separately assembled in low and high copy plasmids respectively and (F) pHrpL-GFP reporter assembled in both low and high copy plasmids. Within the same plasmid vector, the presence of an upstream pHrpL promoter is able to turn on proximate, downstream pHrpL promoter and caused GFP expression even in the absence of HrpRS transcription activators. This genetic context dependent effect can be circumvented by insulating pHrpL expression cassettes in different plasmids. (G) Quantitative characterisation of pHrpL promoter leakiness in different genetic context. Error bars represent the standard deviation of biological triplicates.

6.2 Characterisation of NOT and IMPLY Logic Gates

A



B

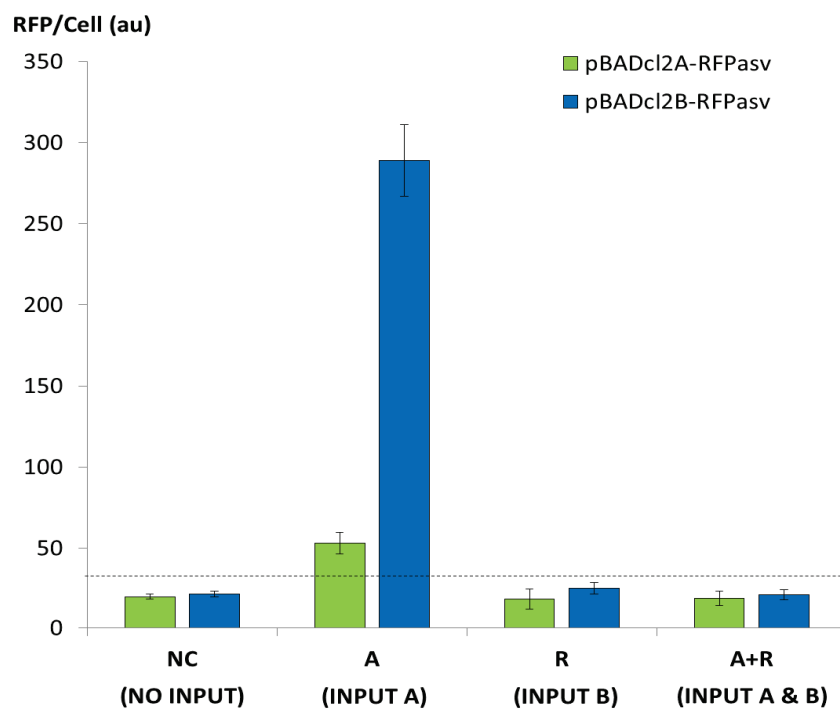


Figure 6-4: Directed evolution and characterisation of lambda repressor binding sites. (A) Layout of genetic circuit used in the mutation and screening of lambda repressor binding sites. Lambda repressor binding sites located downstream of pBAD promoter transcription start site (denoted with +1) are denoted as red and green letters (reverse complement). Key mutations that generated the best repressor in this screen are marked by green boxes. mRNA secondary structures from the both the original and screened repressor binding sites are shown. (B) Characterisation of original (blue bars) and evolved (red bars) lambda repressor binding sites in the engineered genetic circuit. Error bars represent the standard deviation of biological triplicates.

As part of the development of XOR logic operations of the half adder, orthogonal repressor binding sites are required downstream of the OR gate promoters. To examine the minimal number of λ CI repressor binding sites required for effective repression, single λ CI operator site and dual λ CI operator sites of perfect dyad symmetry downstream of pBAD promoter were fused before the RFP gene. The repressibility of both circuits were tested by generating λ CI repressors from HrpRS AND gate in a separate plasmid¹⁷⁵. Negligible repression was observed when only one λ CI repressor operator site was present. In the presence of two operator sites of perfect dyad symmetry, RFP expression from pBAD promoter was greatly attenuated – even when λ CI repressor was not synthesized. I postulate that the observed reduction of RFP expression might be caused by the presence of secondary hairpin structures immediately downstream of TSS acting as *pseudo* transcription terminator or locking RBS in conformations that translation initiation (**Figure 6-4A**).

In order to examine this further, random mutagenesis on the natural sequence of the λ CI repressor operator sites were performed and screened for mutants with significant difference in RFP expression levels, in the absence and

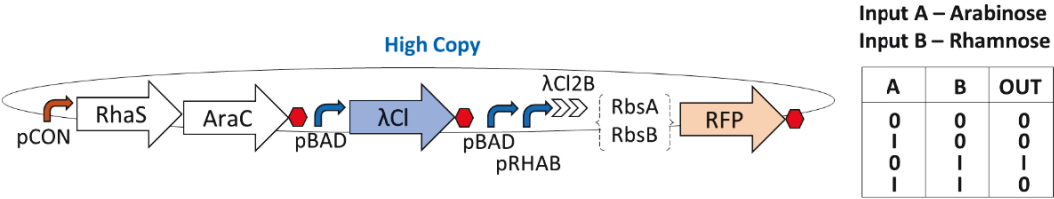
presence of λ CI repressor. Accordingly, an evolved candidate (CI2B) with 4 mutations in the inverted sequence of the λ CI repressor binding (**Figure 6-4B**) was obtained. Sequence comparison of the original λ CI repressor binding sites (CI2A) with the evolved candidate revealed that the directed evolution process had relieved secondary hairpin structures from 7 to 3. Next, the efficiency of λ CI-mediated transcription termination in the context of a genetic IMPLY gate was studied. This was achieved by placing repressor binding sites directly downstream of tandem pBAD-pRHAB promoters and generating λ CI repressors from a separate pBAD expression cassette. Two IMPLY logic circuits were developed which generated RFP transcripts with strong and weak RBS (**Figure 6-5A**). These were tested in both systems in the presence and absence of input A overtime with input B, both above switch point. The results showed that while IMPLY logic can be achieved from both circuits, the system with strong RBS exhibited higher order of expression leakiness as compared to that which translated RFP from weaker RBS.

We characterised the steady state transfer function of the IMPLY gate with strong RBS by titrating cells that were activated for RFP expression by a fixed input of B above switch point with variable concentration of input A (**Figure 6-5C**). The result shows that the IMPLY gate can be described mathematically by a Hill-like equation of the form

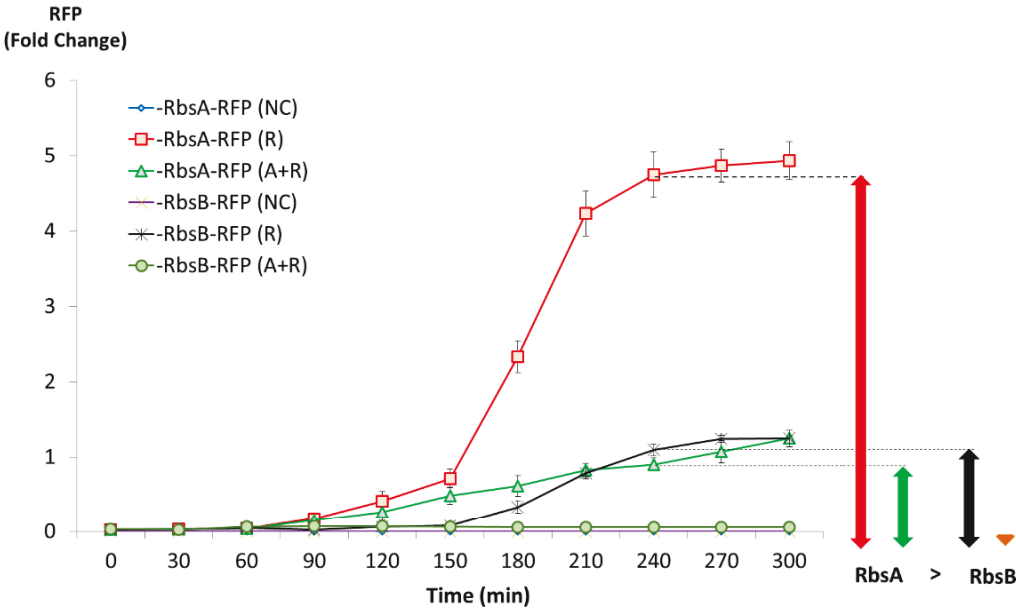
$$\text{Output } Y = A + \frac{\alpha B(C^n)}{C^n + X^n}$$

Where A , B , C and n are empirically derived parameters, X is input A concentration, and $\alpha = 1$ in presence and 0 in absence of input B above switch point.

A



B



C

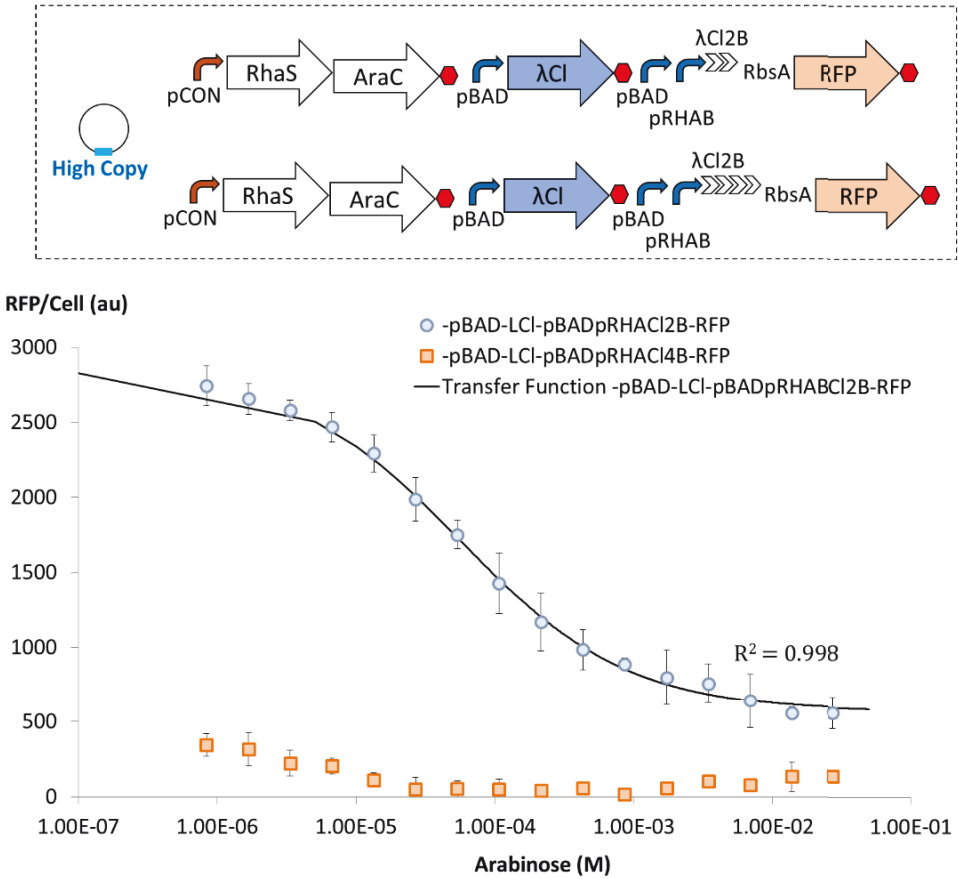


Figure 6-5: Design and characterisation of IMPLY gate. (A) Genetic blueprint and true table of IMPLY gate. The IMPLY gate is designed by incorporating synthetic λ CI binding sites downstream of OR gate promoters and regulating the expression of λ CI repressors through the pBAD promoter. (B) Characterisation of IMPLY gate with different ribosome binding sites. At steady state, the IMPLY gate with a weaker ribosome binding site (RbsB) upstream of the RFP reporter (black stars, orange circles and purple crosses) exhibits reduced expression leak as compared to IMPLY gate design with a stronger ribosome binding site (red squares, green triangles and blue diamonds). Induction with input B, inputs A and B, or uninduced are represented by R, A+R and NC. (C) Characterisation of IMPLY gates with two (blue circles) and four (red squares) lambda repressor binding sites. Black line represents empirically-derived transfer function for construct with dual λ CI binding sites. Error bars represent standard deviation of biological triplicates.

Drawing insights from earlier results, we hypothesized that parameters A and B are parameters that are most sensitive to the overall behaviour of IMPLY gates when the RBS that initiate the expression of the RFP reporter are changed. To test this hypothesis, we simulated the output of IMPLY gates whereby the RFP expressions are initiated from RBS of different strengths (**Figure 6-6**). The simulation aligned with observed experimental results and implied that the choice of RBS can be employed as a signal moderation technique to achieve balance between precision tuning and output gain in layered logic gates. In an attempt to alleviate expression leakiness from the IMPLY gate with strong RBS, an additional pair of λ CI repressor binding sites with imperfect dyad symmetry were introduced downstream of pBAD-pRHAB-CI2B and before the RBS-RFP module. However, the presence of 4 λ CI binding sites completely inhibited RFP expression, resulting in failure of the IMPLY gate. It is likely that this failure could be an effect of pronounced 5' UTR secondary structures formed due to the repeated use of identical λ CI repressor binding sites.

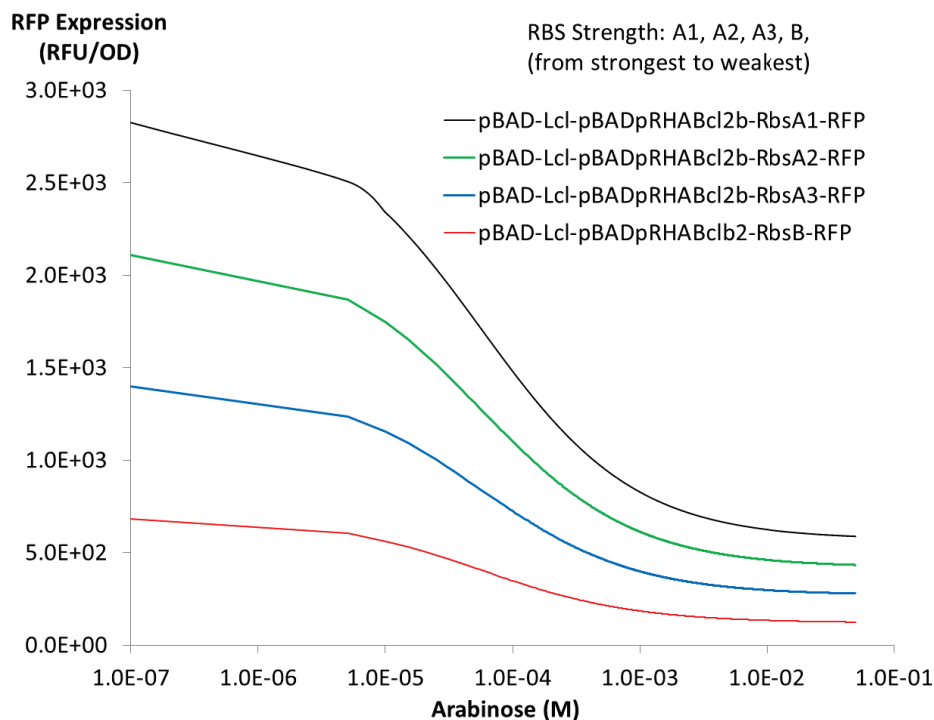


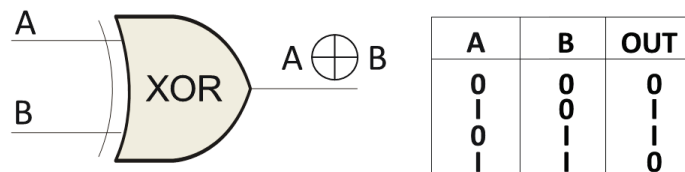
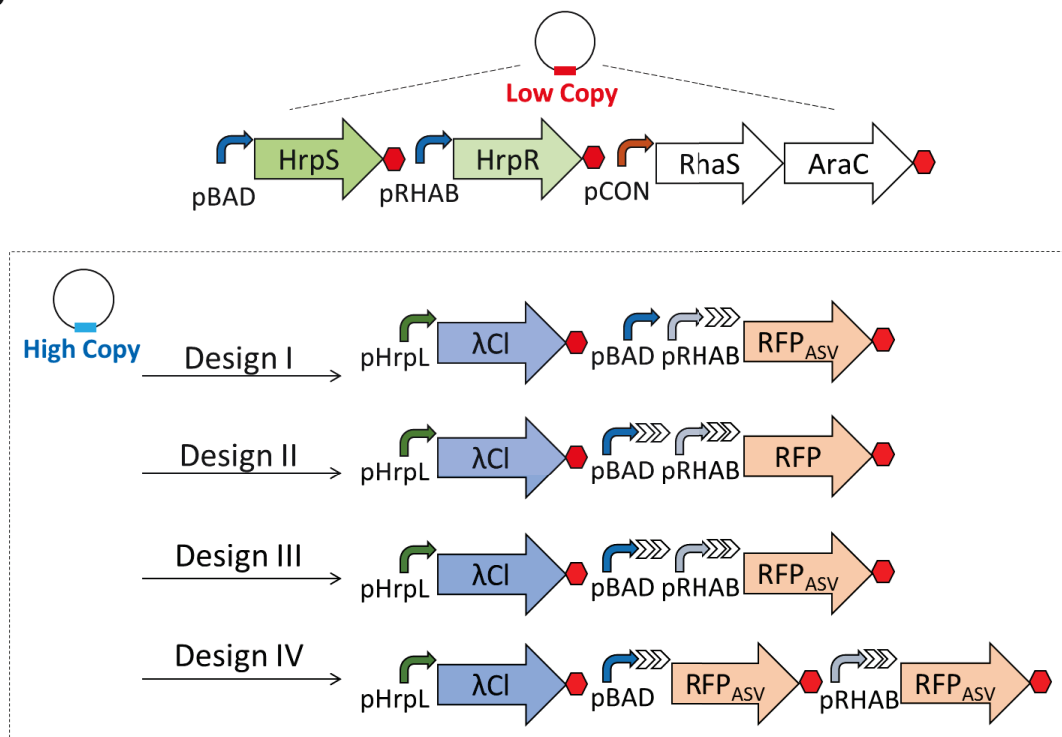
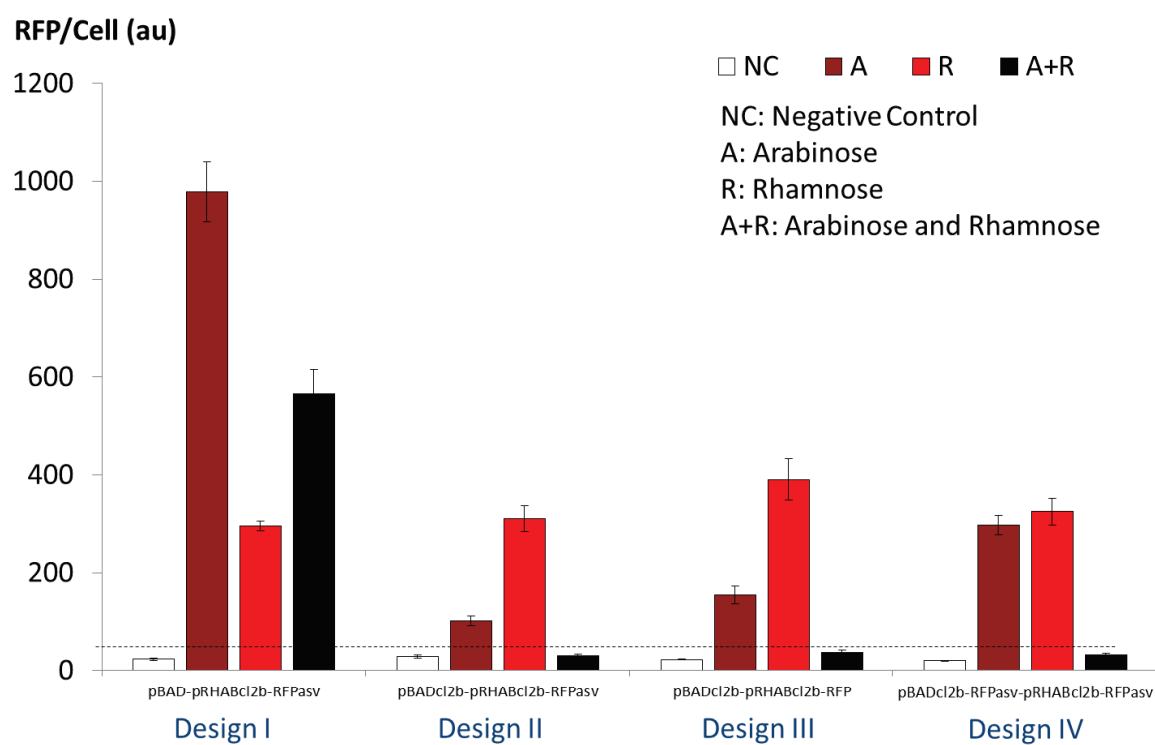
Figure 6-6: Predicted transfer function of IMPLY gate with different ribosome binding sites at steady state. Transfer function of IMPLY gate with the strongest ribosome binding site RbsA1 was obtained by empirical fitting.

6.3 Characterisation of XOR Logic Gate

In order to develop the XOR component of the half adder, we assimilated and tested a combination of AND, OR, and NOT logic gates in four different genetic circuits. In all the designs, HrpRS transcription activators were expressed from low copy plasmid to drive the synthesis of λ CI repressors from pHrpL promoter in high copy plasmids (**Figure 6-7B**). OR and NOT biological modules were assembled in the same high copy plasmid downstream of pHrpL- λ CI module. In design I, an OR gate comprising a tandem arrangement of pBAD, pRHAB and λ CI repressor binding sites was used to express *ssrA*-tagged RFP (RFP_{asv}) I – one of the most well characterised protein degradation system in *E. coli*¹⁷⁶. In design II, we created hybrid promoters of

pBAD and pRHAB by incorporating λ CI binding sites downstream of both promoters before connecting them in tandem to elicit hypothetical OR logic as similar to design I. Design III was slightly altered from design II to express untagged, long-lived RFP. To overcome possible complication from 5'UTR secondary structures due to presence of multiple λ CI binding sites within the same mRNA transcript, design IV which comprised of synthetic hybrid promoters of pBAD-CI2B and pRHAB-CI2B expressing RFP_{asv} in two discrete expression cassette was also developed.

Accordingly, only design IV was able to achieve well-balanced outputs which accurately described XOR logic operations (**Figure 6-7C**). While design I demonstrated strong suppression of RFP output in the presence of both inputs (arabinose and rhamnose) when characterised as an IMPLY gate (as described earlier), the same design failed to function in the context of XOR gate in which a weaker pHrpL promoter was used to drive the synthesis λ CI repressors instead of the strong pBAD promoter. Interestingly, the results implied that when employing transcription repressors as molecular blockers to mRNA elongation, higher concentration of λ CI molecules is needed to completely suppress transcription as λ CI binding sites are engineered further away from the transcription start site. This observation may be an effect of RNAP gaining momentum as it runs down template DNA to perform transcription, inadvertently enabling RNAP to continue its course of action in the inadequacy of “molecular brakes”.

A**B****C**

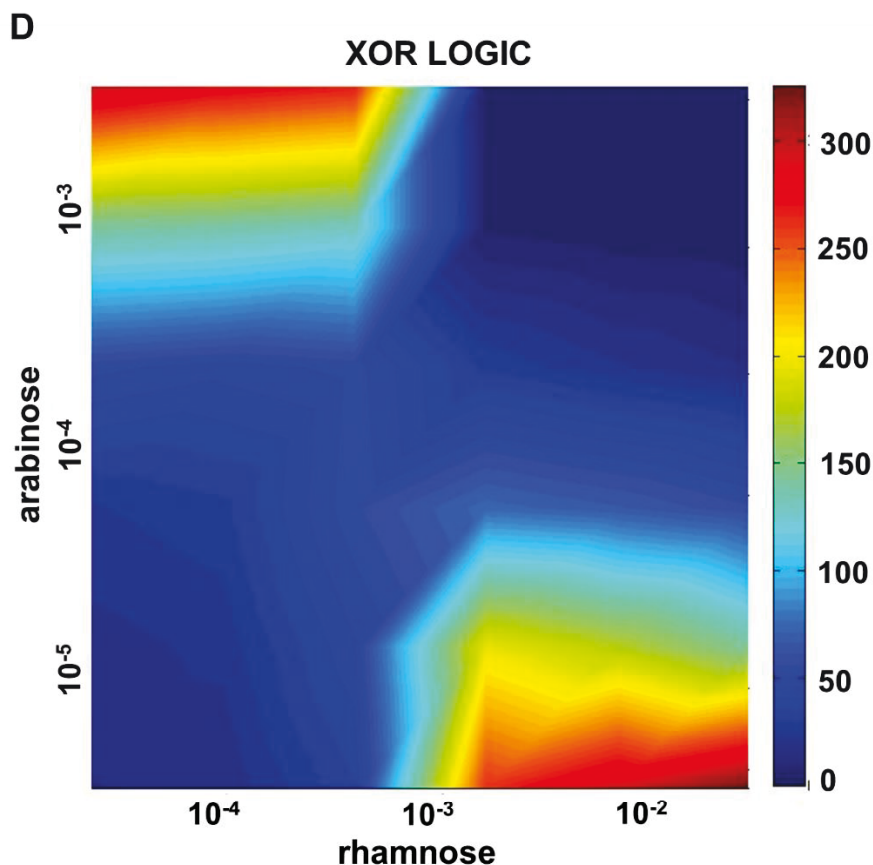


Figure 6-7: Design and characterisation of biological XOR gates. (A) Logic output of XOR gate. (B) Genetic blueprint of four biological XOR gate designs. The XOR gate comprises of serially layered AND, NOT and OR gates. HrpRS transcription factors are carried in low copy plasmid while pHrpL- λ CI and distinct modules of OR gates with lambda repressor binding sites expressing RFP reporter are carried in high copy plasmids. Design I comprises of tandem promoters with repressor binding sites downstream of pRHAB promoter and a RFP reporter engineered with the ASV protein degradation tag. Design II and III comprises of tandem promoters with repressor binding sites downstream of each promoter and RFP with and without the ASV degradation tag respectively. Design IV is modified from design II with RFP expressed in two disparate transcripts. (C) Digital performance of various designs of biological XOR gates at steady state. (D) Steady state profile of XOR gate IV in varying concentration of arabinose (input A) and rhamnose (input B). Error bars represent the standard deviation of four independent experiments.

While both design II and III that were developed with λ CI binding sites downstream of both pBAD and pRHAB promoters, exhibited slight semblance of XOR logic operations, the presence of multiple, repeated sequences of λ CI binding sites in the transcript generated from the pBAD promoter greatly reduced the RFP output from Input A. Of note, using untagged RFP gene in design III led to slight increase in overall RFP output but did not alleviate the signal balancing issue due to pronounced 5'UTR structural effects. In order to increase the range of application for the XOR gate in the implementation of the half adder, design IV was characterised for its steady state profile by titrating with varying concentration of arabinose and rhamnose as shown in **Figure 6-7D**.

6.4 Characterisation of Single Cell Half Adder and Half Subtractor

The half adder computes dual inputs with both AND and XOR logic operations to generate CARRY and SUM output respectively. Building on bio-logical devices that were modularised and rigorously characterised earlier, we co-transformed constructs which produce GFP (CARRY) from HrpRS AND gate in low copy plasmid, RFP_{asv} (SUM) from hybrid promoters pBAD-CI2B and pRHAB-CI2B and λ CI repressors from pHrpL promoter in high copy plasmid into *E. coli* (**Figure 6-8A**). To study the digital performance of the single cell half adder, we characterised the system at both population wide and single cell level by microplate fluorescent assay (**Figure 6-8B**) and flow cytometry (**Figure 6-8C**) for four different logic conditions.

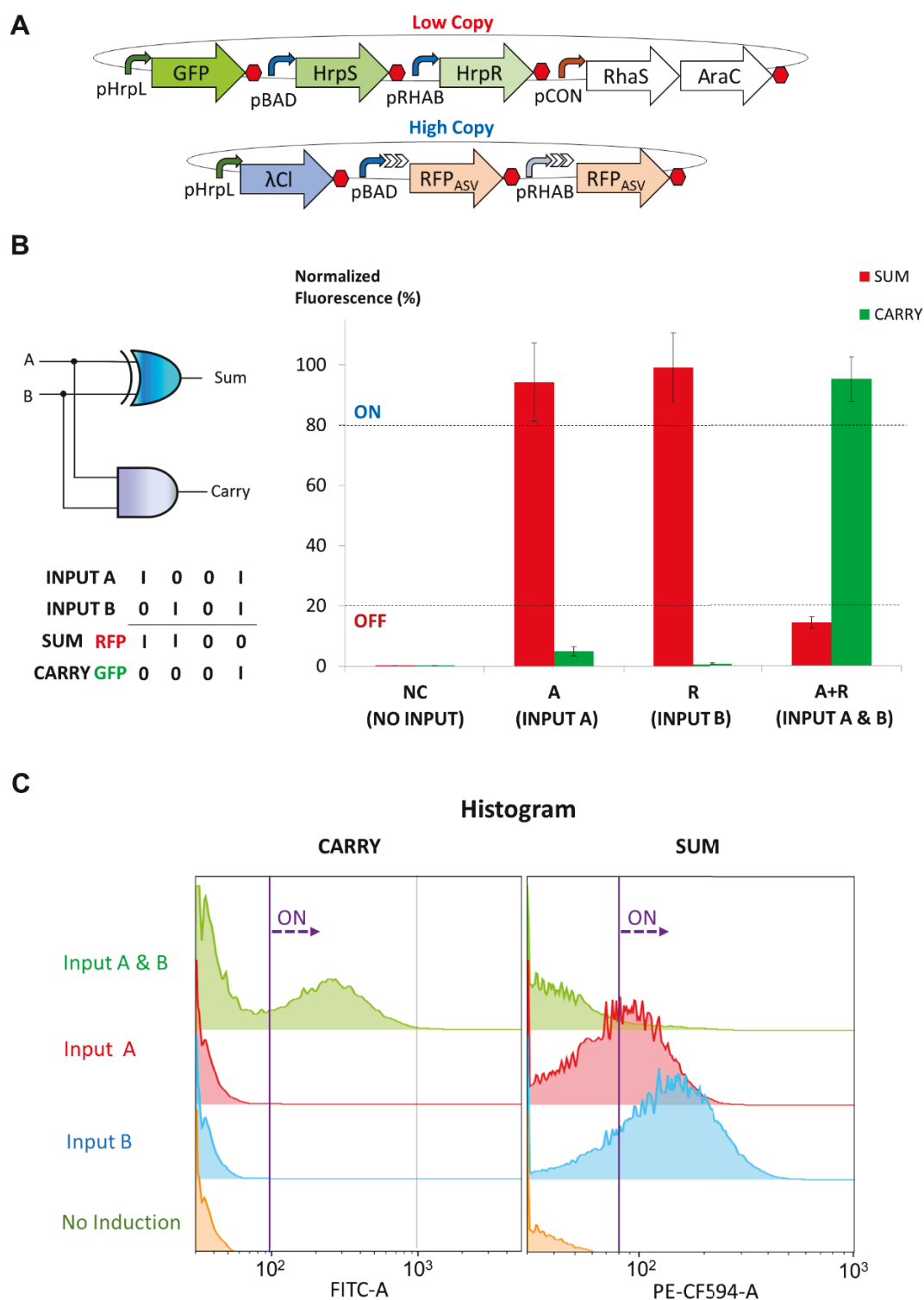


Figure 6-8: Design and characterisation of biological half adder. (A) Genetic blueprint of half subtractor. (B) Digital performance of half adder at steady state. Error bars represent the standard deviation of four independent experiments. (C) Flow cytometry analysis of half subtractor. Y axis represents population count, while FITC-A and PE-CF594-A represent channels that detect GFP and RFP fluorescence respectively. Population shifts to the right represent ON behaviour.

The results showed that the engineered cells exhibited robust and digital-like performance with minor expression leak ($< 20\%$) in XOR output when both inputs were present. While previous characterisation with standalone XOR gate displayed near perfect XOR outputs, parallel implementation of both AND and XOR logic gates in half adder led to probable competition for HrpRS transcription activators by pHrpL promoters in both low and high copy plasmids – which is suggestive of expression shunting in competitive transcription dynamics ¹³². In other words, the availability of HrpRS activators are now divided between pHrpL-GFP module in low copy plasmid and pHrpL- λ CI module in high copy plasmid, causing both AND and XOR gates to perform below par than when they are operating individually. To affirm the hypothesis, the AND output of standalone AND gate and the AND output of half adder were examined using microplate fluorescent assay. The results showed that the GFP output of isolated AND gate was approximately 7 times stronger than that of half adder's AND gate, thus confirming my hypothesis (**Figure 6-9**).

It is noteworthy, that the reduced expression of GFP did not affect the overall performance of the half adder as effective half adder logic operations were still achieved. In the current single cell half adder, the engineered cells exhibited relatively healthy growth with the same order of viable cells ($\sim 10^9$ cfu/ml) in both induced and uninduced cell cultures (**Figure 6-10**). Nonetheless, as genetic complexity and heterologous expression increases, concomitant increase in the metabolic burden of *E. coli* was also observed.

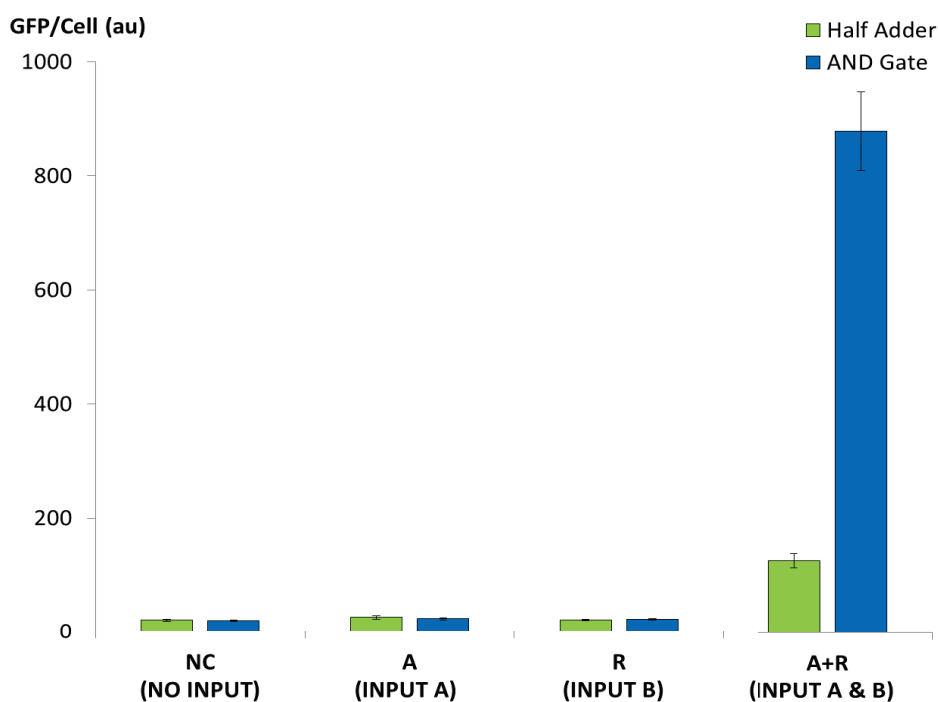


Figure 6-9: Effect of plasmid copy number in the shunting and sequestering of transcription factors. GFP output of pure AND gate is higher than the GFP output of half adder's AND gate. Error bars represent the standard deviation of four independent experiments.

To demonstrate the modularity of this approach, I also developed single cell half subtractor by performing slight modifications to the genetic circuits that formed the basis of the half adder. Specifically, GFP which exemplifies BORROW output was produced from hybrid promoter pBAD-CI2B in low copy plasmid instead of the pHrpL promoter (**Figure 6-11A**). As above, the construct which generated BORROW output (GFP) and that which generated the DIFFERENCE output (RFP) were co-transformed into *E. coli* cells. Characterisation was undertaken at both population wide and single cell level by microplate fluorescent assay (**Figure 6-11B**) and flow cytometry (**Figure 6-11C**) under four different logic conditions. The results showed that the engineered cells functioned as effective biological half subtractors, producing

GFP only in the presence of input A and RFP in the presence of input A or B but not when both inputs were present.

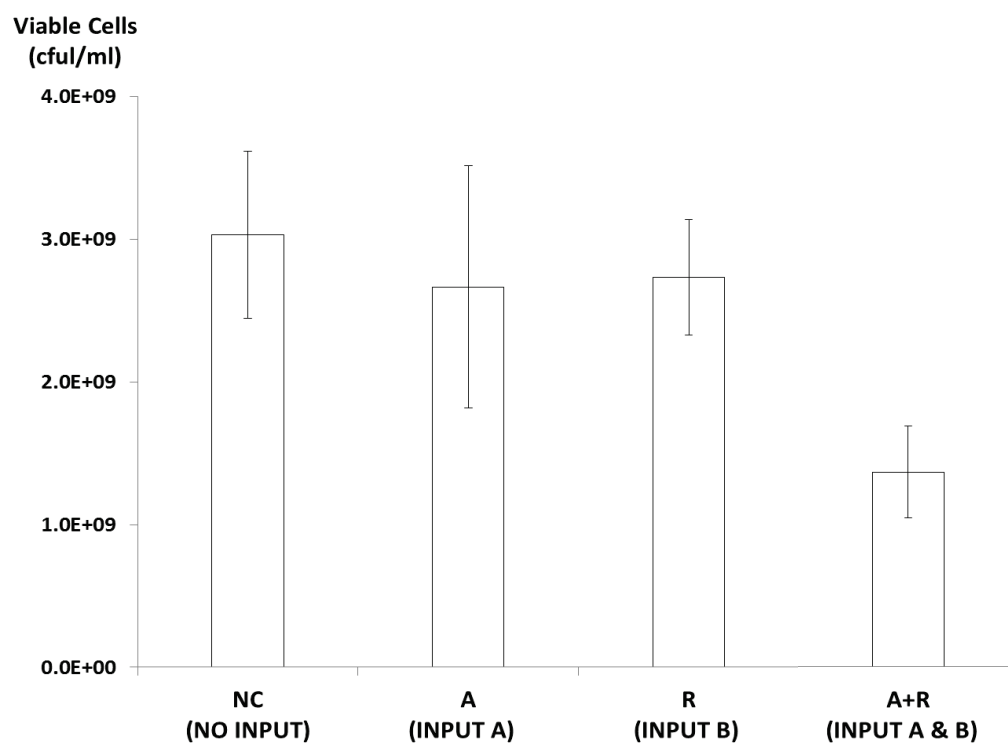


Figure 6-10: Cell viability profile of biological half adder after 4 hours of induction. Error bars represent the standard deviation of four independent experiments.

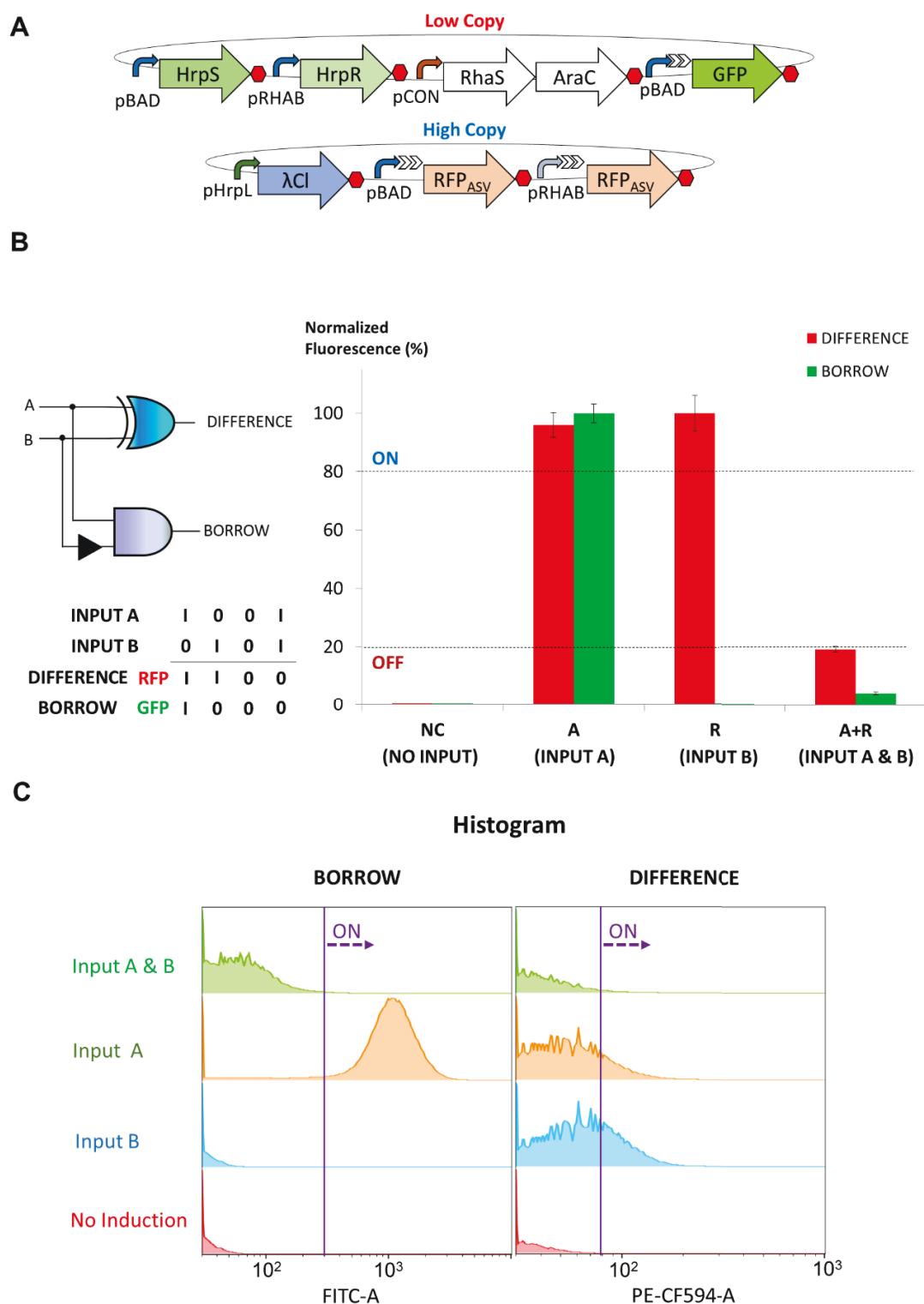


Figure 6-11: Design and characterisation of biological half subtractor. (A) Genetic blueprint of half subtractor. (B) Digital performance of half subtractor at steady state. Error bars represent the standard deviation of four independent experiments. (C) Flow cytometry analysis of half subtractor. Y axis represents population count, while FITC-A and PE-CF594-A represent channels that detects GFP and RFP fluorescence respectively. Population shifts to the right represent ON behaviour.

6.5 Conclusion

In this work, we created single cell half adder and half subtractor in prokaryotic system by applying classical engineering theorems (standardisation, modularisation, characterisation and abstraction) during the design process prior to system assembly. The first stage of this process was to systematically assemble and characterise independent modules that enable programmable digital operations in prokaryotic cells, including simple genetic switches, AND, OR and NOT logic operations. Using a forward engineering approach that is distinguished by rigorous characterisation and model-aided expression tuning, AND, OR and NOT logic gates were layered in both parallel and serial arrangements to generate a repertoire of cellular logic operations that includes IMPLY, XOR, half adder and half subtractor logic operations. Using a bottom up approach for constructing biological systems of increasing complexity we assessed genetic architectures that led to genetic context dependent effects. On this basis, the significance of each design on the overall digital performance of programmable logic gates in engineered cells was studied.

Of all, the presence of secondary structures in 5'-UTR of mRNA affects genetic expression the most. We discovered that the presence of seven consecutive hairpins immediately downstream of promoter transcription start site would cause severe impediment of gene expression. Although OR gate design made up of tandem promoters can be subjected to the undesirable effects of 5'-UTR secondary structure, we showed that that the effect is not pronounced in the digital performance of OR logics when the promoters and DNA operator sites involved are of markedly different DNA sequences. The

OR gate design that comprises a separate gene expression cassette also reliably demonstrates digital operation. However, the involvement of larger DNA modules and repetitive use of transcription terminators that are rich in secondary hairpin structures may impede system assembly in terms of construction efficiency and accuracy. Where identical DNA sequences are incorporated in a single mRNA transcript, as shown in design II and III of XOR gate, the effect of 5'-UTR secondary structure preventing gene expression is significantly more pronounced. Thus, it is proposed that XOR gate logics in layered genetic circuits should be designed with two discrete expression cassette instead of employing a tandem promoter circuit design. It would also be interesting to test if RNA processing tools can be employed in multiplex mode to insulate the myriad of biological devices from RNA genetic context dependent effects in layered genetic circuits concurrently.

Transcription terminators are important insulators to modularise independent expression cassettes. Still, if transcriptional overrun is present, even with the use of the strongest known transcription terminator, or when the choice of parts is limited by DNA length or synthesis capability, the problem can be overcome elegantly from an architecture perspective. This can be achieved by designing the most sensitive component in a reverse direction or placing it directly upstream of other expression modules – such that transcription overrun can never occur. In this study, the second method was applied, leading to the successful development of a biological AND gate. This was done by having the pHrpL-GFP reporter device upstream of other modules which generated HrpRS transcription factors.

Perhaps of particular interest, I discovered that $\sigma 54$ promoters can exhibit genetic context dependent effects if two $\sigma 54$ promoters are placed close to each other. Previously, $\sigma 54$ -dependent NtrC-binding promoters have been reported which permit transcription *in vitro* in the absence of enhancer-binding proteins and ATP. These include conditions that promote DNA melting, such as DNA supercoiling, temperature rise and lower ionic strength, or when characteristic point mutations are implemented on the $\sigma 54$ protein^{177, 178}. Here, I show that an upstream $\sigma 54$ pHrpL promoter could also activate downstream pHrpL promoter *in vivo* if the two promoters are of close proximity – possibly as a result of plasmid DNA supercoiling. This undesired switched-on activity can be avoided by designing pHrpL expression modules in different plasmids, i.e. to use plasmid as genetic buffers to insulate such genetic context dependent effects. As perturbations in genetic expression may propagate sizable effects on genome-wide transcriptional responses, it is worth considering if an additional role of plasmid in the course of evolution is to provide more robust and stringent control to genetic expression as with the occurrence of multiple chromosomes.

While recombinases have been intelligently crafted into Boolean logic gates with DNA-encoded memory functions, it is important to note that biosensors connected in AND, OR and XOR operations with recombinase-based logic gates may not be able to distinguish inputs from different environment and provide the desired response. For example, a probiotic that is genetically programmed in AND logic to sense two inputs such as hypoxia and low pH may be activated for hypoxia and low pH signals in two different location, as

compared to sensing both signals *in situ*. The same may be applicable for other logic operations with recombinase-based logic gates. Thus, layered genetic circuits that are capable of sensing and providing location-sensitive Boolean logic operations are still useful in programming cellular behaviours. Of particular interest is a combination of layered genetic circuits with the synthesis of recombinases as intermediary output, this may provide a novel and better platform for programmable cellular behaviour with both accuracy and memory.

The implementation of synthetic genetic circuits with the complexity of half adder and half subtractor may represent the extent to which synthetic biologists can currently engineer such circuits in single *E. coli* cell without causing an excessive metabolic load onto the cell. Nature, over many years of evolution, has produced specialised cells that perform specific and differentiated roles. Drawing inspiration, perhaps a practical way towards the development of synthetic biological systems that could perform sophisticated logic operations such as full adders and ripple adders is to implement a network of intercommunicating cells, each capable of performing specialised half adder logic operations.

Chapter Seven

7. OVERVIEW AND FUTURE WORKS

7.1 Summary of Contributions

In this thesis, I made the following contributions

A) Genetic engineering of commensal microbe to sense and eradicate a common superbug pathogen, *Pseudomonas aeruginosa*. A prototype of designer probiotic to combat superbug infection was developed by reprogramming human commensal *E. coli* with genetic devices that enabled *E. coli* to sense quorum sensing molecules from *P. aeruginosa*, produce corresponding killing molecules, and release the accumulated killing molecules through self-lysis to eradicate the pathogen. The sensing, killing, and lysing devices were characterised to elucidate their functionalities, which subsequently aided in the construction of the final system and the verification of its designed behaviour. Based on the characterisation and modelling results, the sensing device was verified to display an optimal activity approximately in the range of $1.0\text{E-}7$ to $1.0\text{E-}6$ M $3\text{OC}_{12}\text{HSL}$, which was in line with the concentration of $3\text{OC}_{12}\text{HSL}$ secreted by *P. aeruginosa*. The activities of the killing and lysing devices were also confirmed in response to this range of $3\text{OC}_{12}\text{HSL}$ concentration. The engineered *E. coli* with the complete system

was effective in killing planktonic *P. aeruginosa* by 99% and prevented its transition into sessile state by 90% when those two microbes were grown together.

B) Development of genetic interfaces that could integrate multiple input signals for enhanced biosensing capabilities.

The influence of ribosome binding sites on the output expression of biosensors or switch devices were elucidated and could be accurately modelled using Hill-like equation – a logistical function which describes output genetic expression as a function of input inducer concentration. A forward engineering framework involving characterisation of only the essential information of input biosensors and model-aided design principles were created to guide the predictable and reliable engineering of AND & OR logic gates in commensal *E. coli* bacteria. Using this novel design approach, the normalised outputs of actual AND & OR logic gates assembled in this study were comparable to outputs predicted from the steady state transfer function models, with least square regression fittings of at least 0.90. Single cell, single plasmid AND & OR logic gates were constructed in commensal bacteria and characterised to study how genetic context dependent effects could occur through choices of genetic architectures.

C) Programming autonomous decision-making in commensal microbe with biological half adder.

Using a forward engineering approach that is supported by rigorous characterisation and model-aided expression tuning, AND, OR and NOT logic gates were layered in both parallel and serial arrangements to generate a repertoire of cellular logic operations that includes

IMPLY, XOR, half adder and half subtractor logic operations. Through bottom up approach of constructing biological systems of increasing complexity, genetic architectures that led to genetic context dependent effects were carefully assessed. On this basis, the significance of each design on the overall digital performance of programmable logic gates in engineered cells was studied. The design heuristics established in this study will be invaluable guidelines to future designs and implementations of synthetic genetic circuits.

7.2 Future Work

Probiotic engineering represents a novel antimicrobial strategy. The proposed research presents a systematic and rational framework to treat microbial infections through the engineering of probiotic *E. coli*. Issues that previously reduce the effectiveness of conventional antibiotic treatments are addressed with the introduction of functionalities such as biofilm disruption and pyocin antimicrobial modules. Pyocin-based antimicrobial strategies are narrow spectrum and effective only against closely related species. Moreover, pyocin production can be rewired to be under the regulation of multiple input signals using the biological AND & OR genetic interfaces developed earlier. As a result, exclusive killing of *P. aeruginosa* could be achieved without collateral damage to beneficial gut flora during the treatment process. These signals can be other biomarkers of the pathogen, including other quorum sensing signals 4-hydroxy-2-alkylquinolines and N-butanoyl-L-homoserine lactone, and toxins, or signals that are deliberately introduced via edible prebiotic sugars.

One way to improve the efficacy of designer probiotics in treating superbug infection is to infuse commensal bacteria with the ability to swim and seek out elusive bacterial infections that are unreachable by conventional antibiotics. To mediate this mechanism, a biological half adder can be coupled to regulate the motility of engineered commensal bacteria. Specifically, biosensors that recognise two different input quorum signals will drive the propulsion of engineered commensal towards the pathogen in three dimensional space. Then, when positioned within proximity to target pathogen whereby concentration of both input signals are maximum, the motility mechanism in

engineered commensal is deactivated as exhibited in the output of XOR gate. The AND gate command can then be activated for the production and release of pyocin antimicrobial modules. For protection against gastrointestinal tract infections in immunocompromised and surgical patients, the probiotic-based infection defence can be either consumed as a health supplement or encapsulated within a specialised pharmaceutical carrier.

It is envisioned that the framework developed in this study could be readily applied to the treatment of other infectious diseases such as *Vibrio cholera* and *Staphylococcus aureus*, and ultimately lead us toward the development of a probiotic strain that is able to diagnose and treat multiple infections. This can be achieved by rewiring probiotic bacteria with novel biological devices to sense and analyse quorum signals from various pathogens, leading to the production of specific antimicrobials against the superbugs. Through this research, I hope to provide an exemplary model on the engineering of probiotic with multiple functionalities and intensify the global research efforts toward bacterial therapy. The development of probiotic strains that are equipped with user-defined functions for treating infectious disease requires the amalgamation of microbiology and functional genomics with the novel field of synthetic biology. Henceforth, synthetic biology approaches, such as system assembly and bioinformatics analysis, will be employed in concert with conventional microbiology and functional genomics techniques, such as transcriptome and proteome profiling and gene knockout.

7.2.1 Identification of biological parts that impart bacteria motility and biofilm disruption

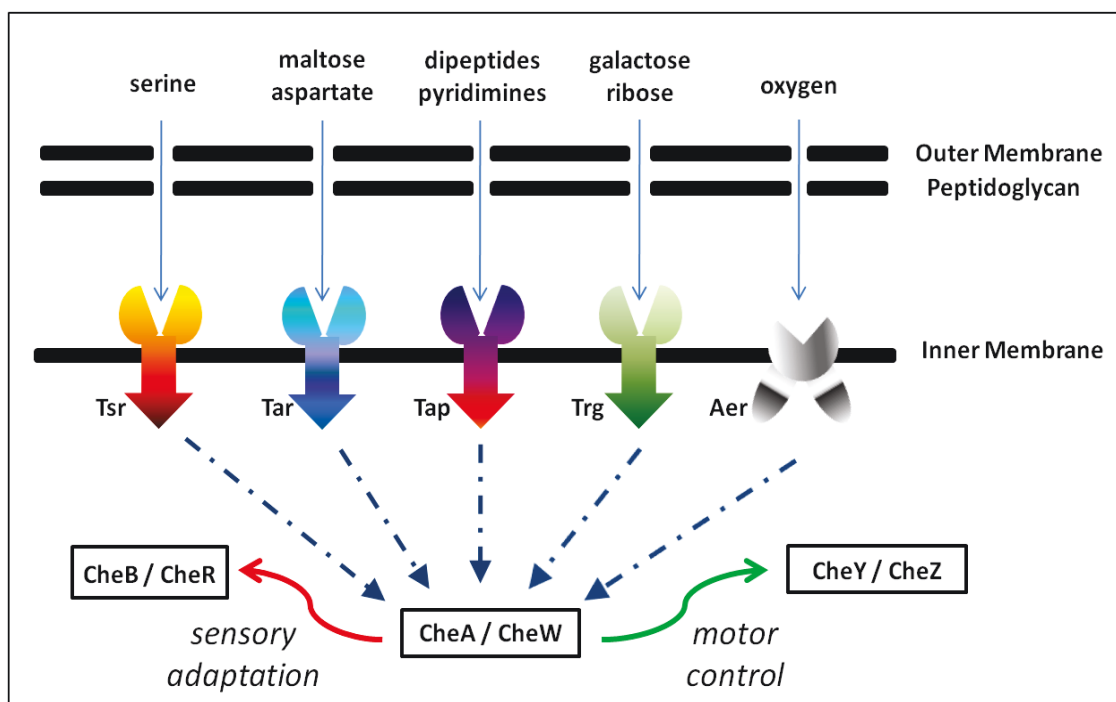


Figure 7-1: Sensory cascade of *E. coli* chemotaxis. *E. coli* detect chemoeffector gradients through five transmembrane proteins known as the methyl-accepting chemotaxis proteins (MCPs). MCPs form a signalling complex with CheA and CheW at the cell poles, which in turns phosphorylate CheY to initiate flagella for forward propulsion. CheR interfaces with CheB to retune the signalling state of MCP through methylation and demethylation of the receptors respectively.

Motivation: Engineering bacteria with the capability to swim toward *Pseudomonas* and destroy its protective biofilm matrix will enhance the killing efficiency of existing drugs and novel antimicrobials. Chemotaxis is the phenomenon whereby cells navigate their movements in response to chemical signals from the surrounding in a series of prolong straight “runs” and short intermittent “tumbles”. In *E. coli*, this process is effectuated through an ion-driven molecular complex of about twenty motility proteins, known as the bacterial flagella motor¹⁷⁹. Despite the widespread interest in motility research,

only a handful of studies had successfully engineered micro-organism with the ability to swim towards specific molecules that are not natural chemo-attractants of *E. coli*^{180, 181}. This limitation is perpetuated by two reasons; firstly, work in rewiring genetic circuits to control bacterial motility is considered laborious and costly due to a lack of assembly framework to facilitate systematic construction of advanced biological system. Secondly, because bacteria chemotaxis is regulated by other innate chemotaxis signalling cascade, a synthetic chemotaxis system might be subjected to noise and failure in an environment when both natural and non-natural chemotaxis molecules are present¹⁸². Similarly, the lack of studies on soluble enzymes which are effective in breaking the biofilm of both mucoid and non-mucoid strains of *Pseudomonas* is a major obstacle in the development of probiotic-based therapy.

Proposed Approach:

(a) To develop an *E. coli* strain that is isolated from its native chemotaxis signalling cascade, gene knockout can be performed with the targetron-based gene knockout technique to remove component that is interconnected with innate chemoeffectors¹⁸³. **Figure 7-1** shows the sensory cascade of *E. coli* chemotaxis and a list of major transmembrane chemoreceptor proteins (MCPs) involved in chemotactic response. As downstream chemotactic proteins are able to self-phosphorylate¹⁸⁴, the deletion of motility proteins such as CheA and CheW will decouple the influence of native MCPs to bacterial motility without disabling the flagella motor. Further, to confirm the decoupling, primer walking and chemotaxis assay on motility agar will be implemented¹⁸⁵.

(b) Bacterial stators are torque-generating devices for flagella rotation, powered by either proton motive force (pmf) or sodium motive force (smf). Given that proton energetic is crucial to *E. coli* metabolism and that vastly different pH environment exists along the GI tract, smf-driven bacterial stators can be employed in place of native pmf-driven stator to avoid dysregulation of critical life processes in the engineered biological system.

(c) To enable the engineered *E. coli* to swim towards *P. aeruginosa*, proteins that regulate cell motility such as CheZ can be rewired under the regulation of *Pseudomonas* quorum sensing controller. Bacterial motility can be analysed using techniques as described above, as well as phase contrast microscopy in specially designed capillary tubes.

(d) *Pseudomonas* biofilm matrix is encased in an extracellular polymeric substance comprising of nucleic acids, proteins and polysaccharides¹⁸⁶. Consequently, genes encoding for biofilm degradation proteins can be independently tested for biofilm degradation. For high throughput biofilm degradation assay, *Pseudomonas* biofilms can be cultivated on microtiter plate and treated with lysed supernatant of recombinant proteins. Biofilm can be quantified in terms of film dry mass and CFU count of viable biofilm cells after treatment. **Table 7-1** presents a list of biological parts which can be developed and characterised for the proposed research.

Table 7-1. List of potential biological parts for engineered motility and *P. aeruginosa* biofilm disruption.

Biological Part	Function	Reference
Stator component <i>pomA</i>	Component of sodium-driven flagella stator derived from <i>V. alginolyticus</i>	187
Stator component <i>potB</i>	Component of sodium-driven flagella stator derived from <i>E. coli</i> and <i>V. alginolyticus</i>	187
Direction controller CheZ	Modulate bacteria tumbling and forward propulsion through rotation of bacteria flagella	188
Bovine Pancreatic DNase I	Catalyse hydrolysis of phosphodiester bonds in nucleic acids	189, 190
Alginate Lyase <i>algL</i>	Catalyse cleavage of 4-O-linked glycosidic bonds between the urinate residues of alginate	191

Potential Difficulties:

(a) Production and degradation of motility proteins in the engineered *E. coli* may be too slow to effectuate a sensitive and responsive *pseudotaxis*. For this, the transcriptional regulation of motility proteins can be coupled to a genetic amplifier to enhance protein synthesis rate. In addition, motility proteins can be tagged with protease-recognition tag to promote faster protein turnover.

(b) Biofilm degradation protein may not be soluble in the engineered *E. coli*, leading to poor yield of active forms during over expression. To enhance

soluble protein production, folding chaperon or solubility tags such as GST fusion tag can be co-expressed with the protein of interests¹⁹².

7.2.2 Engineer genetic circuits to regulate the bacterial motility and biofilm disruption for *in vitro* studies.

Motivation: While individual biological parts can be assembled and characterised by traditional genetic engineering techniques, the assembly or analysis of larger biological systems are challenged by the constraint of available cloning sites on plasmid vectors. That is, cloning sites are expended with the inclusion of each promoter, ribosomal binding site, protein encoding gene and terminator sequence, in turn supporting only the development of small biological systems. Clearly, to enhance the effectiveness of the proposed treatment strategy, the engineered *E. coli* will entail laborious addition and optimisation of multiple genes. Moreover, as the concerted expression of various genes regulated by the same quorum sensing receiver may be lower than when they are expressed in isolation, *in vitro* studies are required to assess the performance of engineered bacterial motility and biofilm disruption before integration into the final system (sensing, swimming, killing and biofilm disruption).

Proposed Approach:

(a) Higher-ordered devices and system can be mathematically modelled using elementary data collected from parts' characterisation. Results from the

computational models will aid in the optimisation of the overall system and identify devices that require further refinement.

(b) The proposed biological system, integrated with both motility and biofilm disruption functions, can be assembled using the standardised synthetic biology framework. This framework involves the assembly of biological parts into sub-system constructs known as 'devices' and finally the assimilation of multiple devices into an advanced biological system.

(c) To test the engineered *E. coli* for directional motility, synthetic acyl homoserine lactone molecules (AHL) can be layered onto agarose plate to create motility agars with differential AHL gradient. Next, the engineered *E. coli* can be spotted onto different points of the motility agar and assessed for bacterial movement. Results of swimming distance and direction can be captured and analysed with relevant imaging software. Further, to test motility on liquid media, specially designed microfluidic device can be fabricated¹⁹³. The device consists of an input for engineered *E. coli* and three sampling output, containing different concentration of AHL. Cells at each sampling output will be quantified by CFU count.

(d) To test the engineered *E. coli* for *Pseudomonas* biofilm disruption function, mature biofilms of *P. aeruginosa* isolate In7 can be cultivated on reconstituted human epithelium tissues and treated with engineered *E. coli* cells. The efficiency of biofilm disruption can be quantified by CFU count and visualised by confocal laser scanning microscopy.

(e) To test whether the engineered *E. coli* can sense, swim towards and destroy *Pseudomonas* biofilm, a mutant strain of In7 (In7 Δ LasI) will be created by knocking out LasI gene encoding for AHL synthase. Next, biofilms of In7 and In7 Δ LasI will be allowed to develop at the sampling output of the microfluidic device before the engineered *E. coli* is introduced at the input. The hypothesis is that only the biofilm of In7 will be disrupted as the engineered *E. coli* will only swim toward In7.

Potential Difficulties:

(a) Engineered genetic circuit that shares a common input for divergent outputs may result in output signals below desired values. For this, signal amplification by means of orthogonal promoters may be utilised to circumvent the problem.

(b) Homogenous samples of *Pseudomonas* biofilms on reconstituted human tissue maybe difficult to cultivate. For this, special maintenance medium and incubator with precise air quality, humidity and temperature controllers may be required.

7.2.3 Transfer the engineered biological system into a probiotic *E. coli* host for *in vivo* murine studies.

Motivation: Promising treatment strategies for human diseases such as cancer, HIV and bacterial infections, must be tested in animal models before they are endorsed for further evaluation on a human body. This legislative

requirement is fulfilled so as to enhance the understanding of potential benefits and complications associated with the novel treatment method, and ensure that human health is not compromised for human clinical trials later. Further, because the environmental and growth condition of animals are easy to control, animal studies provide relatively accurate insights on the *in vivo* treatment efficiencies analogous to that of human vertebrates. Animals for pre-human clinical trials are selected in terms of their size, lifespan, cost and genetic likeness to human. For this reason, mice are the most widely utilised mammalian animals for clinical research and will be employed for the testing of the engineered probiotic in treating *P. aeruginosa* infections.

Proposed Approach:

(a) For treatment against *P. aeruginosa* in the mature biofilm state, a probiotic strain of *E. coli* (*Nissle 1917*), can be chromosomally engineered with sensing and therapeutic features discussed in this thesis (Chapter 4 – 7). Two reasons underline the choice of this probiotic strain; firstly, strains from the *E. coli* genus are well characterised for diverse microbiological properties. With relevant bioinformatics and metabolic pathways fully accessible in online databases, the adoption of *E. coli* for probiotic engineering is more predictable and cost-effective. Secondly, because *Nissle 1917* is originally a human isolate, the strain is able to colonise the gastrointestinal tract more readily, further advocating the use for treatment against GI infections.

(b) Murine studies should be performed following strict compliance to protocols and guidelines as approved by relevant bioethics committee. Germ-free mice

may be used as relevant murine models. To enable pathogen colonization of the GI tract, overnight cultures of *P. aeruginosa* clinical isolate In7 can be fed to mice in sterile environment. Following, the infected mice are treated with the engineered probiotic at periodic intervals after infection. The efficacy of treatment may be quantified using viable cell counts of the target pathogen from homogenised mice tissues¹⁷⁴.

(c) To trace the colonization of *P. aeruginosa* and engineered probiotic in the GI tract of infected mice, whole-body imaging techniques may be applied¹⁹⁴. For this, a strain of In7 that is able to constitutively express green fluorescent protein (In7-GFP) can be used. To visually differentiate the engineered probiotic from *P. aeruginosa*, a plasmid that constitutively express red fluorescent protein can be transformed into the engineered probiotic (EC-RFP). Subsequently, to monitor the extent of biofilm development, the infected mice can be viewed under fluorescent microscope at regular interval until adequate fluorescence is observed. Finally, to visualise the effectiveness of the proposed treatment, the infected mice can be examined with dual-color imager at regular intervals.

Potential Difficulties:

(a) Gene knockout: gene knockout in *E. coli* Nissle 1917 maybe exhibit low efficiency due to poor competency of Nissle 1917 in vector transformation. This issue may be addressed by using CRISPR technology and mediating vector transfer by inter-bacterial conjugation.

(b) Chromosomal insertion may lead to many false positives due to complication in removing the antibiotic selection marker. This issue can be addressed by increasing the frequency of marker removal through additional rounds of antibiotic removal and selection.

(c) Fluorescent reporters from *P. aeruginosa* and engineered probiotic may be too weak to be detectable in the infected mice. Consequently, the removal of mice surface hair or skin, or the use of stronger fluorescent protein such as the superfolder GFP may be necessary¹⁹⁵.

7.3 Socio-Ethical Aspects of Designer Probiotic and Its Adoption

In many countries, probiotics are classified as functional foods and made available in the form of encapsulated health supplements or yogurt. As such, these products are not subjected to the tight regulation associated with pharmaceutical drugs and medical food. With the advent of synthetic biology and the human microbiome project, newly acquired knowledge about the human health and gut microbiota may see the development of probiotics being genetically functionalised with novel therapeutic features and control elements – designer probiotics. Just like genetically modified (GM) foods, designer probiotics would inadvertently be under tighter scrutiny and regulation, leading to divisive opinions about their use and relevance. Perhaps one contentious issue about designer probiotics is whether the products would introduce unintended health repercussions in the long run. To the common folks, genetically modifying foods introduce an unnatural and potentially harmful flavour into them. These concerns are rightfully justified, given the untended

outcomes of GM crops which surfaced only years after their adoption. These includes gene flow through horizontal gene transfer, development of pesticide resistance, and uncontrolled escape of modified crops into the environment.

To maximise the potential of designer probiotics in advancing human health, it is imperative that the product development cycle is accompanied by a detailed analysis of the potential benefits and risks associated with its use. An ethical assembly consisting of clinicians, health professionals, synthetic biologists, social scientists, and representatives from food industry and government could be formed to brainstorm and develop safety nets on the testing and adoption of designer probiotics. Ideally, probiotics should be modified as little as possible, introducing only essential features that either boost therapeutic efficacy or enable human-imposed control. Human-imposed control such as the design and implementation of genetic kill-switch in this thesis serves as secondary control to prevent the unintentional escape of designer probiotics. As with medical foods and pharmaceutical products, designer probiotics ought to be subjected to tight regulation and testing before being endorsed for clinical adoption. Human clinical trials should be performed in gradual phases of at least 20, 100 and 1000 people in double-blinded, randomised, placebo-controlled trials. Results of clinical trials should be transparently communicated to the public while avoiding the use of technical jargons that may otherwise lead to more distrust and confusion. Scientists should also be ready to acknowledge public views that are based on socio-cultural aspects rather than scientific accuracy and be patient to engage in constructive dialogues with the public.

Chapter Eight

8. APPENDIX A - RECIPES

Appendix A provides the list of materials that were used to perform each experiment as described in Chapter 3.

8.1 Recipes – Therapeutic Microbe against *Pseudomonas* Infection

8.1.1 Gibson Assembly

1. Thermocycler
2. Gel electrophoresis setup
3. UV transilluminator
4. Heating block
5. Nanodrop (Thermo Scientific)
6. Electroporator
7. Isothermal water bath
8. Shaking incubator
9. Sterile scalpel
10. Microcentrifuge tubes 1.5 ml
11. PCR reaction tubes
12. Electroporation cuvette 2.0 mm
13. L-shape cell spreader
14. Round bottom cell culture tubes with cap

15. Phusion High Fidelity DNA polymerase (New England Biolabs), 5× Phusion HF buffer, DMSO, dNTPs.
16. 1× TAE buffer (40 mM Tris base, 40 mM acetic acid, 1 mM EDTA) from 10× Tris-Acetate-EDTA buffer. Top up to 1L with sterile deionized water.
17. 0.8% w/v agarose gel with 8-wells in 55 ml 1× TAE and 1× nucleic acid gel stain. This is sufficient for loading of up to 50 µl of PCR product.
18. QIAquick Gel Extraction Kit (Qiagen)
19. Gibson Assembly Master Mix (New England Biolabs). Alternatively, a 5× isothermal reaction buffer (ISO) and 1.33× assembly master mix can be prepared with the recipe formulated by Gibson as described in **Table 8-1** and **8-2**¹⁵⁰. The 5× ISO buffer and 1.33× assembly master mix can be stored at -20 °C in aliquots of 350 µl and 15 µl for up to a year respectively.
20. SOC medium (0.5% w/v yeast extract, 2% w/v tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose). Glucose is added last after autoclaving the other ingredients. Sterilize the solution with a 0.20 µm filter and stored at 4 °C.
21. Chemically competent *E. coli* cells
22. Antibiotic-supplemented LB agar plate

Table 8-1. Preparation of 5× isothermal buffer (ISO)

Initial Concentration	Volume in 6 ml	Final Concentration	Source
Sterile ddH ₂ O	Top up to 6 ml		
Tris-HCl pH 7.5 (1 M)	3 ml	500 mM	
MgCl ₂ (2 M)	150 µl	50 mM	
dNTP mix (10 mM of ea. dNTP)	600 µl	1 mM of ea. dNTP	
DTT (1 M)	300 µl	50 mM	Thermo Scientific
NAD (100 mM)	300 µl	5 mM	Applchem Lifescience
PEG-8000	1.5 g	25% w/v	Affymetrix

Table 8-2. Preparation of 1.33× assembly master mix

Initial Concentration	Volume in 1.2 ml	Final Concentration	Source
Sterile ddH ₂ O	Top up to 1.2 ml		
ISO buffer (5×)	320 µl	500 mM	
T5 exonuclease (10 U/µl)	0.64 µl	6.4 U	New England Biolabs
Phusion polymerase (2 U/µl)	20 µl	40 U	New England Biolabs
Taq Ligase (40 U/µl)	160 µl	6400 U	New England Biolabs

8.1.2 Characterisation of Quorum Sensing Device

1. Shaking incubator
2. Microplate reader with programmable time-based fluorescence and absorbance measurement functions. (Biotek, Synergy HT Multi-Mode Microplate Reader recommended)
3. Gen5 Data Analysis software (Biotek, software for Synergy HT microplate reader)

4. Matlab with Curve Fitting Toolbox (The Mathworks, Natwick)
5. MS Excel (Microsoft Office 2010 or equivalent)
6. Multi-channel pipette
7. Vortex
8. Microplate sealing film
9. Round bottom cell culture tubes with cap
10. Petri dishes
11. 1 L of supplemented M9 characterisation media (1× M9 salts, 1 mM thiamine hydrochloride, 0.4% v/v glycerol, 0.2% w/v casein hydrolysate, 2 mM MgSO₄, and 0.1 mM CaCl₂, **Table 8-3**). Sterilize the solution with a 0.20 µm filter and store at 4 °C away from light.
12. 3OC₁₂ homoserine lactone. Dissolved in absolute DMSO to a stock concentration of 10 mM. Obtain 3OC₁₂HSL test solutions (0.1 µM, 1 µM, 10 µM, 0.1 mM and 1 mM) by sequential 10× dilution in supplemented M9 characterisation medium (10 µl 3OC₁₂HSL solutions to 90 µl supplemented M9)
13. Fresh plate of *E. coli* that expresses green fluorescent protein (GFP) when induced with 3OC₁₂HSL (QS-GFP, Amp^r)

Table 8-3. Preparation of characterisation media (M9S)

Initial Concentration	Volume in 1 L	Final Concentration
5× M9 minima salts	5.64 g	1×
Thiamine hydrochloride	150 mg	1 mM
MgSO ₄ (0.1 M)	20 ml	2 mM
CaCl ₂ (0.5 M)	200 µl	0.1 mM
Casein hydrolysate (10% w/v)	20 ml	0.2% w/v
Glycerol (50% v/v)	8 ml	0.4% v/v
Sterile ddH ₂ O	Top up to 1 L	

8.1.3 Estimation of Native Autoinducer Produced by *P. aeruginosa*

1. Shaking incubator
2. Microplate reader with programmable time-based fluorescence and absorbance measurement functions. (Biotek, Synergy HT Multi-Mode Microplate Reader recommended)
3. Gen5 Data Analysis software (Biotek, software for Synergy HT microplate reader)
4. MS Excel (Microsoft Office 2010 or equivalent)
5. Multi-channel pipette
6. Vortex
7. Microplate sealing film
8. Round bottom cell culture tubes with cap
9. Membrane filters 0.2 µm
10. Syringes 5 ml

- 11.1 L of supplemented M9 characterisation media (1×M9 salts, 1 mM thiamine hydrochloride, 0.4% v/v glycerol, 0.2% w/v casein hydrolysate, 2mM MgSO₄, and 0.1mM CaCl₂, **Table 8-3**). Sterilize the solution with a 0.20 µm filter and store at 4°C away from light.
12. Clinical isolates of *P. aeruginosa* In3, In4, In5, In7, In8 and InB (Amp^r)
13. Fresh plate of *E. coli* that expresses green fluorescent protein (GFP) when induced with 3OC₁₂HSL (QS-GFP, Amp^r)

8.1.4 Characterisation of Lysis Device

1. Shaking incubator
2. Microplate reader with programmable time-based absorbance measurement functions. (Biotek, Synergy HT Multi-Mode Microplate Reader recommended)
3. Gen5 Data Analysis software (Biotek, software for Synergy HT microplate reader)
4. Mini-PROTEAN Tetra Cell electrophoresis chamber and power supply
5. MS Excel (Microsoft Office 2010 or equivalent)
6. Incubator
7. Microcentrifuge
8. Multi-channel pipette
9. Vortex
10. Microplate sealing film
11. Round bottom cell culture tubes with cap
12. Petri dishes

- 13.1 L of supplemented M9 characterisation media (1× M9 salts, 1 mM thiamine hydrochloride, 0.4% v/v glycerol, 0.2% w/v casein hydrolysate, 2 mM MgSO₄, and 0.1 mM CaCl₂, **Table 8-3**). Sterilize the solution with a 0.20 µm filter and store at 4°C away from light.
14. 3OC₁₂ homoserine lactone. Dissolved in absolute DMSO to a stock concentration of 10 mM. Obtain 3OC₁₂HSL test solutions (0.1 µM, 1 µM, 10 µM, 0.1 mM and 1 mM) by sequential 10× dilution in supplemented M9 characterisation medium (10 µl 3OC₁₂HSL solutions to 90 µl supplemented M9)
15. Membrane filters 0.2 µm
16. Syringes 5 ml
17. Ice bath
18. Absolute trichloroacetic acid
19. Reconstitution solvent 20 ml (1× PBS buffer pH 7.0, 30 mM imidazole, 4 M urea pH 6.0)
20. Vivapure miniprep MC (Sartorius Stedim Biotech GmbH)
21. Bradford protein assay kit (Thermo Scientific)
22. Precast SDS PAGE gel (Biorad)
23. Fresh plate of *E. coli* with Sense-Release functions when induced with 3OC₁₂HSL (QS-E7, Amp^r)
24. Fresh plate of *E. coli* with Sense-Kill-Release functions (QS-E7, Amp^r)
25. Fresh plate of *E. coli* with Sense-Kill functions (QS-S5, Amp^r)

8.1.5 Electron Microscopy Analysis of *E. coli* Cytoplasmic Release

1. Shaking incubator
2. Centrifuge
3. Microcentrifuge with refrigeration function
4. Vacuum dryer
5. Osmium plasma coater
6. Field emission scanning electron microscope (FESEM)
7. Round bottom cell culture tubes with cap
8. Microcentrifuge tubes 2.0 ml
9. Membrane filters 0.2 μm
10. Syringes 5 ml
11. 24-well microplate plate
12. Tweezer
13. 10 ml of LB broth with ampicillin (100 $\mu\text{g/ml}$).
14. PEI-coated silicon slide. To prepare a silicon substrate for SEM application, first clean the surface of a silicon slide with 70% v/v ethanol solution and blow free of dust with an air hose. Immerse the slide in polyethyleneimine (PEI) for 30 min before rinsing completely with sterile deionized water 2 – 3 times. Leave to dry on a clean bench for at least 3 h or until use. PEI-coated slides can be stored in for up to two weeks at room temperature.
15. 0.1 M sodium cacodylate, pH 7.4
16. 2.5% w/v glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4
17. 1% w/v osmium tetroxide in 0.1 M sodium cacodylate, pH 7.4
18. Ethanol solutions (37%, 67%, 95% and 100%), 5 ml of each.
19. SEM coating powder, gold-palladium alloy (60:40)

20. Fresh plate of engineered *E. coli* with Sense-Kill-Release functions (QS-S5-E7, Amp^r)

21. Control plate of engineered *E. coli* with only Sense-Kill functions (QS-S5, Amp^r)

8.1.6 Overlay Inhibition Assay

1. Kitchen microwave
2. Incubator with shaking function
3. Isothermal water bath
4. Bio-imager (Biorad ChemiDoc XRS or equivalent)
5. Round bottom cell culture tubes with cap
6. Microcentrifuge tubes 2.0 ml
7. Membrane filters 0.2 μ m
8. Syringes 5 ml
9. Tryptic soy agar plates (3% w/v Bacto tryptic soy broth and 1.5% w/v Bacto agar in 400 ml of deionized water). Autoclave the mixture and transfer 12 ml aliquots of the resultant solution into sterile petri dishes. Solidified tryptic soy agar (TSA) plates can be stored at 4 °C for up to a month.
10. 100ml of soft agar (1% w/v peptone and 0.5% w/v Bacto agar in 100 ml deionized water). Autoclave the mixture and cool to 45 °C in a water bath for immediate use. Otherwise, the solution can be stored at room temperature for up to a month.
11. Ice bath
12. Fresh plate of *P. aeruginosa* clinical isolate In7 which produces 3OC₁₂HSL

13. Fresh plate of engineered *E. coli* with Sense-Kill-Release functions (QS-S5-E7, Amp^r)
14. Control plate of engineered *E. coli* with only Sense-Kill functions (QS-S5, Amp^r)

8.1.7 Live and Dead Fluorescent Microscopy

1. Incubator with shaking function
2. Round bottom cell culture tubes with caps
3. Membrane filters 0.2 µm
4. Syringes 5ml
5. Bacterial viability kit (Invitrogen)
6. Fluorescent microscope with high resolution camera (Zeiss Axio Scope A1).

8.1.8 Co-culture Inhibition Assay of Engineered *E. coli* and *P. aeruginosa*

1. Microplate reader with programmable time-based fluorescence functions.
(Biotek, Synergy HT Multi-Mode Microplate Reader recommended)
2. MS Excel (Microsoft Office 2010 or equivalent)
3. 96-well microplate (Greiner 96-well CellStar®, black)
4. Round bottom cell culture tubes with caps
5. 10 ml of LB broth with ampicillin (100µg/ml).
6. Optional. LB agar plate with chloramphenicol (100µg/ml)

7. Fresh plate of *P. aeruginosa* clinical isolate In7 carrying plasmid pMC-PA_{GFP/CM} (see **Note 1**)
8. Fresh plate of engineered *E. coli* with Sense-Kill-Release functions (QS-S5-E7, Amp^r)

8.1.9 Biofilm Inhibition Assay

1. Incubator with shaking functions
2. 12-well microplate (Nunc, clear)
3. Microplate sealing films
4. Thin glass coupons
5. MHB with chloramphenicol
6. MHB with ampicillin
7. LB agar plates with chloramphenicol (50µg/ml)
8. Fresh plates of *P. aeruginosa* clinical isolate In7 and PAO1 carrying plasmid pMC-PA_{GFP/CM}. PAO1 is used as a negative control in this experiment as it has resistant to pyocin S5
9. Fresh plates of engineered *E. coli* with full Sense-Kill-Release functions (QS-S5-E7, Amp^r)
10. Fresh plates of engineered *E. coli* with partial Sense-Kill or Sense-Release functions. (QS-S5 or QS-E7, Amp^r)

8.2 Recipes – Engineering Digital Logic Operations in Microbe

8.2.1 BioBrick Assembly

1. Thermocycler
2. DNA Nanodrop
3. Microcentrifuge
4. Heat block
5. Amplified PCR insert
6. Biobrick vectors pSB4A5 (Amp^R) or pSB1C3 (Cm^R)
7. Restriction enzymes EcoRI-HF, XbaI, SpeI, PstI (New England Biolabs)
8. rAPid alkaline phosphatase (Roche)
9. T4 DNA ligase
10. Minelute gel purification kit (Qiagen)
11. PCR purification kit (Qiagen)

8.2.2 Characterisation and Orthogonal Testing of Input Switches

1. Microplate reader with fluorescence and absorbance measurement functions. (BMG Lab Technologies)
2. Matlab with Curve Fitting Toolbox (The Mathworks, Natwick)
3. MS Excel (Microsoft Office 2010 or equivalent)
4. Microplate incubator with heated lid (Biofrontier)
5. Multi-channel pipette
6. 100× arabinose and rhamnose (2.8 M).
7. Fresh plate of *E. coli* that expresses RFP when induced with input inducer (pBAD-RFP and pRHAB-RFP, Amp^r)

8. Fresh plate of *E. coli* with orthogonal testing construct (pRHAB-RFP-pBAD-GFP, Amp^R)

8.2.3 Characterisation of AND, OR, IMPLY Gates

1. Microplate reader with fluorescence and absorbance measurement functions. (BMG Lab Technologies)
2. Matlab with Curve Fitting Toolbox (The Mathworks, Natwick)
3. MS Excel (Microsoft Office 2010 or equivalent)
4. Microplate incubator with heated lid (Biofrontier)
5. 96-well microplate
6. Multi-channel pipette
7. 100× arabinose and rhamnose (2.8 M).
8. Fresh plates of *E. coli* with AND (pSB4A5, Amp^R) and OR (pSB1A2, Amp^R) constructs

8.2.4 Parts Mutagenesis of λ CI Repressor Binding Sites

1. Thermocycler
2. Gel electrophoresis setup
3. Phusion DNA polymerase
4. Forward primer 5'-TTCGAATTCGCGGCCGCTTCTAGAGGCCGGATTAT
5. Reverse primer 5'-
CTACTAGTATATNNNNNNNNCCGGTGATATATGGAGAAACAGTA
6. Microplate incubator with heated lid (Biofrontier)
7. LB agar plate with chloramphenicol

8. 96-well microplate
9. Microplate reader with fluorescence and absorbance functions (BMG Lab Technologies)
10. Autoclaved toothpicks
11. 100× Arabinose stock (0.35 M)
12. 100× Rhamnose stock (2.8 M)
13. Plasmid template pBAD_{Cl2A}-RFP (pSB1C3, Cm^R)
14. Plasmid pHrpL-λCI (pSB1C3, Cm^R)
15. Competent cells with pBAD-HrpS-pRHAB-HrpR (pSB4A5, Amp^R)

8.2.5 Characterisation of XOR Gate, Half Adder and Half Subtractor

1. Microplate reader with monochromatic fluorescence and absorbance measurement functions. (Synergy H1m, Biotek Instruments)
2. Matlab with Curve Fitting Toolbox (The Mathworks, Natwick)
3. MS Excel (Microsoft Office 2010 or equivalent)
4. Thermomixer (Eppendorf)
5. 1.5ml microcentrifuge tubes
6. 100× arabinose and rhamnose (2.8 M).
7. Fresh plates of *E. coli* with AND (pSB4A5, Amp^R) and OR (pSB1A2, Amp^R) constructs

8.2.6 Flow Cytometry of Half Adder and Half Subtractor

1. Thermomixer (Eppendorf)
2. LSR Fortessa X-20 with 488nm excitation laser (BD Bioscience)

3. FlowJo (TreeStar)
4. 1.5ml microcentrifuge tubes
5. Flow cytometry tubes (BD Bioscience)
6. 100× Arabinose stock (0.35 M)
7. 100× Rhamnose stock (2.8 M)
8. Fresh plates of *E. coli* with half adder and half subtractor constructs

Chapter Nine

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